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INVESTIGATIONS IN THE ENDOCRINOLOGY OF REPRODUCTION

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D.Sc. UNIVERSITY OF EDINBURGH 1980

CONTENTS

		PAGE NUMBER
ABSTRACT		1 - 2
STATEMENT		3 - 6
LIST OF PUBLICATIONS		7 - 12
		PUBLICATION NUMBER
CHAPTER 1.	LUTEINIZING HORMONE RELEASING HORMONE (LH-RH)	1 - 16
CHAPTER 2.	THE HYPOTHALAMO-PITUITARY-OVARIAN SYSTEM IN DOMESTIC ANIMALS	17 - 37
CHAPTER 3.	ARTIFICIAL CONTROL OF REPRODUCTION IN DOMESTIC ANIMALS	38 - 43
CHAPTER 4.	METHODOLOGY	44 - 49
CHAPTER 5.	MISCELLANEOUS	50 - 53

ABSTRACT

The research papers submitted were published between 1964 and 1980 on the subjects of luteinizing hormone-releasing hormone (LH-RH), the hypothalamo-pituitary-ovarian system and artificial control of reproduction in domestic animals, methods of hormone assay and miscellaneous topics. Work was carried out in the Department of Physiology and Environmental Studies, University of Nottingham, in the Department of Physiology, University of Texas Southwestern Medical School at Dallas, Texas, U.S.A. and in the Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, Jouy-en-Josas, France.

Chapter 1 contains publications on LH-RH and certain peptides related to LH-RH. These investigations were begun in Dallas in 1966 and have been continued to the present day at Nottingham. Included are papers on the localization of LH-RH within the hypothalamus and the synthesis, biological activity, plasma elimination and tissue degradation of LH-RH and fragments and analogues of the LH-RH molecule.

Chapter 2 describes studies on the hypothalamo-pituitary-ovarian systems of female domestic animals. This research was carried out at Nottingham and included is work on the control of the lactational anoestrus of the sow and of gonadotrophin secretion and ovulation in the ewe.

Chapter 3 consists of publications on the artificial control of reproduction in domestic animals. Studies on the sow arose from the work on lactational anoestrus described in Chapter 2. In the ewe, the possibility of using synthetic LH-RH for the induction of ovulation during seasonal anoestrus was investigated. The final paper in this chapter is a review of the endocrinology of meat production written at Jouy-en-Josas in 1979.

Chapter 4 includes papers on the development and use of methods of hormone assay; pituitary tissue incubation for the assessment of LH-RH and both biological and radioimmunological methods for the gonadotrophins. These techniques have been the basis of much of the work described in other Chapters.

Chapter 5 consists of several papers on aspects of endocrinology out of the main streams of the candidate's research.

STATEMENT

The research work described in these papers was carried out in the Department of Physiology and Environmental Studies, University of Nottingham and during a period of one year leave of absence in the Department of Physiology, University of Texas Southwestern Medical School at Dallas, U.S.A. Paper 43 was written during a period of six months leave of absence in the Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, Jouy-en-Josas, France. Work described in publications 17 and 35 was accepted for the degree of Ph.D. of the University of Nottingham in 1966. None of the other work has been submitted previously for any degree or diploma.

The publications divide into five chapters.

Publications

1 - 16 CHAPTER 1. Luteinizing hormone-releasing hormone (LH-RH)

The work on this hormone was begun at Dallas in 1966 under the supervision of Professor S. M. McCann and supported by a Post-Doctoral Fellowship from the Ford Foundation. Dr. S. Watanabe and Dr. A.P.S. Dhariwal were colleagues already established in the Department. Dr. H.P.G. Schneider was a Post-Doctoral Fellow who replaced the candidate in 1967 and contributed to studies on the localization of LH-RH which had been initiated and largely completed by the candidate during the year of post-doctoral work. Studies on LH-RH were continued on the candidate's return to Nottingham University. Dr. D. J. Schafer, Dr. A. D. Black and Dr. J. D. Bower were chemist colleagues at Reckitt and Colman Pharmaceutical Division who were responsible for the synthesis of the molecules referred to in papers 6 and 14. All of the biological testing of these molecules was carried out by the candidate at Nottingham. J. P. Foster and A. D. Swift were Ph.D. students supervised solely by the candidate. Brenda Siddall was a graduate technician.

17-37 CHAPTER 2. The hypothalamo-pituitary-ovarian system in domestic animals.

The work included in this chapter was carried out at Nottingham University. Papers 17 and 35 arose from the candidate's Ph.D. project which was supervised by Professor (then Dr.) G. E. Lamming. The degree was awarded in 1966. R. B. Land is a colleague working at the Agricultural Research Council Animal Breeding Research Organization, Edinburgh who provided the pituitary glands from experimental sheep which were bio-assayed for follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the candidate.
B. M. Hartley was a Ph.D. student supervised by the candidate.
Diane T. Holland and Dr. S.L. Jeffcoate were colleagues from the Department of Chemical Pathology, St. Thomas's Hospital, London with whom an extensive co-operative project was carried out on the hypothalamic control of LH secretion in the ewe. The animal experimentation and radioimmunoassay of LH was carried out by post-graduate student J. P. Foster and the candidate at Nottingham and the radio-immunoassay of LH-RH-like immunoactivity originally at St. Thomas's and later also at Nottingham.
W. Haresign was a Ph.D. student and Susan A. Scott was a final year B.Sc. student in the Department.

38-43 CHAPTER 3. Artificial control of reproduction in domestic animals.

The research work in papers 38-42 was carried out at the University of Nottingham, in some cases by the candidate alone and in others with colleagues, some of whom have been referred to previously. I. A. Jeffcoate is a Ph.D. student supervised solely by the candidate and P.D. Webb was a final year B.Sc. student in the Department. Paper 43 was written by the candidate during six months leave of absence to carry out research in the Laboratoire de Physiologie de la Lactation, Institut National de la Recherche Agronomique, Jouy-en-Josas, France in 1978-79.

44-49 CHAPTER 4. Methodology.

The six papers included here (nine with the addition of the relevant parts of papers 7, 17 and 21) represent the candidates contribution to the evolution of methods of assay for hormones. During the course of the candidate's Ph.D. study it was necessary to set up biological assays for LH and FSH, neither of which was available in the Department although the methods had been published previously by others. For the immunological study carried out by the Ph.D. student M.A. Saji it was necessary to set up a biological assay for prolactin. The biological assay for LH was used subsequently in an assessment of a new cytochemical assay for the hormone in co-operation with Rosanne Kramer, Dr. A. S. McNeilly, Dr. Lesley H. Rees and Professor T. Chard of the Department of Chemical Pathology, St. Bartholomew's Hospital, London. The radio-immunoassays for ovine LH and FSH were developed by the candidate with a post-graduate student (J. P. Foster) and a technician (Brenda Siddall). Some thousands of ovine pituitary glands were collected at Nottingham, subjected to physico-chemical purification by Dr. Anne Stockell Hartree in the Department of Biochemistry, University of Cambridge and the resulting LH and FSH rich fractions assessed at each stage of purification by biological assays for LH and FSH by the candidate at Nottingham.

Antisera to LH and FSH were raised at Nottingham and a suitable anti-LH serum used in the radio-immunoassay for LH. While a suitable FSH preparation for iodination was obtained, specificity problems rendered all anti-FSH sera raised at Nottingham useless and eventually a heterologous assay system using an anti-human FSH serum donated by Dr. W. Butt, Birmingham Women's Hospital was employed for the assay of ovine FSH. Both the ovine LH and ovine FSH radio-immunoassays are currently used as standard methods at Nottingham University and in other Institutes.

50-53 CHAPTER 5. Miscellaneous.

The four papers included here represent studies outside the main streams of the candidate's research work. Paper 50 resulted from the early interest in the setting up and application of biological assays for hormones.
D. A. Pickard was a final year B.Sc. student in the Department. Papers 51 and 52 arose from work carried out by K. J. Cooper, a Ph.D. student with the assistance of final year B.Sc. Honours students Maxine Griffiths and A. S. McNeilly both of whom were supervised by the candidate. Paper 53 arose from one aspect of work by a Ph.D. student I. J. Shearer whose study was jointly supervised by a colleague Dr. N. B. Haynes and the candidate.

Signed

D. B. Crighton

LIST OF PUBLICATIONS

CHAPTER 1. Luteinizing hormone-releasing hormone (LH-RH).

Papers in scientific journals (refereed)

1. CRIGHTON, D.B., WATANABE, S., DHARIWAL, A.P.S. & McCANN, S.M. (1968). Failure of inhibitors of protein synthesis to affect the LH-releasing action of hypothalamic extracts in vitro. Proceedings of the Society for Experimental Biology and Medicine 128, 537-540.

2. CRIGHTON, D.B., SCHNEIDER, H.P.G. & McCANN, S.M. (1969). Possible interaction of luteinizing hormone-releasing factor with other hypothalamic releasing factors at the level of the adenohypophysis. Journal of Endocrinology 44, 405 - 410.

3. SCHNEIDER, H.P.G., CRIGHTON, D.B. & McCANN, S.M. (1969). Suprachiasmatic LH-releasing factor. Neuroendocrinology 5, 271-280.

4. CRIGHTON, D.B., SCHNEIDER, H.P.G. & McCANN, S.M. (1970). Localization of LH-releasing factor in the hypothalamus and neurohypophysis as determined by an in vitro method. Endocrinology 87, 323-329.

5. CRIGHTON, D.B. (1973). The effects of synthetic gonadotrophin releasing factor on the release of luteinizing hormone and follicle-stimulating hormone from ovine pituitary tissue in vitro. Journal of Endocrinology 58, 387-391.

6. SCHAFER, D.J., BLACK, A.D., BOWER, J.D. & CRIGHTON, D.B. (1975). Synthesis and biological activity of luteinizing hormone-releasing hormone and related peptides. Journal of Medicinal Chemistry 18, 613-619.

7. SIDDALL, B. & CRIGHTON, D.B. (1977). Effects of certain analogues of synthetic luteinizing hormone-releasing hormone on the release of luteinizing hormone and follicle-stimulating hormone in the anoestrous ewe. Journal of Endocrinology 75, 49-57.

8. SWIFT, A.D. & CRIGHTON, D.B. (1979). Relative activity, plasma elimination and tissue degradation of synthetic luteinizing hormone releasing hormone and certain of its analogues. Journal of Endocrinology 80, 141-152.

9. SWIFT, A.D. & CRIGHTON, D.B. (1979). The effects of certain steroid hormones on the activity of ovine hypothalamic luteinizing hormone-releasing hormone (LH-RH)-degrading enzymes. FEBS Letters 100, 110-112.

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10. CRIGHTON, D.B. & SCHNEIDER, H.P.G. (1969). Localization of luteinizing hormone-releasing factor in the pre-optic area. Journal of Reproduction and Fertility 18, 166.

11. CRIGHTON, D.B. (1972). The effects of a synthetic decapeptide on the release of luteinizing hormone and follicle-stimulating hormone from ovine pituitary tissue in vitro. Journal of Physiology 226, 68P-69P.

12. CRIGHTON, D.B. & FOSTER, J.P. (1972). The effects of a synthetic preparation of gonadotrophin releasing factor on gonadotrophin release from the ovine pituitary in vitro and in vivo. Journal of Endocrinology 55, xxiii-xxiv.

13. CRIGHTON, D.B. (1973). Review of releasing hormones in domestic animals. Veterinary Record 93, 254.

14. CRIGHTON, D.B. & SCHAFER, D.J. (1974). Effects of various synthetic fragments of the decapeptide molecule of gonadotrophin releasing factor on the release of luteinizing hormone from ovine pituitary tissue in vitro. Journal of Endocrinology 61, xi.

15. SWIFT, A.D. & CRIGHTON, D.B. (1978). Relative activity, plasma elimination and hypothalamic and pituitary degradation of synthetic luteinizing hormone releasing hormone and certain of its analogues. Journal of Endocrinology 77, 35P.

Contributions to books

16. CRIGHTON, D.B. (1978). Effects of certain analogues of synthetic luteinizing hormone-releasing hormone on luteinizing hormone and follicle-stimulating hormone release in the anestrous ewe. In 'Hypothalamic hormones - Chemistry, physiology and clinical applications' Eds. D. Gupta and W. Voelter, Verlag Chemie GmbH, Weinheim pp. 387-393.

CHAPTER 2. The hypothalamo-pituitary-ovarian system in domestic animals

Papers in scientific journals (refereed)

17. CRIGHTON, D.B. & LAMMING, G.E. (1969). The lactational anoestrus of the sow: The status of the anterior pituitary-ovarian system during lactation and after weaning. Journal of Endocrinology 43, 507-519.

18. LAND, R.B., CRIGHTON, D.B. & LAMMING, G.E. (1972). Gonadotrophin content of the pituitaries of sheep of differing fertility at three stages of the oestrous cycle. J. Reprod. Fert. 30, 313-316.

19. CRIGHTON, D.B., HARTLEY, B.M. & LAMMING, G.E. (1973). Changes in the luteinizing hormone releasing activity of the hypothalamus, and in pituitary gland and plasma luteinizing hormone during the oestrous cycle of the sheep. Journal of Endocrinology 58, 377-385.

20. CRIGHTON, D.B., FOSTER, J.P., HOLLAND, D.T. & JEFFCOATE, S.L.(1973). Simultaneous determination of luteinizing hormone and luteinizing hormone releasing hormone in the jugular venous blood of the sheep at oestrus. Journal of Endocrinology 59, 373-374.

21. FOSTER, J.P. & CRIGHTON, D.B. (1974). Luteinizing hormone (LH) release after single injections of a synthetic LH-releasing hormone (LH-RH) in the ewe at three different reproductive stages and comparison with natural LH release at oestrus. Theriogenology 2, 87-100.

22. CRIGHTON, D.B., FOSTER, J.P., HARESIGN, W. & SCOTT, S.A. (1975). Plasma LH and progesterone levels after single or multiple injections of synthetic LH-RH in anoestrous ewes and comparison with levels during the oestrous cycle. Journal of Reproduction and Fertility 44, 121-124.

23. FOSTER, J.P. & CRIGHTON, D.B. (1976). Pituitary responsiveness to a single injection of synthetic luteinizing hormone releasing hormone before and after the natural preovulatory plasma luteinizing hormone peak in the sheep. Journal of Endocrinology 71, 269-270.

24. FOSTER, J.P., JEFFCOATE, S.L., CRIGHTON, D.B. & HOLLAND, D.T. (1976). Luteinizing hormone and luteinizing hormone releasing hormone-like immunoreactivity in the jugular venous blood of sheep at various stages of the oestrous cycle. Journal of Endocrinology 68, 409-417.

25. CRIGHTON, D.B. & FOSTER, J.P. (1977). Luteinizing hormone release after two injections of synthetic luteinizing hormone releasing hormone in the ewe. Journal of Endocrinology 72, 59-67.

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26. CRIGHTON, D.B., HARTLEY, B.M. & LAMMING, G.E. (1972). Changes in the luteinizing hormone-releasing factor (LH-RF) content of the hypothalamus of the sheep during the oestrous cycle. Journal of Physiology 223, 26P-27P.

27. FOSTER, J.P. & CRIGHTON, D.B. (1973). Preliminary observations on the administration of a synthetic preparation of gonadotrophin releasing factor to cyclic and anoestrous ewes. Journal of Endocrinology 57, xxv.

28. FOSTER, J.P. & CRIGHTON, D.B. (1973). Comparison of LH levels in lactationally anoestrous and cycling ewes and the effects of synthetic gonadotrophin releasing factor. Journal of Reproduction and Fertility 35, 599-600.

29. CRIGHTON, D.B., FOSTER, J.P., HARESIGN, W., HAYNES, N.B. & LAMMING, G.E. (1973). The effects of a synthetic preparation of gonadotrophin releasing factor on pituitary and ovarian function in anoestrous ewes. Journal of Physiology 231, 98P-99P.

30. FOSTER, J.P., HOLLAND, D.T., JEFFCOATE, S.L. & CRIGHTON, D.B. (1974). Simultaneous determination by radioimmunoassay of luteinizing hormone and luteinizing hormone releasing hormone at various stages of the oestrous cycle in the sheep. Journal of Endocrinology 61, lxiii.

31. CRIGHTON, D.B., SCOTT, S.A. & FOSTER, J.P. (1974). An attempt to simulate, by injection of luteinizing hormone releasing hormone in the anoestrous sheep, the pattern of release observed at oestrus and the effects of this on luteinizing hormone release. Journal of Endocrinology 61, lxiii-lxiv.

32. FOSTER, J.P. & CRIGHTON, D.B. (1976). Luteinizing hormone release after injection of synthetic luteinizing hormone releasing hormone at various stages of the oestrous cycle in the sheep. Journal of Endocrinology 68, 41P-42P.

33. CRIGHTON, D.B. & FOSTER, J.P. (1976). Effects of duplicate injections of synthetic luteinizing hormone releasing hormone at various intervals on luteinizing hormone release in the anoestrous ewe. Journal of Endocrinology 69, 36P-37P.

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34. CRIGHTON, D.B. (1967). Effects of lactation on the pituitary gonadotrophins of the sow. In 'Reproduction in the Female Mammal'. Eds. G. E. Lamming and E. C. Amoroso. Butterworths, London,pp. 223-238.

35. CRIGHTON, D.B. (1971). Lactational anoestrus and the effects on lactation of the induction of varying levels of ovarian and uterine activity. In 'Lactation'. Ed. I. R. Falconer. Butterworths, London, pp. 105-121.

36. CRIGHTON, D.B. (1975). The natural control of ovulation in the sheep. In 'University of Nottingham School of Agriculture Report, 1974/1975,' Echo Press, Loughborough pp.53-65.

37. CRIGHTON, D.B. & LAMMING, G.E. (1975). Control of FSH and LH secretion in animals. In 'Some aspects of hypothalamic regulation of endocrine functions', Symposia Medica Hoechst 7,Schattauer Verlag, Stuttgart pp. 325-328.

CHAPTER 3. Artificial control of reproduction in domestic animals

Papers in scientific journals (refereed).

38. CRIGHTON, D.B. (1970). Induction of pregnancy during lactation in the sow. Journal of Reproduction and Fertility 22, 223-231.

39. CRIGHTON, D.B. (1970). The induction of pregnancy during lactation in the sow: The effects of a treatment imposed at 21 days of lactation. Animal Production 12, 611-617.

40. HARESIGN, W., FOSTER, J.P., CRIGHTON, D.B., HAYNES, N.B. & LAMMING, G.E. (1975). Progesterone levels following treatment of seasonally anoestrous ewes with synthetic LH-releasing hormone. Journal of Reproduction and Fertility 43, 269-279.

41. JEFFCOATE, I.A., FOSTER, J.P. & CRIGHTON, D.B. (1978). Effects of active immunisation of ewes against synthetic luteinizing hormone releasing hormone. Theriogenology 10, 323-335.

Papers in 'Proceedings' etc.

42. FOSTER, J.P., WEBB, P.D. & CRIGHTON, D.B. (1977). Effects of active immunization of sheep against synthetic luteinizing hormone releasing hormone. Journal of Endocrinology 72, 17P.

Contributions to books

43. CRIGHTON, D.B. (1980). Endocrinology of meat production. In 'Developments in Meat Science - 1'. Ed. Ralston Lawrie. Applied Science Publications Ltd. London, pp.1-36.

CHAPTER 4. Methodology

Papers in scientific journals (refereed)

44. CRIGHTON, D.B. (1964). Breeding mice for the biological assay of follicle-stimulating hormone. In 'University of Nottingham School of Agriculture Report, 1964,' Echo Press, Loughborough pp.73-78.

45. CRIGHTON, D.B. (1968). Depletion of rat ovarian ascorbic acid by a factor other than luteinizing hormone present in the blood of the pig. Journal of Reproduction and Fertility 15, 457-459.

46. SAJI, M.A. & CRIGHTON, D.B. (1968). A study of the antihormonal activity of an antiserum to ovine prolactin using the local lactogenic response in the rabbit. Journal of Endocrinology 41, 555-561.

47. HARTLEY, B.M., CRIGHTON, D.B. & LAMMING, G.E. (1973). The specificity of an ovine pituitary incubation system linked with gonadotrophin assays for detecting gonadotrophin releasing activity. Journal of Endocrinology 58, 363-375.

48. KRAMER, R., HOLDAWAY, I.M., CRIGHTON, D.B., McNEILLY, A.S., REES, L.H. & CHARD, T. (1976). Comparison of the redox bioassay with other assays for luteinizing hormone. Journal of Endocrinology 69, 205-211.

Papers in 'Proceedings' etc.

49. CRIGHTON, D.B., HARTLEY, B.M. & LAMMING, G.E. (1972). An in vitro technique for the study of hypothalamic control of gonadotrophin secretion in the sheep. Journal of Physiology 222, 175P-176P.

CHAPTER 5. Miscellaneous

Papers in scientific journals (refereed)

50. PICKARD, D.A. & CRIGHTON, D.B. (1967). An investigation into the possible oestrogenic effect of kale. British Veterinary Journal 123, 64-69.

51. McNEILLY, A.S., COOPER, K.J. & CRIGHTON, D.B. (1970). Modification of the oestrous cycle of the under-fed rat induced by the proximity of the male. Journal of Reproduction and Fertility 22, 359-361.

52. GRIFFITHS, MAXINE, COOPER, K.J. & CRIGHTON, D.B. (1972). The effects of castration and androgen replacement therapy on the ability of the male to influence the oestrous cycle of the underfed rat. Journal of Reproduction and Fertility 30, 481-483.

Papers in 'Proceedings' etc.

53. SHEARER, I.J. HAYNES, N.B. & CRIGHTON, D.B. (1971). Peripheral steroid hormone levels and nitrogen retention in the gilt from prepuberty to parturition. Journal of Reproduction and Fertility 27, 491-492.

CHAPTER 1. LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH)

Localization, synthesis, biological activity, plasma elimination and tissue degradation of LH-RH.

Synthesis, biological activity, plasma elimination and tissue degradation of certain peptides related to LH-RH.

Papers in scientific journals (refereed)

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1968, v128, 537-540

Failure of Inhibitors of Protein Synthesis to Affect the LH-Releasing Action of Hypothalamic Extracts *in Vitro** (33060)

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The role of the hypothalamus in controlling the secretion of luteinizing hormone (LH) has been well established. The final common pathway through which the hypothalamus exerts its influence is undoubtedly the LH-releasing factor (LHRF), the existence of which was first reported by McCann *et al.* (1). This factor has been shown to be effective in increasing LH release both *in vivo* (2–4) and *in vitro* (5,6). It has also been suggested that the hypothalamus may influence the synthesis of LH as well as its release (7, 8).

The present paper describes studies made on rat anterior pituitary glands incubated *in vitro* in the presence and absence of 2 antibiotics, actinomycin D and puromycin, which are inhibitors of protein synthesis. Actinomycin D is an inhibitor of the synthesis of ribonucleic acid (RNA) and consequently that of protein (9, 10) and puromycin acts as a relatively specific inhibitor of protein synthesis, apparently by preventing the final condensation of activated amino acids to form a complete protein (11,12). The 2 antibiotics were used to obtain information on the mechanism of action of purified LHRF in increasing LH secretion and specifically to determine if protein synthesis is required for its action.

Materials and Methods. Preparation of purified LHRF. The purified LHRF was prepared from ovine hypothalami using gel filtration on Sephadex G-25 (13). The fraction to be tested had been shown to be active in releasing LH by biossay *in vivo* (1). The LHRF was still dissolved in the 0.1 *M* (pH 5.5) ammonium acetate (AmAc) buffer which was used to elute fractions from the Sephadex column.

In vitro incubations. Anterior pituitary glands from male Holtzman rats weighing 250–300 gm were used. The rats were killed by stunning and decapitation. The posterior lobe of the extirpated pituitary was removed and discarded and the anterior lobe was cut in half along the midline. One half of each gland was transferred to a 25-ml Erlenmeyer control flask containing 2.0 ml of medium 199 (Difco Laboratories) at pH 7.2 and the opposite half was placed in an experimental flask containing 2.0 ml of medium 199 plus actinomycin D or puromycin in concentrations of 10–80 μg/ml. Each flask contained 12 anterior pituitary halves.

Incubation was carried out in a Dubnoff metabolic shaker at 37°C under an atmosphere of 95% O_2, 5% CO_2. After a preincubation period of 30 min the media were replaced with identical fresh aliquots and the incubation was continued for an additional 6-hour period. The 20 μg/ml concentrations of puromycin and actinomycin D were found to be effective in blocking protein synthesis and RNA synthesis, respectively, by pituitaries incubated *in vitro* under these conditions (14).

In experiments on LH release in response to added LHRF, purified LHRF was added after preincubation where appropriate. A volume of 0.2 ml of extract was added into the control and experimental flasks which represented opposite halves of the same pituitaries. The experimental flasks contained the antibiotic to be evaluated. The ability of the LHRF to increase LH release in the absence of the antibiotics was verified concurrently

* Supported by a grant from the Ford Foundation and USPHS Grant No. AM 10073.

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TABLE I. Effects of Actinomycin D and Puromycin on the Release of LH by Anterior Pituitaries *in Vitro*.

Replicate	Dose of antibiotic (μg/ml)	LH release[a] (μg/mg of anterior pituitary/6 hours)			
		Control	Actinomycin D	Control	Puromycin
1	10	0.12 (0.08–0.17) $\lambda = 0.15$[b]	0.09 (0.06–0.14) $\lambda = 0.15$	0.07 (0.05–0.11) $\lambda = 0.13$	0.07 (0.04–0.12) $\lambda = 0.16$
2	80	0.10 (0.07–0.17) $\lambda = 0.16$	0.08 (0.05–0.12) $\lambda = 0.14$	0.10 (0.07–0.14) $\lambda = 0.13$	0.06 (0.04–0.10) $\lambda = 0.14$
3	80	0.39 (0.26–0.60) $\lambda = 0.17$	0.48 (0.29–0.99) $\lambda = 0.21$	0.12 (0.09–0.19) $\lambda = 0.13$	0.15 (0.08–0.24) $\lambda = 0.20$

[a] Values in parentheses are fiducial limits at $p = 0.95$.

[b] λ = Index of precision of the assay.

with each experiment by comparing a flask containing medium plus 0.2 ml of purified LHRF with one containing medium plus 0.2 ml of the AmAc buffer as a control.

At the conclusion of the incubation the media were decanted, centrifuged to remove any red blood cells and fragments of pituitary tissue and stored at −15°C. The bulked anterior pituitary halves from each flask were blotted and weighed.

Measurement of LH release. The media were assayed for LH using the ovarian ascorbic acid depletion (OAAD) method (15). A 3-point design was used throughout, employing doses of standard (NIH-LH-S9)[3] of 0.4 and 2.0 μg and dilutions of medium of $\times 5$ or $\times 10$ chosen in order to produce a response between those of the 2 doses of the standard. Immature female rats of the Holtzman strain, 27 days old and weighing 60–70 gm received a s.c. injection of 75 IU of pregnant mares' serum gonadotrophin (PMS) (Ayerst Laboratories)[4] followed 70 ± 2 hr later by a s.c. injection of 33 IU human chorionic gonadotrophin (HCG).[4] The assay was performed 5–7 days after the injection of HCG. The rats were anesthetized with ether and the standard or unknown preparations were injected into the jugular vein. Each dose was given in a volume of 1.0 ml and injected into 6 rats. The rats were killed in ether 4 hours ± 5 min later; the left ovary was dissected free of extraneous tissue and was weighed to the nearest 0.1 mg on a torsion balance. The ascorbic acid content of each ovary was determined by the 2,6-dichlorophenolindophenol reaction and the results were expressed as μg of ascorbic acid/100 mg of ovary. The relative potency, fiducial limits of error at $p = 0.95$ and index of precision (λ) of each assay were calculated by established methods (16). The values for LH release were expressed as μg of NIH-LH equivalent/1.0 mg of anterior pituitary/6 hours. Each experiment was replicated at least 3 times.

Results. The results of 3 replicates of an experiment to examine the effects of actinomycin D and puromycin on the release of LH by anterior pituitaries in the absence of LHRF are shown in Table I. Neither antibiotic had any effect at the doses used on the release of LH by anterior pituitary glands. The mean amount of LH released in the presence of actinomycin D was 92.9% of control release and in the presence of puromycin was 95.2% of control release.

The results of 5 replicates of an experiment to examine the effects of actinomycin D and puromycin on the release of LH by anterior pituitaries stimulated by LHRF are shown in Table II. In each replicate (except no. 3 where the data are incomplete) LHRF produced a significant increase in LH release over control. Neither antibiotic had any effect at the doses used on the release of LH by anterior pituitary glands stimulated by

[3] This standard was a gift from the Endocrinology Study Section of the Public Health Service.

[4] We are grateful to the Ayerst Pharmaceutical Company and Dr. J. A. Jewell for supplying us with PMS and HCG.

TABLE II. Effects of Actinomycin D and Puromycin on the Release of LH by Anterior Pituitaries Stimulated *in Vitro* by LHRF.

Replicate	Dose of antibiotic (µg/ml)	LH release[a] (µg/mg of anterior pituitary/6 hours)					
		Control	LHRF	LHRF	LHRF plus actinomycin D	LHRF	LHRF plus puromycin
1	10	0.20 (0.13–0.31) λ = 0.17[b]	1.03 (0.75–1.42) λ = 0.11	0.77 (0.56–1.07) λ = 0.12	0.70 (0.53–0.91) λ = 0.11	0.82 (0.57–1.18) λ = 0.14	0.56 (0.41–0.78) λ = 0.15
2	10	0.10 (0.07–0.15) λ = 0.14	0.57 (0.40–0.82) λ = 0.14	0.33 (0.20–0.52) λ = 0.18	0.33 (0.21–0.51) λ = 0.17	0.33 (0.20–0.56) λ = 0.15	0.48 (0.34–0.69) λ = 0.15
3[c]	20	—	0.41 (0.33–0.51) λ = 0.09	0.35 (0.26–0.47) λ = 0.12	0.37 (0.29–0.46) λ = 0.09	0.31 (0.23–0.42) λ = 0.12	0.31 (0.24–0.41) λ = 0.11
4	40	0.26 (0.19–0.35) λ = 0.12	0.64 (0.47–0.86) λ = 0.12	0.47 (0.33–0.67) λ = 0.14	0.41 (0.30–0.57) λ = 0.13	0.46 (0.32–0.65) λ = 0.14	0.52 (0.37–0.75) λ = 0.14
5	80	0.13 (0.05–0.27) λ = 0.21	1.10 (0.55–2.07) λ = 0.18	0.71 (0.46–1.11) λ = 0.17	0.74 (0.46–1.35) λ = 0.19	0.76 (0.46–1.37) λ = 0.20	0.85 (0.54–1.41) λ = 0.18

[a] Values in parentheses are fiducial limits at $p = 0.95$. [b] λ = Index of precision of the assay. [c] Data incomplete due to failure of one assay.

LHRF. The mean amounts of LH released in the presence of actinomycin D and puromycin were 97.3 and 108.0% of the release in the presence of LHRF alone.

The possible effect of the antibiotics on the response to LH dissolved in the incubation medium was ascertained in a final control experiment. Neither actinomycin nor puromycin at a dose of 16 µg, which was equivalent to the highest dose injected in the incubations, affected the ovarian ascorbic acid depletion induced by a dose of 2 µg of the reference standard LH.

Discussion. Watanabe *et al.* (14) reported that actinomycin D and puromycin in doses of 10–100 µg/ml of medium prevented the enhancement of follicle stimulating hormone (FSH) release evoked by FSHRF when rat anterior pituitary glands were incubated *in vitro* in a system similar to the one described in the present paper. Neither antibiotic had any effect on the release of FSH in the absence of FSHRF. These FSH results and the present LH results were obtained concurrently in the same laboratory using the same antibiotic samples.

That sufficient doses of antibiotic were used in our experiments is indicated by the observation that at concentrations of 20 µg/ml puromycin gave a 93% inhibition of protein synthesis, whereas actinomycin gave a 73% inhibition of RNA synthesis (14). Our earlier results suggest strongly that *de novo* protein synthesis is required for the FSHRF to act on the pituitary cell. Either the FSHRF promotes the synthesis of FSH which is then released, or protein synthesis is required for stored FSH to be released from the cell under the influence of FSHRF. On the other hand from the present results LHRF appears to act fully even when protein synthesis has been inhibited and must have a direct effect on the release of preformed stores of LH from the LH-secreting cells.

Recently, data have been reported by Jutisz and his colleagues (17) of the addition of actinomycin D at a concentration of 10 µg/ml and puromycin at a concentration of 2×10^{-4} M (100 µg/ml) to rat anterior pituitaries in a 2-hour *in vitro* incu-

bation. While no effect of actinomycin D was observed, puromycin was reported to inhibit by 54% the LH release stimulated by purified LHRF. The present results are not in accord with this observation. While the conditions differed in a number of respects in the work cited and the present experiments, the disagreement may be related more to the lack of replication in the work of Jutísz and his colleagues. It is considered essential by the present authors that experiments of this nature are adequately replicated in view of the variation encountered in incubation and assay systems.

The difference in duration of the incubation can hardly be a factor in explaining the difference in results between our group and that of Justisz, since in preliminary experiments no inhibition in response to LHRF was observed when a shorter period of 1 hour was used for incubation (unpublished data).

One study on the effect of puromycin on LH release by rat anterior pituitaries *in vitro* has appeared since the present work was completed. Samli and Geschwind (18) found no effect of puromycin in a concentration of 4×10^{-4} *M* on the LH release from rat anterior pituitaries *in vitro* either in the presence or absence of crude extracts of rat hypothalami although the dose of puromycin used inhibited by 94% the incorporation of ^{14}C-labeled leucine into LH. The present results, obtained with purified LHRF are in agreement with those of Samli and Geschwind (18). They suggest a different mechanism of action of FSHRF and LHRF in that the former appears to promote FSH synthesis directly or require the synthesis of an essential protein before it can evoke release of FSH, whereas the latter, if responsible for LH synthesis, may produce it only indirectly by depleting pituitary stores of the hormone.

Summary. Anterior pituitary halves from adult male rats were incubated *in vitro* for 6 hours following a preincubation of 30 min in tissue culture medium 199. The LH release from the glands was determined by the ovarian ascorbic acid depletion assay. Neither puromycin nor actinomycin in doses of 10–80 μg/ml interfered with the "basal" release of LH from the pituitaries. Purified LH-releasing factor (LHRF) enhanced the release of LH, and this enhancement was not influenced by the presence of either of the antibiotics. The LHRF can thus act when protein synthesis is blocked and must facilitate release of preformed LH from the cell. The present results with LHRF are different from those previously obtained with FSHRF whose action in increasing FSH release was blocked by the antibiotics and therefore appeared to require protein synthesis.

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POSSIBLE INTERACTION OF LUTEINIZING HORMONE-RELEASING FACTOR WITH OTHER HYPOTHALAMIC RELEASING FACTORS AT THE LEVEL OF THE ADENOHYPOPHYSIS

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SUMMARY

Anterior pituitary halves from adult male rats were incubated *in vitro* for 6 hr. in tissue culture Medium 199. Luteinizing hormone (LH) released from these glands under the influence of purified preparations of growth hormone-releasing factor (GH-RF), growth hormone-inhibiting factor (GH-IF), corticotrophin-releasing factor (C-RF) and follicle-stimulating hormone-releasing factor (FSH-RF) was determined by the ovarian ascorbic acid depletion (OAAD) assay. The effects of these factors, both alone and together with purified luteinizing hormone-releasing factor (LH-RF), were examined and compared with the response to purified LH-RF alone. While LH-RF consistently produced significant increases in LH release, none of the other factors did so, although FSH-RF showed some indication of LH-releasing activity, probably due to incomplete separation from LH-RF on the Sephadex gel filtration column used for purification. The LH released in response to LH-RF was not affected by the presence of any of the other factors. An apparent slight augmenting effect of FSH-RF could be accounted for by its contamination with LH-RF. The results are discussed in relation to the physiological mechanisms concerned in modifying LH release from the adenohypophysis.

INTRODUCTION

The role of the hypothalamus in controlling the release of hormones from the adenohypophysis is well established. This control is believed to be mediated by the hypothalamic releasing factors which either stimulate or inhibit release of the appropriate adenohypophysial hormone, there being at least one factor for each hormone (Harris, Reed & Fawcett, 1966; McCann, Dhariwal & Porter, 1968). The existence of a luteinizing hormone-releasing factor (LH-RF) was reported by McCann, Taleisnik & Friedman (1960). LH-RF has been purified and separated from other releasing factors by gel filtration on Sephadex and ion exchange chromatography

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on carboxymethylcellulose (see review by McCann *et al.* 1968). Dhariwal, Grosvenor, Antunes-Rodrigues & McCann (1968) reported that difficulties had been encountered in separating LH-RF from prolactin-inhibiting factor (P-IF) by these means although Arimura, Saito, Müller, Bowers, Sawano & Schally (1967) have claimed to have effected this separation, obtaining porcine LH-RF devoid of P-IF activity.

It is important to establish whether changes in the rate of luteinizing hormone (LH) release are due to proportional fluctuations in the release of LH-RF. The elucidation of this problem has been hampered by the difficulty of detecting the levels of LH-RF in hypophysial portal blood, although one group of workers have claimed to have succeeded in this (Fink, Nallar & Worthington, 1966). It is not known whether LH-secreting cells in the adenohypophysis respond with LH release solely to the levels of LH-RF in the blood reaching them or whether LH release may be influenced by the presence of other hypothalamic factors in addition to LH-RF. For this reason it was decided to examine the effects of releasing factors other than LH-RF, both alone and in the presence of LH-RF on the release of LH by rat anterior pituitaries incubated *in vitro*.

MATERIALS AND METHODS

Preparation of purified releasing factors. The factors were prepared from ovine or rat hypothalami by preliminary extractions followed by gel filtration on Sephadex G-25 (Dhariwal, Nallar, Batt & McCann, 1965). Each factor used had been shown previously to be active in promoting or inhibiting release of its particular adenohypophysial hormone either *in vivo* or *in vitro*. All factors were dissolved in the 0·1 M-ammonium acetate buffer (pH 5·5) used to elute fractions from the Sephadex columns. The factors tested in these experiments in addition to LH-RF (ovine) were growth hormone-releasing factor (GH-RF) (rat), growth hormone-inhibiting factor (GH-IF) (ovine), corticotrophin-releasing factor (C-RF) (ovine) and follicle-stimulating hormone-releasing factor (FSH-RF) (ovine).

Incubations in vitro. Anterior pituitary glands from male Holtzman or Simonsen rats, weighing 230–300 g., were used. The rats were killed by stunning and decapitation. The posterior lobe of the pituitary gland was removed and discarded and the anterior lobe was cut in half along the mid-line. Each half of the gland was transferred to one of a pair of 25 ml. Erlenmeyer flasks containing 2·0 ml. of Medium 199 (Difco Laboratories), the pH of which was adjusted to 7·2 with 5·6 % sodium bicarbonate.

Incubation was carried out in a Dubnoff metabolic shaker at 37° in an atmosphere of 95 % O_2:5 % CO_2. Each flask contained 12 anterior pituitary halves. After pre-incubation for 30 min. the medium was replaced with the same volume of fresh medium. The appropriate purified releasing factors were added at this stage. Purified LH-RF was added in a volume of 0·2 ml. throughout. Other factors were added in volumes ranging from 0·2 to 0·5 ml. Control flasks received an equivalent volume of 0·1 M-ammonium acetate buffer. The pH of each flask was again adjusted to 7·2 with 5·6 % sodium bicarbonate. Incubation was carried out for 6 hr. The media were then decanted, centrifuged to remove any red blood cells and fragments of pituitary tissue and stored at −15°. The combined anterior pituitary halves from each flask were blotted and weighed.

Measurement of LH release. The incubation medium from each flask was assayed

for LH by the ovarian ascorbic acid depletion (OAAD) method of Parlow (1958, 1961) with minor modifications. Intact, immature female rats of the Holtzman strain, 27 days old and weighing 60–70 g., received an s.c. injection of 75 i.u. pregnant mare serum gonadotrophin (PMSG, Ayerst Laboratories) followed 68–72 hr. later by an s.c. injection of 33 i.u. human chorionic gonadotrophin (HCG, Ayerst Laboratories). The assay was performed 5–7 days after the injection of HCG. The standard or unknown preparations were injected in a volume of 1 ml. into a jugular vein under ether anaesthesia. The rats were killed with ether 4 hr. ± 5 min. after injection, the left ovary only was removed, dissected free of extraneous tissue and weighed. The ascorbic acid content of each ovary was determined using the 2,6-dichlorophenol-indophenol reaction and the results were expressed as μg. ascorbic acid/100 mg. ovary.

Design of assays. A three-point design, employing two doses of standard and one dose of unknown, was used throughout. Six rats were used for each dose. The LH standard used was NIH-LH-S9 (National Institutes of Health), the doses being 0·4 and 2·0 μg. Doses of medium of 0·1 or 0·2 ml. were chosen, depending upon the expected potency, to produce a response between those of the two doses of the standard.

Statistical analysis. The relative potency, fiducial limits of error at $P = 0{\cdot}95$ and the index of precision (λ) of each assay were calculated as recommended by Gaddum (1953). The results were expressed as μg. NIH-LH equivalent released/1·0 mg. anterior pituitary tissue/6 hr.

RESULTS

The effects of purified GH-RF, GH-IF, C-RF, and FSH-RF in the in-vitro incubation system are shown in Table 1. While purified LH-RF consistently provoked significant increases over ammonium acetate controls in the release of LH, purified preparations of the other factors when added alone failed to influence release, although in the case of GH-RF (2 out of 4 experiments) reductions in release which approached significance were observed and in the case of FSH-RF (2 out of 3 experiments) increases which approached significance were observed. When the mean release rates in the presence of each of the releasing factors tested were expressed as percentages of ammonium acetate controls, the values obtained were 229, 92, 93, 95 and 136 % for LH-RF, GH-RF, GH-IF, C-RF and FSH-RF, respectively.

When the other releasing factors were added in the presence of LH-RF they failed to influence the LH-RF-induced release in any experiment (Table 1). When mean release rates provoked by LH-RF in the presence of each of the other releasing factors tested were expressed as percentages of LH-RF controls, the values obtained were 92, 94, 100 and 113 % for GH-RF, GH-IF, C-RF and FSH-RF, respectively.

DISCUSSION

There is excellent evidence derived from assays of pituitary and plasma LH that a variety of physiological and experimental situations lead to alterations in the rate of LH secretion from the adenohypophysis of the rat. Many of these alterations have now been correlated with changes in the levels of LH-RF in the hypothalamus. While

Table 1. *Effect of added hypothalamic releasing factors on the release of LH by rat anterior pituitaries* in vitro

	LH release* (μg./mg. anterior pituitary/6 hr.)					
Expt	Control	LH-RF	Control	GH-RF	LH-RF	LH-RF + GH-RF
1	0·19 (0·10–0·31)	0·52 (0·29–0·91)	0·24 (0·11–0·51)	0·29 (0·17–0·45)	0·61 (0·38–1·02)	0·59 (0·36–0·97)
2	—	—	0·16 (0·14–0·18)	0·15 (0·12–0·18)	—	—
3	—	—	0·17 (0·13–0·23)	0·13 (0·11–0·16)	0·37 (0·31–0·45)	0·32 (0·26–0·41)
4	0·16 (0·13–0·21)	0·36 (0·32–0·44)	0·17 (0·14–0·19)	0·13 (0·10–0·17)	—	—
			Control	GH-IF	LH-RF	LH-RF + GH-IF
5	0·21 (0·09–0·44)	0·55 (0·30–1·01)	0·26 (0·21–0·52)	0·22 (0·16–0·30)	0·48 (0·24–0·88)	0·31 (0·16–0·52)
6	0·10 (0·07–0·15)	0·22 (0·20–0·24)	0·25 (0·15–0·41)	0·25 (0·15–0·39)	0·22 (0·20–0·25)	0·27 (0·18–0·41)
			Control	C-RF	LH-RF	LH-RF + C-RF
7	0·19 (0·10–0·31)	0·52 (0·29–0·91)	0·27 (0·18–0·39)	0·23 (0·12–0·40)	0·62 (0·34–1·23)	0·80 (0·49–1·44)
8	—	—	0·27 (0·20–0·37)	0·28 (0·18–0·44)	0·67 (0·45–1·16)	0·68 (0·46–1·18)
9	—	—	—	—	0·96 (0·62–1·79)	0·66 (0·45–1·14)
			Control	FSH-RF	LH-RF	LH-RF + FSH-RF
10	0·31 (0·19–0·51)	0·49 (0·33–0·71)	0·24 (0·17–0·36)	0·36 (0·25–0·51)	0·66 (0·46–0·95)	0·79 (0·59–1·13)
11	0·09 (0·02–0·17)	0·21 (0·09–0·38)	0·11 (0·08–0·19)	0·13 (0·05–0·23)	0·34 (0·16–0·63)	0·47 (0·19–1·20)
12	—	—	0·23 (0·16–0·33)	0·32 (0·24–0·44)	0·88 (0·60–1·47)	0·71 (0·48–1·24)

* Figures in parentheses are fiducial limits at $P = 0{\cdot}95$.

λ in the above assays ranged from 0·06 to 0·28. In 73 % of assays λ was less than 0·2. See text for abbreviations.

values for hypothalamic content alone are not easy to interpret, these changes must be examined together with evidence that LH-RF promotes LH release and ovulation *in vivo* and LH release *in vitro*. These findings have led to the conclusion that LH-RF is the final link in the chain of events ending in LH release from the adenohypophysis of the rat (see reviews by McCann & Ramirez, 1964; McCann *et al.* 1968).

Using the in-vitro system described above it was demonstrated that purified LH-RF promotes increased LH release by incubated rat anterior pituitaries (see also Crighton, Watanabe, Dhariwal & McCann, 1968). It has also been possible to construct a dose–response curve showing increasing amounts of LH release with the addition of increasing amounts of crude extracts of rat stalk-median eminences (D. B. Crighton & S. M. McCann, unpublished observations). While other workers have employed similar systems in the study of LH-RF (Schally & Bowers, 1964; Piacsek & Meites, 1966; Minaguchi & Meites, 1967) using both crude hypothalamic extracts and purified LH-RF, no studies have been reported previously in which the effects of other purified releasing factors on the response to purified LH-RF have been evaluated.

The present results show that none of the factors tested other than LH-RF itself had inherent significant LH-releasing activity *in vitro* or caused any change in the response of incubated anterior pituitaries to purified LH-RF. The slight increases in LH release observed in the presence of FSH-RF, both alone and when FSH-RF was added to LH-RF, although they failed to reach significance in any of the experiments, may represent slight contamination of the FSH-RF preparation with LH-RF. FSH-RF and LH-RF are eluted consecutively from columns of Sephadex G-25.

The level of LH-RF in the hypophysial portal blood at any time presumably is the resultant of numerous external and internal stimuli acting on the hypothalamic centres concerned with the synthesis and release of LH-RF. Although the present study was not exhaustive in that a number of known releasing factors were not tested, it appears probable that in a particular physiological situation in which LH-RF is released, the response of the adenohypophysis is not modulated by the interaction of other hypothalamic factors with LH-RF at the level of the adenohypophysial cells.

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Suprachiasmatic LH-Releasing Factor[1]

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Hypophysiotropic principles appear to have a discrete localization in the hypothalamus [MCCANN *et al.,* 1968]. Information has been obtained by two techniques, one of which is to implant the anterior pituitary in the hypothalamus and study the effect on pituitary cytology. Pituitaries were seen to maintain basophilic cells and produce some maintenance of sex organs when they were grafted into an area that included the arcuate nucleus as well as part of the anterior hypothalamic and suprachiasmatic nuclei [HALÁSZ *et al.,* 1962, 1965; KNIGGE, 1962]. Presumably, neurons in these areas secrete gonadotropin-releasing factors. Definitive evidence for this localization of LRF has come from studies in which regions of the hypothalamus have been extracted and assayed for such activity. Early work suggested that LRF could be extracted from a medial, basal hypothalamic zone that extended as far rostrally as the optic chiasm [MCCANN, 1962]. Recently, we have localized this activity more precisely by sectioning frozen rat hypothalami and assaying the extracts prepared from discrete areas by means of the *in vitro* assay for LRF [CRIGHTON *et al.,* 1969b]. Although it confirmed the earlier localization, our study could not determine the localization of the cell bodies of the neurons that presumably secrete LRF. Do they all have their cell bodies

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in the suprachiasmatic region with long axons that project to the median eminence, there to release LRF into the portal vessels, or is there a mixed population of cells, some being of the type just described and others with cell bodies located more caudally, a shorter distance from the median eminence? To answer this question the effect of lesions in the suprachiasmatic region on the content of LRF stored in the stalk-median eminence was evaluated.

Materials and Methods

Experimental Animals

Adult virgin female rats of the Sherman strain (body wt 200–220 g) were housed in an air-conditioned room with controlled lighting (14 h of light per day), and supplied with Purina laboratory chow and water *ad libitum*. Vaginal smear cytology was recorded 6 days a week. Animals were anesthetized with ether prior to all operative procedures. Bilateral electrolytic lesions were placed stereotaxically in the suprachiasmatic region on the day of estrus or the first day of diestrus of 4-day cycling rats that had been checked over 6 consecutive cycles. A cathodal current of 3 mA was passed for 15 sec, using a nichrome electrode that was insulated except at the tip. Similar lesions had been found to induce constant estrus in approximately 90% of rats in previous experiments [ANTUNES-RODRIGUES and MCCANN, 1967; TALEISNIK and MCCANN, 1960, 1961]. Some of the animals were ovariectomized 2 h prior to placement of lesions.

The rats were stunned and decapitated 6 to 8 weeks after the operative procedure. Animals with more than 90% estrous smears over a period of 4 weeks were considered to be in constant estrus. The median eminence and stalk (SME) from those brains in which visual control showed the lesion to be located in the desired area were dissected and the brains preserved in 10% formaldehyde. SME's were extracted with 0.1 ml of 0.1 N HCl/SME. After low speed centrifugation for 20 min, the pH of the supernatant was adjusted with 5.6% $NaHCO_3$ to 7.2, and the precipitate that formed was removed by centrifugation for 10 min.

In several experiments, brains from estrous females or adult males were frozen on dry ice. The SME's were removed and discarded, and a small, about 2 mm^3 ($1 \times 2 \times 1$) cube was then cut out of the medial preoptic area overlying the optic chiasm, with a scalpel. A crude extract from this part of the hypothalamus was prepared in the same way as from SME's. The equivalent of ¼ cube/AP was then assayed for possible LRF stores. The supernatant from all extracts was tested for LRF activity in an *in vitro* system by adding the equivalent of ¼ SME per anterior pituitary to the medium. Incubation was performed exactly as previously described [CRIGHTON *et al.*, 1968]. Male Sprague-Dawley rats (body wt 240–300 g) served as donors of anterior pituitaries for the incubation.

LH Assay

The incubation media were assayed for LH released from the anterior pituitary by the ovarian ascorbic acid depletion (OAAD) method of PARLOW [1961] with minor modifications [CRIGHTON *et al.*, 1968].

Design of Assay

A 3-point design with 2 doses of standard and 1 dose of unknown was used throughout. The LH standard (NIH-LH-S_9) was injected in doses of 0.4 and 2.0 μg. The medium was diluted by a factor of 5 to 10 and injected at a dose that produced a response in between the 2 doses of the standard. Each dose was injected into 6 animals.

Statistical Analysis

The relative potency, fiducial limits of error at $P = 0.95$, and the index of precision (λ) of each assay were calculated as recommended by GADDUM [1953]. The results were expressed as μg NIH-LH equivalent released by 10 mg pituitary tissue per 6 h or as a percentage of the control LH release, the control being set at 100%. Part of the calculation was done with a modified Gaddum Program with the true standard deviation, using a 1620 IBM computer.

Histology

Selected brains were serially sectioned in the frontal plane at 10 μ and every third section was stained with thionin [CHOWERS and MCCANN, 1965].

Results

Hypothalamic LRF Content in Normal Female Rats and Those with Suprachiasmatic Lesions

SME's from both normal females in estrus (E) and from rats showing hypothalamic constant estrus (CE) as a result of hypothalamic lesions possessed LRF activity, as indicated by a highly significant increase in LH release by pituitary halves incubated in the presence of SME in comparison with the paired control halves incubated with diluent alone (table I). The LH release obtained from SME's of rats with lesions appeared to be less than that obtained with SME's from the normal females. Consequently, the activity of SME's from the two types of rats was compared directly in two experiments. In both instances, the LRF activity from SME's of CE rats was less than that of normal animals ($P<0.01$).

Hypothalamic LRF Content in Ovariectomized Rats with and without Suprachiasmatic Lesions

Since the lowered LRF stores in the CE rats might have resulted from constant levels of estrogen secreted by their ovaries, it was

Table I. LRF content of hypothalami in normal female rats and those with suprachiasmatic lesions

Experiment no.	Type of hypothalamus	LRF activity of ¼ SME/AP (μg LH released/ 10 mg AP/6 h)[a]	LH release (% of control)
2	Control	1.61 (1.20– 2.13)[b]	
	Intact, estrus	5.01 (3.76– 7.11)[c]	311.2
	Control	1.85 (1.41– 2.39)	
	Lesion, constant estrus	3.51 (2.80– 4.49)[c]	189.7
	Intact, estrus	5.34 (4.19– 7.11)	
	Lesion, constant estrus	3.25 (2.58– 4.19)[c]	60.9
4	Intact, estrus	5.35 (4.59– 6.31)	
	Lesion, constant estrus	3.55 (3.17– 4.00)[c]	66.3
9	Control	1.60 (0.60– 3.40)	
	Intact, estrus	5.40 (3.40–11.70)[c]	337.5
	Control	1.10 (0.30– 2.20)	
	Lesion, constant estrus	3.30 (1.30–10.90)[d]	300.00

[a] lambda, index of precision, range = 0.09–0.30.
[b] Mean (95% confidence limits).
[c] $P<0.01$ versus control.
[d] $P<0.05$ versus control.

Table II. LRF content of hypothalami in ovariectomized rats with and without suprachiasmatic lesions

Experiment no.	Type of hypothalamus	LRF activity of ¼ SME/AP (μg LH released/ 10 mg AP/6 h)[a]	LH-release (% of control)
4	Control	1.09 (0.88–1.32)[b]	
	Ovariectomized	2.85 (2.43–3.35)[c]	261.5
	Control	1.15 (0.92–1.39)	
	Lesion, ovariectomized	1.95 (1.66–2.27)[c]	169.6
	Ovariectomized	2.98 (2.60–3.15)	
	Lesion, ovariectomized	2.05 (1.79–2.33)[c]	68.8
3	Control	1.21 (0.87–1.66)	
	Lesion, ovariectomized	1.86 (1.30–2.56)[d]	153.7
5	Control	2.30 (1.78–2.93)	
	Ovariectomized	4.80 (4.07–5.74)[c]	208.7
	Control	2.29 (1.80–2.86)	
	Lesion, ovariectomized	3.81 (3.08–4.77)[c]	166.4
	Ovariectomized	4.62 (3.79–5.76)	
	Lesion, ovariectomized	3.45 (2.89–4.18)[d]	74.7

[a] lambda, index of precision, range = 0.06–0.13.
[b] Mean (95% confidence limits).
[c] $P<0.01$ versus control.
[d] $P<0.05$ versus control.

Table III. LRF content of suprachiasmatic (SCH) cubes in normal rats

Experiment no.	Tissue	LRF-activity (µg LH released/ 10 mg AP/6 h)[a]	LH-release (% of control)
7	Control	2.10 (1.60–2.70)[b]	
	¼ SME, male	6.98 (5.81–8.60)[c]	332.4
5	Cortex	2.12 (1.74–2.55)	
	¼ SCH, cube, estrus	3.32 (2.71–4.09)[c]	156.6
6	Cortex	1.88 (1.54–2.28)	
	¼ SCH, cube, estrus	3.07 (2.54–3.77)[c]	163.3
	Cortex	2.02 (1.68–2.57)	
	¼ SCH, cube, estrus	3.25 (2.75–3.87)[c]	160.9
8	Cortex	1.99 (1.68–2.35)	
	¼ SCH, cube, male	3.12 (2.45–4.00)[c]	156.8
	Cortex	2.19 (1.77–2.69)	
	¼ SCH, cube, male	2.96 (2.44–3.61)[d]	135.2

[a] lambda, index of precision range = 0.07–0.11.
[b] Mean (95% confidence limits).
[c] $P<0.01$ versus control.
[d] $P<0.05$ versus control.

important to determine LRF stores in spayed rats with lesions. The concentrations of LRF in ovariectomized (O) and lesioned ovariectomized (LO) rats differed significantly from each other at the 1 or 5% level, when estimated in two experiments (table II).

LRF Content of Suprachiasmatic Cubes in Normal Rats

In all three experiments with suprachiasmatic (SCH) cubes obtained from E animals and both experiments with SCH cubes from males, LRF activity could be detected ($P<0.05$–0.01) (table III). Cerebral cortical extracts of the same animals served as controls. The relative potency of these cubes was less than ¼ of one SME from male donors.

Location of Lesions

Histologic study of serial sections through the lesions indicated that they involved the optic chiasm and overlying medial preoptic area and suprachiasmatic nuclei, but did not extend caudally into the ME.

Discussion

Hypothalamic LRF content in rats with SCH lesions was decreased in comparison with that of control animals, regardless of the presence or absence of the ovaries (fig. 1). It was important to determine the LRF content in ovariectomized animals in order to eliminate possible effects of constant estrogen secretion in animals with constant estrus, which might feedback to the hypothalamus and alter the LRF content [CHOWERS and McCANN, 1965; PIACSEK and MEITES, 1966]. Since the stored LRF was reduced in these animals with SCH lesions, even in the absence of the gonads, we conclude that some LRF-secreting neurons have their cell bodies in the SCH region, with axons that project to the ME. Recent studies of TEJASEN and EVERETT [1967] suggest that the axons from these LRF neurons probably project to the ipsilateral portion of the ME. After destruction of this area the LRF stored in the axons from these neurons dissipates with axonal degeneration, and this leads to a fall in the LRF content of the ME. This conclusion is buttressed by the finding of LRF in extracts from the SCH region in both male and female rats. The concentration of LRF in this region was less than in the SME, as is logical if LRF is synthesized in the cell bodies and then transported to axon terminals in the SME, which are the main storage site for the RF. This would be exactly analogous to the situation in the neurohypophysis, where only small amounts of hormone are extractable from the supraoptic and paraventricular nuclei and the main store of hormones is in the neural lobe [VAN DYKE *et al.,* 1955].

Since LRF activity did not disappear after SCH lesions, these experiments lead to the further conclusion that some of the LRF neurons were not injured and presumably have cell bodies caudal to the chiasm in the medial, basal tuberal region. But there is a weakness in this argument, for it is quite possible that the lesions led to incomplete destruction of the suprachiasmatic area. That some LRF neurons must lie caudally is an almost inescapable conclusion to be drawn from experiments with hypothalamic islands that have demonstrated some LH secretion even after separation of the medial basal tuberal region from the SCH region [HALÁSZ and GORSKI, 1967]. As in the case of SCH lesions, these knife cuts lead to constant vaginal estrus and failure to ovulate.

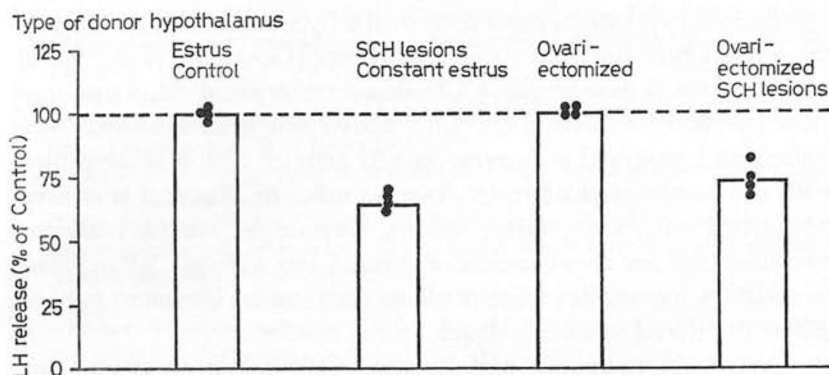

Fig. 1. LH-releasing activity *in vitro* of stalk-median eminence extracts from various types of donor rats. All values for hypothalamic extracts from experimental animals were compared with the activity of controls tested on paired pituitary halves at the same time and arbitrarily assigned a value of 100%.

Other lines of evidence also indicate that the SCH region is involved in the induction of ovulation. For example, EVERETT [1961] found that stimulative lesions in the septal or preoptic area could induce ovulation, while BARRACLOUGH [1966] has reviewed evidence that favors an inductive action of neonatal androgen in the SCH region in eliminating, in the male, the cyclic discharge of gonadotrophin that is characteristic of the female. It is tempting to speculate that the ovulatory stimulus in the female causes a liberation of LRF from LRF neurons whose cell bodies lie in the SCH region. The more caudally located LRF neurons might then be concerned with the 'so-called' tonic discharge of LRF that occurs during the rest of the estrous cycle and after gonadectomy [BARRACLOUGH, 1966].

It was interesting to note that LRF could be extracted from the SCH region, even in the male. This is in accord with previous evidence that electrical stimulation of this region can induce ovulation in males bearing ovarian grafts [QUINN, 1966; MOLL and ZEILMAKER, 1966]. Apparently, the rostral LRF system is present in the male, but the positive feedback effects of gonadal steroids that lead to the ovulatory surge of gonadotrophin discharge are lacking.

Since portions of the hypothalamus other than the SCH region and SME were not assayed in the present experiments, it could be argued that LRF has a wide distribution in the hypothalamus. This was ruled out in a study in which frozen sections of rat hypothalamus were assayed *in vitro* for LH-releasing activity. The activity

was limited to a medial basal zone of the hypothalamus that included the SCH region and stalk-median eminence [CRIGHTON *et al.,* 1969a].

Using the *in vivo* pituitary LH depletion method, MARTINI *et al.* [1968] recently estimated the LRF activity in male rats with SCH lesions and observed a decrease in this activity that is in agreement with our results in the female. As the studies of MARTINI *et al.* were performed only 5 days after lesions, they might not have allowed adequate time for degeneration of severed axons from LRF neurons; in addition, no studies were made in castrates to eliminate possible effects of altered steroid feedback on the results.

Earlier, MOSZKOVSKA and KORDON [1965] had concluded that preoptic lesions decreased the LRF content in female hypothalami, as indicated by an *in vivo* test involving implantation of hypothalami into hypophysectomized male rats with hypophyseal grafts. Their lesions appeared to lie more caudally than those of the present study and may have encroached on the median eminence. These authors also failed to employ gonadectomized animals.

Summary

Hypothalamic LH-releasing factor (LRF) concentrations were estimated by an assay that employs, as the end point, the LH released into the medium by anterior pituitaries incubated *in vitro*. The LRF concentration in the stalk-median eminence of rats with suprachiasmatic lesions that induced constant vaginal estrus was compared with that in intact estrous rats. There was a significant decrease of LRF activity in the rats with lesions, suggesting a localization of the LRF-secreting neurons as far rostrally as the optic chiasm. Since the animal showing hypothalamic constant estrus is constantly subjected to high levels of endogenously secreted estrogen, the different hypothalamic LRF concentrations might have been caused by endogenous estrogen feeding back to reduce LRF levels, rather than by a destruction of LRF-secreting neurons in the rats with lesions. Consequently, lesions similar to those that produce constant estrus in intact rats were produced in spayed rats. Again, hypothalamic LRF concentrations were significantly lower than in spayed rats without lesions. Finally, a small cube was dissected from the supraoptic area of frozen brains from intact estrous females or males and tested for LRF activity. The crude acid extract of the suprachiasmatic cubes caused a marked increase in release of LH. It is concluded that LRF-secreting neurons may be located in the suprachiasmatic area and be responsible for the discharge of ovulatory amounts of LH.

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Localization of LH-Releasing Factor in the Hypothalamus and Neurohypophysis as Determined by an *in Vitro* Method

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ABSTRACT. The LH-releasing action of male rat hypothalamic extracts was assessed by an *in vitro* method using male anterior pituitaries incubated in tissue culture medium. The end-point of the assay was the increase produced in the LH content of the medium after a 6 hr period of incubation with hypothalamic extract when compared with cerebral cortex extract. LH was assayed by the rat ovarian ascorbic acid depletion method. LH-releasing activity was absent from the neurohypophysis and vasopressin was inactive in promoting LH release. Frozen sections were cut in 3 planes through the hypothalamus to determine the localization of the LH-releasing factor. Activity was found in the medial, basal tuberal region, an area which included the median eminence and arcuate nucleus, and more rostrally in an area which included the supra-chiasmatic nucleus, and all but the most rostral portions of the preoptic nucleus. The relevance of these findings to the hypothalamic control of LH release is discussed. (*Endocrinology* **87:** 323, 1970)

IT IS WELL established that the hypothalamus exerts control over the secretion of LH by the anterior pituitary gland. This control is mediated by a hypothalamic luteinizing-hormone-releasing factor (LH-RF) which has been purified and separated from other releasing factors (1). In the present paper the topographic distribution of LH-RF in the hypothalamus and neurohypophysis of the rat has been determined by an *in vitro* method and compared to the hypothalamic localization of follicle stimulating hormone-releasing factor (FSH-RF) (2).

Materials and Methods

Experimental animals. Male rats of the Holtzman strain (Holtzman Co., Madison, Wis.) weighing 230-300 g were used as donors of hypothalami and pituitaries for incubation. Immature female rats of the Holtzman strain were used for LH assay. All rats were housed in an air-conditioned room with controlled lighting (14 hr of light and 10 hr of darkness) and supplied with laboratory chow and water *ad lib.*

Preparation of hypothalamic extracts. To prepare extracts of different parts of the hypothalamus, the animals were killed by stunning and decapitation, the brain was frozen on dry ice, and a block of tissue consisting of the hypothalamus, pituitary stalk and overlying brain including the corpus callosum was placed in a cryostat. Serial sections were cut at −20 C at 100 μ in 1 of 3 planes, sagittal, frontal or horizontal. The horizontal sections were cut parallel to the plane of the corpus callosum, whereas frontal sections were cut at right angles to this plane. To obtain sagittal sections, the hypothalamus was first transected sagittally in the midline and serial sections were then made from the midline to the lateral border of the hypothalamus. The sections were pooled to give tissue equivalent to a hypothalamic section 0.7-1.5 mm in thickness. In order to determine the exact location of each pooled section, a section 16 μ in thickness was cut just prior to beginning each pool and was stained with 0.1% toluidine blue for subsequent microscopic examination. The procedure was essentially that described by Watanabe and McCann (2) for the localization of FSH-RF. The pooled sections were extracted with 0.5 ml of 0.1N HCl/section and were assayed for LH-releasing activity *in vitro*. The sections from a given region were pooled and extract obtained from 3 hypothalami was added to each flask. This is considered to represent a dose of 1/2 hypothal-

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amic equivalent/pituitary since there were 12 pituitary halves/flask.

Incubation of pituitaries. Anterior pituitaries were hemisected and alternate halves were placed in alternate 25 ml Erlenmeyer flasks which contained 2 ml of medium 199 (Difco Laboratories). The medium contained 25 IU/ml of penicillin and 50 μg/ml of streptomycin as bacteriostatic agents. Each flask contained 12 pituitary halves. Usually the pituitary halves in one flask were subjected to a given treatment, the opposite halves of the same glands in another flask serving as controls. In a few instances, the pituitary halves in both flasks of a pair received different doses of the same extract.

Incubation was carried out in a Dubnoff metabolic shaker in an atmosphere of 95% O_2, 5% CO_2, at 60 cycles/min and 37 C and at a pH of 7.2. Following a 30 min preincubation of all flasks, the medium was decanted and replaced by an equal volume of fresh medium. The appropriate crude extracts were added at this stage and the medium was readjusted to pH 7.2 by dropwise addition of 5.6% $NaHCO_3$ solution. Incubation was continued for an additional 6 hr. At the conclusion of the experiment the pituitary halves from each flask were weighed and the individual media were centrifuged and stored at −20 C until assayed. The method is a slight modification of that of Piacsek and Meites (3).

Assay. The stored media were assayed for LH content by the ovarian ascorbic acid depletion method of Parlow (4) with minor modifications (5). Six rats were assigned to each dose of the standard preparation and unknown.

Three-point assays were performed with 2 doses (0.4 and 2.0 μg) of NIH-LH ovine S9 standard[4] and 1 dose of unknown. Doses of medium of 0.1 and 0.2 ml were chosen, depending upon the expected potency, to produce a response between those of the 2 doses of the standard. The results were calculated according to standard statistical procedures (6) and expressed as a percentage of the release in control flasks.

Results

Localization of LH-RF. Preliminary experiments with crude rat stalk-median eminence (SME) and cortical extract revealed that there was no change in LH release by the incubated pituitaries in the presence of cortical extract prepared from tissue equivalent in weight to 1/64 to 1 SME pituitary. Release was increased up to 3-fold by SME extract at doses of $\frac{1}{8}$ to 1 SME pituitary. The minimal effective dose (MED) was $\frac{1}{8}$ since 1/16 SME pituitary failed to induce a significant increase in LH release in either of two experiments.

The slope of the regression line between log-dose and response was significant ($p < .05$) in the range of dosage from 1/32 to $\frac{1}{2}$ SME pituitary (*i.e.*, doses of 1/32, 1/16, $\frac{1}{8}$, $\frac{1}{4}$ and $\frac{1}{2}$), but 95% fiducial limits of the effective responses overlapped the means of the responses to the other doses in the effective dosage range so that by this criterion a significant dose-response relationship was not demonstrable. A preparation of Sephadex-purified LH-RF prepared by A. P. S. Dhariwal (7) increased release nearly 4-fold, and the slope of the regression line was significant ($p < .05$); however, again 95% fiducial limits overlapped the means of the responses to higher effective doses.

Since $\frac{1}{8}$ SME/pituitary was required to obtain significant stimulation of LH release, all frozen sections used to determine the localization of LH-RF were tested at a dose of $\frac{1}{2}$ hypothalamic equivalent/gland. When horizontal sections were tested, only the basal zone was found to be active (Fig. 1; Table 1). With the frontal cuts, four sections were active in both experiments. These were the sections from the rostral edge of the optic chiasm to 4.4 mm caudal to it. When both experiments are considered together, these four sections showed approximately equal activity. The fifth section corresponding to the region 4.4 to 5.8 mm caudal to the rostral edge of the optic chiasm showed no activity in the first experiment and activity which just reached significance in the second. This may have resulted from slight displacement of the sections in the second experiment so that a small part of the more active section

[4] This standard was a gift from the Endocrinology Study Section of the USPHS.

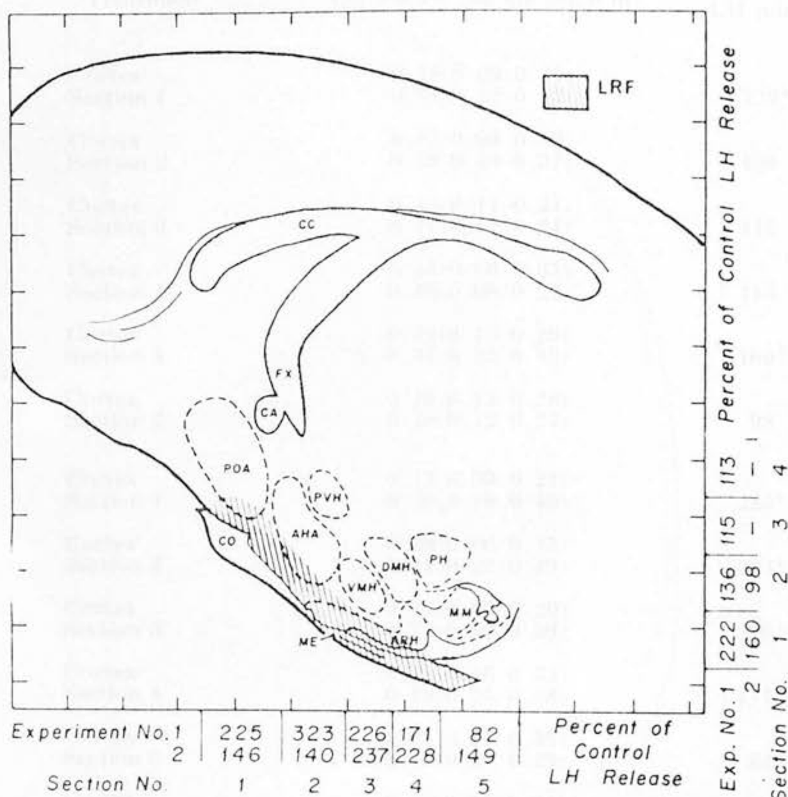

FIG. 1. Localization of LH-RF as determined from horizontal and frontal sections. The diagrams are modified from the atlas of de Groot. Key to abbreviations in Fig. 1 and 2: CC = corpus callosum; FX = fornix; CA = anterior commissure; POA = preoptic area; CO = optic chiasm; AHA = anterior hypothalamic area; CH = hippocampal commissure; VMH = ventromedial nucleus; PVH = paraventricular nucleus; ME = median eminence; ARH = arcuate nucleus; DMH = dorsomedial nucleus; PH = posterior hypothalamic nucleus; MM = medial mammillary nucleus; CI = internal capsule; OT = optic tract; MFB = medial forebrain bundle; LHA = lateral hypothalamic area. Figures are modified from the de Groot atlas (25).

4 could have been included with section 5. This supposition is supported by histological examination of the sections. Activity was found only in the medial zone obtained on cutting sagittal sections in the single experiment conducted (Fig. 2; Table 1). This medial localization was confirmed in three additional experiments in which this region produced a highly significant increase in LH release as compared to control. In these later experiments LH release was determined by radioimmunoassay (8). Thus, the distribution of LH-RF activity in the frontal sections is thought to include medial areas containing the suprachiasmatic nucleus, and all but the most rostral portions of the medial preoptic nucleus as well as more caudal areas extending as far as the stalk and premammillary areas.

Effect of neurohypophysis on LH release in vitro. Extract prepared from the neurohypophysis showed apparent significant LH-releasing activity when tested at a dose of ½ neurohypophysis pituitary. This activity disappeared when the extract was boiled for ten minutes, suggesting that the apparent activity in the first experiment may have been due to contamination with LH.

Lack of effect of vasopressin on LH release. A preparation of synthetic arginine vasopressin (Sandoz, Hanover, N.J.)[a] was inactive in significantly increasing LH release over saline controls when tested at doses of 200 or 1000 mU/ml. The 200 mU/ml dose increased LH release from 0.13 (0.01–0.17)[b] to 0.15 (0.11–0.19) μg/mg pituitary/6 hr, and the 1000 mU/ml dose increased it from 0.12 (0.09–0.16) to 0.16 (0.12–0.21).

[a] We are indebted to Dr. S. Gimple of Sandoz Laboratories for the supply of this hormone.
[b] Mean (95% confidence limits).

TABLE 1. Localization of LH-RF in hypothalamus and neurohypophysis (for key to numbers of sections, see Fig. 1 and 2)

Exp no.	Treatment	LH release (μg/mg pit/6 hr)	% Control LH release
A. Horizontal sections			
1	Cortex[1]	0.14(0.09–0.21)[2]	
	Section 1	0.31(0.22–0.42)	222*
	Cortex	0.13(0.09–0.19)	
	Section 2	0.18(0.13–0.37)	136
	Cortex	0.15(0.11–0.21)	
	Section 3	0.17(0.12–0.24)	115
	Cortex	0.14(0.06–0.35)	
	Section 4	0.16(0.08–0.27)	113
2	Cortex	0.19(0.13–0.29)	
	Section 1	0.31(0.22–0.45)	160*
	Cortex	0.19(0.12–0.28)	
	Section 2	0.18(0.12–0.27)	98
B. Frontal sections			
1	Cortex	0.13(0.09–0.21)	
	Section 1	0.30(0.19–0.49)	225*
	Cortex	0.09(0.06–0.15)	
	Section 2	0.31(0.22–0.43)	323*
	Cortex	0.12(0.07–0.20)	
	Section 3	0.27(0.19–0.39)	226*
	Cortex	0.22(0.16–0.32)	
	Section 4	0.38(0.25–0.58)	171*
	Cortex	0.21(0.11–0.37)	
	Section 5	0.17(0.09–0.29)	82
2	Cortex	0.22(0.16–0.31)	
	Section 1	0.32(0.22–0.43)	146*
	Cortex	0.26(0.19–0.35)	
	Section 2	0.36(0.30–0.52)	140*
	Cortex	0.20(0.14–0.29)	
	Section 3	0.49(0.33–0.71)	237*
	Cortex	0.15(0.10–0.22)	
	Section 4	0.34(0.25–0.46)	228*
	Cortex	0.21(0.14–0.32)	
	Section 5	0.32(0.22–0.45)	149*
C. Sagittal sections			
1	Cortex	0.18(0.12–0.28)	
	Section 1	0.28(0.17–0.48)	154**
	Cortex	0.13(0.08–0.20)	
	Section 2	0.17(0.11–0.26)	132
	Cortex	0.14(0.08–0.28)	
	Section 3	0.10(0.04–0.18)	71
D. Neurohypophysis			
1 Unboiled	Cortex	0.21(0.17–0.25)	
	Posterior lobe	0.30(0.25–0.36)	143*
2 Boiled	Cortex	0.23(0.19–0.29)	
	Posterior lobe	0.22(0.17–0.27)	96

[1] Equivalent amount of cerebral cortical extract, *i.e.*, equal wet weight of tissue in equal volume.
[2] Figures in parentheses are fiducial limits at p = 0.95. The average λ of these assays was 0.15.
* Significantly different from cortex-treated control, p < .05–.01.
** Borderline significantly different from cortex-treated control (p > .05 < .1).

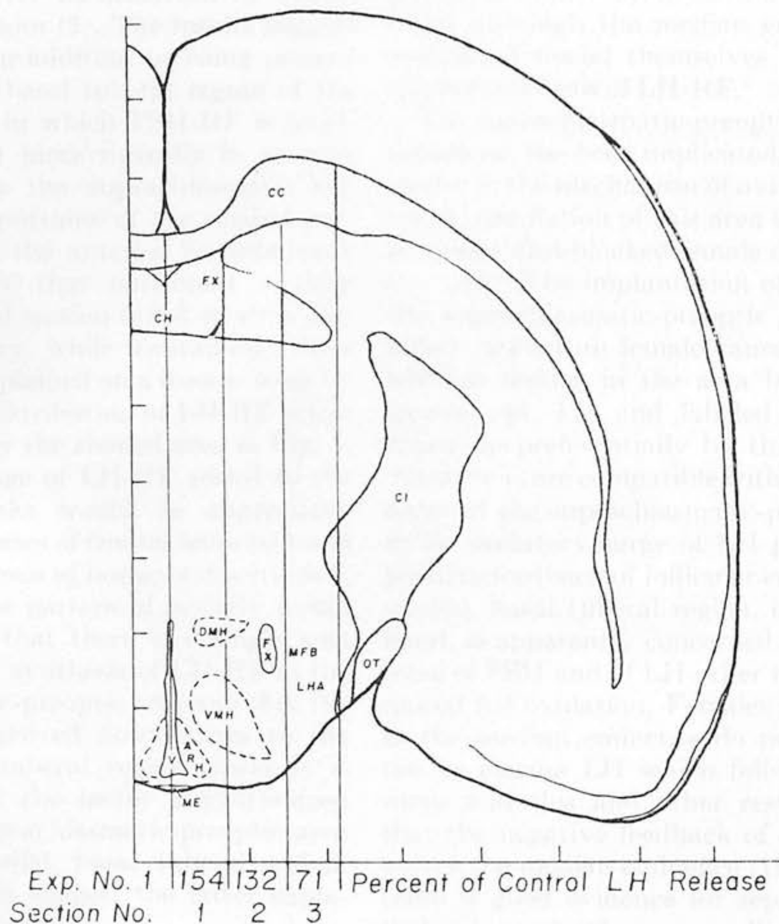

FIG. 2. Localization of LH-RF as determined from sagittal sections. For key to abbreviations see Fig. 1.

Discussion

The *in vitro* method was used to determine the distribution of LH-RF in the present experiments since it has been shown to be a specific and sensitive method for estimating LH-RF (3, 5, 9 12). No response has been obtained with cortical extracts in earlier work (3, 9) and in the present experiments. Furthermore, other releasing factors fail to alter the LH release from pituitaries *in vitro* and do not affect their response to purified LH-RF (10). In additio vasopressin has little or no capacity te mulate LH release as observed earlier (9). ince only a single dose of hypothalamic extract was used and standard LH-RF was not assayed in each case, the results should be considered as indicating the presence or absence of LH-RF and are not an attempt to quantitate the amount of the factor in each tissue section.

The *in vitro* assay employed here required $\frac{1}{8}$ SME pituitary or a total dose of $\frac{1}{4}$ hypothalamus for the six pituitary equivalents. Consequently, it was possible to determine the localization of the factor by sectioning only a single hypothalamus. Three hypothalami were sectioned in the present experiments so that a dose of hypothalamic extract greater than the MED for SME could be evaluated in order to detect the presumably smaller amounts of LH-RF which might be outside the SME.

The present experiments show clearly that LH-RF is much more widely distributed in the hypothalamus of the male rat

than the FSH-RF as described by Watanabe and McCann (2). The results suggest that LH-RF, in addition to being present in the medial, basal tuberal region of the hypothalamus in which FSH-RF is localized, is present more rostrally in an area which includes the suprachiasmatic nucleus, ventral portions of the medial preoptic area and the anterior hypothalamic area. The fact that horizontal sections above the basal section failed to show significant activity while containing these areas can be explained on a dosage basis by postulating a distribution of LH-RF activity as shown by the shaded area in Fig. 1. Thus, the dosage of LH-RF added to the incubation flasks would be appreciably greater in the cases of frontal sections 1 and 2 than in the cases of horizontal sections 2, 3 and 4. Such a pattern of activity would suggest either that there is a single area involved in the synthesis of LH-RF in the suprachiasmatic-preoptic area and that the factor is transported down axons to the medial, basal tuberal region where it is stored, or that the factor is synthesized both in the suprachiasmatic-preoptic area and in the medial, basal tuberal region. Various findings suggest the latter explanation to be correct.

Female rats with lesions in the suprachiasmatic-preoptic area show the condition of hypothalamic constant estrus (for refs. see 13), which is characterized by pronounced follicular development without ovulation. These animals are clearly capable of producing FSH and LH and of increasing LH synthesis and release after ovariectomy although they cannot produce the ovulatory surge of LH. It has been found (14) that LH-RF is present in the suprachiasmatic area of the female and that the placement of lesions in that area causing hypothalamic constant estrus results in a decrease in the LH-RF content of the SME area in both intact and ovariectomized rats although some LH-RF is still detectable in the SME. These facts suggest that the more rostral regions make an appreciable contribution to the level in the SME although the median eminence and associated nuclei themselves constitute a site for synthesis of LH-RF.

The suprachiasmatic-preoptic area in the female rat has been implicated by other evidence in the mechanism of ovulation. Electrical stimulation of this area in the intact, pentobarbital-blocked female causes ovulation (15). The implantation of estrogen in the suprachiasmatic-preoptic area in the intact, immature female causes ovulation, whereas lesions in the area block the response (16, 17) and labeled estradiol is taken up preferentially by this area (18). These facts are compatible with the involvement of the suprachiasmatic-preoptic area in an ovulatory surge of LH provoked by positive feedback of follicular estrogen. The medial, basal tuberal region, on the other hand, is apparently concerned with the release of FSH and of LH other than that required for ovulation. Females with lesions in the median eminence do not show the rise in plasma LH which follows ovariectomy and this and other results suggest that the negative feedback of estrogen involves the median eminence (19–21). Thus, there is good evidence for separate hypothalamic mechanisms controlling FSH and LH secretion and for two levels of hypothalamic control of LH secretion in the female rat as suggested by Barraclough and Gorski (22) and by Flerko (23). From our work on the localization of LH-RF as far rostrally as the suprachiasmatic region, it appears likely that the ovulatory surge of LH is brought about by the rostral LH-RF neurons whose axons presumably project to the median eminence, there to discharge the releasing factor which triggers the ovulatory discharge of LH from the adenohypophysis.

The present work on the hypothalamus of the male rat agrees with and extends the early *in vivo* results of McCann (24), who detected LH-RF activity mainly in the SME region but also in the ventral hypothalamus immediately overlying the me-

dian eminence and in an area designated the chiasmatic zone. The latter included the optic chiasm, the suprachiasmatic and supraoptic nuclei and other portions of the suprachiasmatic region. The present experiments, together with those of Watanabe and McCann (2) which showed a localization of the FSH-RF restricted to the stalk-median eminence region, constitute evidence to suggest that there may be separate FSH- and LH-controlling mechanisms in the hypothalamus.

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THE EFFECTS OF SYNTHETIC GONADOTROPHIN RELEASING FACTOR ON THE RELEASE OF LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE FROM OVINE PITUITARY TISSUE *IN VITRO*

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SUMMARY

A synthetic decapeptide gonadotrophin releasing factor was tested for effects on the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) using an ovine pituitary incubation system. The effects of other synthetic peptides used at similar doses were studied.

The synthetic decapeptide consistently provoked significant increases in the LH content of the incubation medium at doses equal to or in excess of 0·5 ng/flask (0·2 ng/ml medium). Significant increases in the FSH content of the incubation medium at doses equal to or in excess of 0·25 ng/flask (0·1 ng/ml medium) were observed. The other synthetic peptides failed to influence LH or FSH release *in vitro* even at a dose 20–40 times greater.

The results demonstrate that the decapeptide releases both LH and FSH from sheep pituitary tissue, suggesting that it may play a role in the release of both hormones *in vivo* in the sheep.

INTRODUCTION

The incubation of rat pituitary tissue has become established as a means of measuring neurohumoral activity in the hypothalamus (Watanabe & McCann, 1968; Crighton, Schneider & McCann, 1970; Jackson, Roche, Foster & Dziuk, 1971). Pelletier & Ortavant (1968) used pituitary tissue from rams *in vitro* in experiments on the effects of day length on spontaneous and stimulated luteinizing hormone (LH) release. Hartley, Crighton & Lamming (1973) described a technique involving the incubation of pituitary tissue from castrated male sheep which has been applied to the detection of changes in the LH releasing activity of extracts of sheep hypothalami obtained at various stages of the oestrous cycle (Crighton, Hartley & Lamming, 1973). The availability of synthetic gonadotrophin releasing factor (Matsuo, Baba, Nair, Arimura & Schally, 1971; Geiger, König, Wissman, Geisen &

Enzmann, 1971) has since provided a valuable tool for the study of the control of gonadotrophin release in the sheep.

The ability of the synthetic decapeptide to release both LH and follicle-stimulating hormone (FSH) from sheep pituitary tissue *in vitro* has now been studied.

MATERIALS AND METHODS

Incubations

The method was that described by Hartley *et al.* (1973). Synthetic peptides dissolved in 0·5 ml isotonic saline were added to 'treated' flasks and the LH or FSH content of the media compared with that in flasks to which 0·5 ml isotonic saline alone had been added.

Assays

Incubation media were assayed for LH using the ovarian ascorbic acid depletion method (Parlow, 1958) and for FSH using the augmentation method (Steelman & Pohley, 1953). Both assays incorporated minor modifications only. The design in each case was that of a three point assay with two doses of standard (NIH-LH-S16 or NIH-FSH-S7; National Institutes of Health, U.S.A.) as appropriate and one dose of medium designed to produce a response between the responses to the two doses of standard. The results were calculated as recommended by Sakiz & Guillemin (1963) using a computer programme devised by Dr E. Sakiz. The hormone contents of the media were expressed as μg NIH-LH or NIH-FSH equivalents/mg anterior pituitary tissue incubated.

Materials tested

Three synthetic peptides were tested: (1) The decapeptide described as gonadotrophin releasing factor by Matsuo *et al.* (1971) and synthesized by Geiger *et al.* (1971) (Pyroglu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). (2) The tripeptide thyrotrophin releasing factor (TRF) (Pyroglu-His-Pro-NH_2). The structure of this material of ovine origin was originally determined by Burgus, Dunn, Desiderio, Ward, Vale & Guillemin (1970). (3) The tripeptide Pyroglu-Val-Ser-NH_2. These materials were kindly supplied by Mr Julian Best, Hoechst Pharmaceuticals.

The synthetic decapeptide was evaluated in dose–response experiments designed to establish the minimal effective dose of the preparation. The other materials were evaluated at doses similar to those employed for the decapeptide. The doses used in dose–response experiments on LH release for all three preparations were 0·01, 0·10, 1·00 and 10·00 ng/flask in 2·5 ml medium. Subsequently in the case of the decapeptide, a narrower log-dose interval was employed and doses of 0·125, 0·25, 0·50 and 1·00 ng/flask were used. In the case of FSH release the doses of the decapeptide used were 0·125, 0·25, 0·50 and 1·00 ng/flask in 2·5 ml medium. The other preparations were tested at a dose of 10·00 ng/flask only. In experiments on LH release, four doses of material were tested concurrently in 'treated' flasks. In the case of FSH release the relative lack of sensitivity of the FSH assay precluded the testing of more than one dose at a time, this dose being added to all four 'treated' flasks in the experiment. The medium from these four flasks was bulked and subjected to FSH assay as was the case for four 'control' flasks to which saline was added.

RESULTS

Effects on LH release

In the first experiment with the synthetic decapeptide (Table 1) doses of 1·00 and 10·00 ng/flask produced significant increases in the LH content of the medium, while the lower doses used were ineffective. This established the existence of a minimal effective dose between 0·10 and 1·00 ng/flask. In the second experiment (Table 1) doses of 1·00 and 0·50 ng/flask produced significant increases in the LH content of the medium while the lower doses used were ineffective. This established a minimal effective dose between 0·25 and 0·50 ng/flask.

Table 1. *Effects of synthetic decapeptide on luteinizing hormone (LH) release in sheep* in vitro

Experiment no.	Treatment	LH content of medium (μg NIH-LH equiv./mg anterior pituitary tissue incubated)*	Significance of difference†
1	Decapeptide, 0·01 ng	0·49 (0·20–0·87)	NS
	Control	0·45 (0·18–0·77)	
	Decapeptide, 0·10 ng	0·49 (0·22–0·83)	NS
	Control	0·34 (0·15–0·59)	
	Decapeptide, 1·00 ng	3·75 (2·50–5·70)	S
	Control	0·65 (0·33–1·02)	
	Decapeptide, 10·00 ng	5·11 (3·44–8·29)	S
	Control	0·50 (0·21–0·85)	
		$\lambda = 0 \cdot 17$ (for all assays).	
2	Decapeptide, 0·125 ng	1·18 (0·86–1·60)	NS
	Control	0·75 (0·45–1·25)	
	Decapeptide, 0·25 ng	1·43 (1·02–2·02)	NS
	Control	1·08 (0·75–1·54)	
	Decapeptide, 0·50 ng	3·46 (2·41–4·97)	S
	Control	1·07 (0·60–1·68)	
	Decapeptide, 1·00 ng	3·64 (2·46–5·37)	S
	Control	1·49 (1·07–2·06)	
		$\lambda = 0 \cdot 14$ (for all assays).	

* Figures in parentheses are fiducial limits of error at $P = 0 \cdot 95$.
† NS = not significant; S = significant.

In two experiments with synthetic TRF, doses up to 10·0 ng/flask failed to produce a significant increase in the LH content of the medium.

In the case of the tripeptide Pyroglu-Val-Ser-NH_2 similar results to those observed with TRF were obtained. Doses up to 10·00 ng/flask failed to influence LH release in duplicate experiments.

Effects on FSH release

In the case of the decapeptide (Table 2) doses of 0·25, 0·50 and 1·00, but not 0·125 ng/flask significantly increased the FSH content of the medium. With synthetic TRF a dose of 10·00 ng/flask failed to influence FSH release in a single experiment. The synthetic tripeptide Pyroglu-Val-Ser-NH_2 at a dose of 10·00 ng/flask failed to influence FSH release in duplicate experiments.

Table 2. *Effects of synthetic decapeptide on follicle-stimulating hormone (FSH) release in sheep* in vitro

Treatment	FSH content of medium (μg NIH-FSH equiv./mg anterior pituitary tissue incubated)*	λ	Significance of difference†
Decapeptide, 0·125 ng	5·52 (2·63–11·91)	0·25	NS
Control	7·43 (3·98–20·25)		
Decapeptide, 0·25 ng	5·97 (3·38–13·54)	0·23	S
Control	2·86 (1·08–4·86)		
Decapeptide, 0·50 ng	11·23 (6·56–28·24)	0·23	S
Control	3·75 (1·18–6·41)		
Decapeptide, 1.00 ng	8·71 (6·12–14·64)	0·14	S
Control	4·29 (2·89–6·08)		

* Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.
† NS = not significant; S = significant.

DISCUSSION

This study was carried out as a preliminary step in examining the importance of the decapeptide molecule, claimed by Matsuo *et al.* (1971) to be a common LH and FSH releasing factor, in the release of both hormones *in vivo* in the sheep. It was known from previous work that the sheep pituitary incubation system, linked with biological assays for LH and FSH, responded with increased hormone release specifically to LH and FSH releasing activity present in extracts of hypothalamic tissue (Hartley *et al.* 1973). It had been shown that doses of the synthetic octapeptides oxytocin and vasopressin failed to influence LH or FSH release from incubated pituitary tissue in common with a number of other biologically active materials. TRF and Pyroglu-Val-Ser-NH_2 were used for the following reasons: TRF of ovine origin was the first hypothalamic releasing factor for which the structure was determined (Burgus *et al.* 1970) and apart from the postulated LH–FSH releasing factor was the only releasing factor available in synthetic form when this study was begun. The other tripeptide Pyroglu-Val-Ser-NH_2 is similar in structure to TRF.

Only the synthetic decapeptide stimulated LH or FSH release from incubated ovine pituitary tissue at the doses employed and stimulation of release of both hormones occurred (Tables 1 and 2). Increases in LH and FSH output similar to those obtained with extracts of ovine hypothalamic tissue (Hartley *et al.* 1973) were achieved.

Similar results have been obtained with a rat pituitary incubation system (Redding, Schally, Arimura & Matsuo, 1972; Sandow, Schally, Schröeder, Heptner & Enzmann, 1972), doses of synthetic decapeptide in the nanogram range being effective as in the present work. Redding *et al.* (1972) reported that the activity of the synthetic material was similar to that of pure porcine gonadotrophin releasing factor in that both LH and FSH release were affected.

The present results imply that the decapeptide may be a common gonadotrophin releasing factor for the sheep but do not preclude the existence of a separate factor concerned with FSH release.

I wish to thank Miss Anne Lister and Mr J. Corbett for skilled technical assistance. I am grateful to Mr J. Best of Hoechst Pharmaceuticals for providing the synthetic peptides and to Schering Chemicals Ltd. and the National Institutes of Health, U.S.A. for gifts of hormone preparations.

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Synthesis and Biological Activity of Luteinizing Hormone-Releasing Hormone and Related Peptides

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Syntheses of the decapeptide luteinizing hormone-releasing hormone, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 are described. The basic properties of arginine can provide a simple repetitive isolation procedure for arginine-containing peptides. The biological activities of the decapeptide, of a range of fragments and modified fragments, and of two analogs with alteration in the serine at position 4 were measured by in vitro incubation with sheep pituitary slices, measuring the liberated LH by bioassay. None of the compounds of shortened sequence were active, with the exception of <Glu-His-Trp which showed 1% of the activity of the decapeptide in one of four experiments. Neither [Ser(Bu^t)^4]-LH-RH nor [Leu^4]-LH-RH showed significant activity indicating (despite the known activity of [Ala^4]-LH-RH) the importance of this part of the structure for full biological activity.

Following the discovery of the decapeptide structure of luteinizing hormone-releasing hormone (LH-RH) of both porcine[1] and ovine[2] origin, a number of syntheses have been described[3-14] and the structure–activity relationships of the molecule are emerging from the study of synthetic analogs.[15-33] Replacement of single amino acid residues often leads to a dramatic reduction in biological activity, particularly with residues **1**, **3**, and **9** or in replacing glycine at position 6 with L-amino acids. Analogs with a D-amino acid such as D-alanine in position 6 show,[32] in contrast, a remarkably high level of biological activity. Lower but significant activity has resulted by replacing histidine with phenylalanine[19] in position 2, by replacing tyrosine with phenylalanine[23] in position 5, by replacing leucine with isoleucine and other amino-acids[19] in position 7, and by replacing arginine in position 8 with lysine,[19] ornithine,[19] or glutamine.[21] Replacement of serine in position 4 by alanine,[19,22,27] threonine,[19,28] or glutamine[19] gave significantly active analogs, and replacement of the C-terminal glycineamide residue by ethylamido and other groups[19,20,24,28,29] gave analogs with high activity. Smaller peptides or fragments of the decapeptide have generally been inactive[18,33] although the tripeptide amide <Glu-His-Trp-NH_2 was reported as having significant activity,[25] a claim subsequently retracted.[30] There are also conflicting reports about the activity of the corresponding acid.[18,31]

The present work describes our syntheses of LH-RH and of [Ser(Bu^t)^4]- and [Leu^4]-LH-RH and the activity of these compounds and of a range of smaller fragments of LH-RH in releasing luteinizing hormone from ovine pituitary tissue in vitro.

Synthesis. Luteinizing hormone-releasing hormone was synthesized as shown in Charts I–III, using either unprotected serine or *tert*-butyl ether protection for the hydroxy group. In Chart I, the protected heptapeptide **11** corresponding to sequence 4–10 was synthesised by a stepwise active ester approach starting from glycineamide hydrochloride and protecting the arginine side chain with a nitro group. Serine and tyrosine were left unprotected. Benzyloxycarbonyl (Z) groups were used for α-amino protection and were removed by HBr in AcOH. At the heptapeptide stage, hydrogenation removed the nitro and Z groups and tryptophan was introduced using Z-Trp-ONp. Hydrogenation and coupling with <Glu-His-N_3 gave LH-RH. A scheme similar in part to this was adopted by Yanaihara et al.

The dipeptide **1** has been reported as having different melting points, which seem to be best explained by there being two crystalline forms melting at ca. 120°[34] and at ca. 145°,[12] respectively. In our work we obtained initially the form with mp 120°; this was difficult to recrystallize and tended to form a gel. Subsequently the compound crystallized in the higher melting form. The deprotected dipeptide salt **2** analyzed as the dihydrobromide, as did other hydrobromides in this series, possibly by formation of a weak salt with the C-terminal amide group. Countercurrent distribution was used to purify several protected intermediates of Chart I and was carried out either with relatively few transfers using separating funnels (tripeptide **3**, for example) or with more transfers using an automatic (steady state) machine (peptides **11** and **13**). The LH-RH (**15a**) was purified by ion-exchange chromatography on CM-Sephadex C-25 using pyridine–AcOH buffers, followed by partition chromatography on Sephadex LH20. The chromatographically pure decapeptide had the expected amino acid and elemental analyses and optical rotation.

A second approach to the synthesis of LH-RH is shown in Charts II and III. For several stages, use was made of the basic properties of arginine peptides to provide a simple separation of protected peptides from neutral coproducts of the coupling reaction.[35] The approach was based on the similar use of 4-picolyl esters[36-38] and of the basic properties of the histidine side chain[39] when this is present in the peptide. The coupling reaction is carried out with excess acylating agent until no amino component is detected and the product is separated from neutral and acidic coproducts by absorption into an acidic phase. It was found suf-

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Chart I[41,a]

Glu His Trp Ser Tyr Gly Leu Arg Pro Gly

Z–OTcp H–NH$_2$ → Z–NH$_2$ (1)

Z–OPcp (NO_2) H– → Z(NO_2)–NH$_2$ (2)

Z(NO_2)–NH$_2$ (3)

Z–ONp H–(NO_2)–NH$_2$ (4)

Z–(NO_2)–NH$_2$ (5)

Z–OTcp H–(NO_2)–NH$_2$ (6)

Z–(NO_2)–NH$_2$ (7)

Z–ONp H–(NO_2)–NH$_2$ (8)

Z–(NO_2)–NH$_2$ (9)

Z–OPcp H–(NO_2)–NH$_2$ (10)

Z–(NO_2)–NH$_2$ (11)

Z–ONp H–(H^+)–NH$_2$ (12)

Z–(H^+)–NH$_2$ (13)

<–N$_3$ H–(H^+)–NH$_2$ (14)

<–(H^+)–NH$_2$

LH–RH **15a**

^{a} OPcp = pentachlorophenyl; OTcp = 2,4,5-trichlorophenyl.

Chart II

Glu His Trp Ser Tyr Gly Leu Arg Pro Gly

Z–(NO_2)–NH$_2$ (9)

Z–ONp H(Bu^t) –(H^+)–NH$_2$ (18)

<–OMe Z(Bu^t)– (21) (H^+)–NH$_2$ (19)

<–OH H(Bu^t)– (22) (H^+)–NH$_2$ (20)

<–(Bu^t)–(H^+)–NH$_2$

[Ser(Bu^t)^4]–LH–RH **23a**

<–(H^+)–NH$_2$

LH–RH **15b**

Chart III

Glu His Trp Ser Tyr Gly Leu Arg Pro Gly

Z–ONp H(Bu^t)–(H^+)–NH$_2$ (20)

Z–(Bu^t)–(H^+)–NH$_2$ (24)

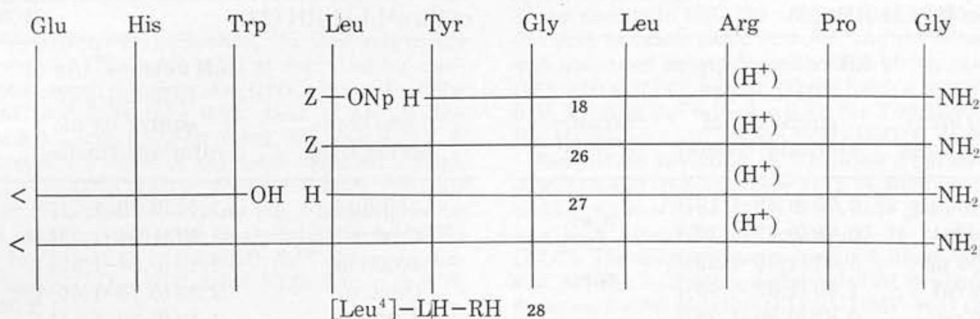
<–N$_3$ H–(Bu^t)–(H^+)–NH$_2$ (25)

<–(Bu^t)–(H^+)–NH$_2$

[Ser(Bu^t)^4]–LH–RH **23b**

ficient to filter the coupling reaction mixture in DMF–H_2O (3:1) through CM-Sephadex resin; the coproducts were washed out with this solvent and the arginine peptides were washed off the resin with pyridine–AcOH–DMF–H mixtures. The crude products frequently had satisfact chromatographic and analytical properties and could

Chart IV



used immediately for the next deprotection and coupling, thus providing a simple repetitive procedure. In some cases, coupling with unprotected arginine gives by-products which are not separated in this way, but use can be made in these cases of ion-exchange chromatography which is well established[40] for the chromatographic purification of arginine peptides. In order to avoid acetylation during coupling reactions, the acetates were converted to pivalates by filtering an aqueous solution through DEAE-Sephadex (pivalate form) and evaporating. The approach was tried by coupling Z-Tyr-ONp to Gly-Leu-Arg-Pro-Gly-NH_2 and then to prepare peptides **19**, **24**, and **23b** (Charts II and III) and **26** (Chart IV).

Coupling of the tripeptide <Glu-His-Trp-OH to **20** with *N,N'*-dicyclohexylcarbodiimide (DCCI) and *N*-hydroxysuccinimide (HOSu) gave [Ser(Bu^t)^4]-LH-RH in 26% yield. The alternative 2 + 8 route (Chart III) gave the same compound in 76% yield. Removal of the *tert*-butyl group with trifluoroacetic acid–H_2O gave LH-RH with only trace impurities on TLC, and a final purification by CM-Sephadex chromatography gave LH-RH with similar properties to the product from Chart I.

Chart IV shows the synthesis of [Leu^4]-LH-RH which was carried out by a route analogous to the synthesis of **23** (Chart II). The final product was purified by preparative TLC and CM-Sephadex chromatography.

Biological Activity. The LH-releasing activity of synthetic LH-RH and related peptides was assessed by incubation of ovine pituitary tissue and biological assay of the incubation medium for LH as described previously.[42–44] The results are expressed in terms of NIH-LH-S17 (National Institutes of Health, Bethesda, Md.). Responses to synthetic peptides were examined at four dose levels. In the case of synthetic LH-RH the doses were 0.01, 0.10, 1.00, and 10.00 ng per incubation flask. All other peptides were tested at doses of 0.10, 1.00, 10.00, and 100.00 ng per flask. The incubation volume in each flask was 2.5 ml in all cases. The data are given in Tables I–V.

Table I. Typical[a] Experiment Showing LH-Releasing Activity of Synthetic LH-RH in Vitro

Dose/incubation flask	LH release[b] (μg of NIH-LH-S17 equiv/mg of pituitary tissue)	Significance[c]
10.00 ng	3.28 (2.39–4.83)	s.
Control	1.41 (1.03–1.92)	
1.00 ng	2.74 (2.01–3.97)	s.
Control	1.39 (1.02–1.89)	
0.10 ng	2.20 (1.63–3.10)	s.
Control	1.28 (0.92–1.74)	
0.01 ng	1.94 (1.43–2.67)	s.
Control	1.37 (1.00–1.85)	

[a]In other experiments the 0.01-ng dose gave no significant response. [b]Figures in parentheses are fiducial limits of error at p = 0.95. λ = 0.13. [c]n.s. = not significant, s. = significant.

Table II. Typical Experiment on LH-Releasing Activity of Trp-Ser(Bu^t)-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (**25**)[b]

Dose/incubation flask	LH release[a] (μg of NIH-LH-S17 equiv/mg of pituitary tissue)	Significance[c]
100.00 ng	0.59 (0.31–0.94)	n.s.
Control	0.44 (0.20–0.72)	
10.00 ng	0.61 (0.32–0.97)	n.s.
Control	0.37 (0.16–0.62)	
1.00 ng	0.33 (0.13–0.57)	n.s.
Control	0.25 (0.10–0.45)	
0.10 ng	0.26 (0.10–0.46)	n.s.
Control	0.26 (0.09–0.47)	

[a]Figures in parentheses are fiducial limits of error at p = 0.95. λ = 0.17. [b]Similar negative results were obtained with peptides **16**, **18**, and **20**. [c]n.s. = not significant, s. = significant.

Table III. Two Experiments on LH-Releasing Activity of <Glu-His-Trp-OH (**22**)

	Dose/incubation flask	LH release[a] (μg of NIH-LH-S17 equiv/mg of pituitary tissue)	Significance[b]
Expt 1[c]	100.00 ng	0.96 (0.41–1.88)	n.s.
	Control	0.97 (0.31–1.93)	
	10.00 ng	0.71 (0.21–1.39)	n.s.
	Control	1.25 (0.57–2.55)	
	1.00 ng	0.70 (0.33–1.37)	n.s.
	Control	1.20 (0.46–2.35)	
	0.10 ng	1.67 (0.79–3.52)	n.s.
	Control	0.86 (0.27–1.68)	
		λ = 0.28	
Expt 2	100.00 ng	2.34 (1.64–3.28)	s.
	Control	1.28 (0.78–1.87)	
	10.00 ng	2.04 (1.38–2.90)	s.
	Control	1.04 (0.63–1.50)	
	1.00 ng	1.08 (0.67–1.55)	n.s.
	Control	0.96 (0.55–1.42)	
	0.10 ng	1.03 (0.59–1.55)	n.s.
	Control	0.85 (0.48–1.27)	
		λ = 0.13	

[a]Figures in parentheses are fiducial limits of error at p = 0.95. [b]n.s. = not significant, s. = significant. [c]Three further experiments gave similar results to expt 1.

Table IV. Two Experiments on LH-Releasing Activity of [Ser(Bu^t)^4]-LH-RH (23)

	Dose/incubation flask	LH release[a] (μg of NIH-LH-S17 equiv/mg of pituitary tissue)	Significance[b]
Expt 1[c]	100.00 ng	0.78 (0.45–1.19)	n.s.
	Control	0.78 (0.47–1.20)	
	10.00 ng	1.44 (1.06–1.99)	n.s.
	Control	1.40 (1.03–1.90)	
	1.00 ng	0.82 (0.50–1.25)	n.s.
	Control	0.91 (0.54–1.37)	
	0.10 ng	0.66 (0.37–1.00)	n.s.
	Control	0.61 (0.35–0.94)	
		λ = 0.18	
Expt 2	100.00 ng	1.25 (0.79–1.80)	s.
	Control	0.66 (0.33–1.03)	
	10.00 ng	0.82 (0.47–1.22)	n.s.
	Control	0.77 (0.44–1.14)	
	1.00 ng	0.81 (0.45–1.20)	n.s.
	Control	0.59 (0.27–0.95)	
	0.10 ng	0.60 (0.30–0.93)	n.s.
	Control	0.83 (0.42–1.30)	
		λ = 0.15	

[a]Figures in parentheses are fiducial limits of error at $p = 0.95$. [b]n.s. = not significant, s. = significant. [c]Two further experiments gave similar results to expt 1.

Table V. Typical Experiment on LH-Releasing Activity of [Leu^4]-LH-RH (28)

Dose/incubation flask	LH release[a] (μg of NIH-LH-S17 equiv/mg of pituitary tissue)	Significance[b]
100.00 ng	1.16 (0.88–1.51)	n.s.
Control	1.37 (1.04–1.76)	
10.00 ng	1.17 (0.86–1.53)	n.s.
Control	1.05 (0.75–1.40)	
1.00 ng	1.10 (0.80–1.44)	n.s.
Control	1.37 (1.00–1.82)	
0.10 ng	2.22 (1.42–3.53)	n.s
Control	1.48 (0.98–2.25)	
	λ = 0.11	

[a]Figures in parentheses are fiducial limits of error at $p = 0.95$. [b]n.s. = not significant, s. = significant.

The results of Table I show typical activity of LH-RH in the in vitro system. In the experiment shown all doses down to 0.01 ng/incubation flask (0.004 ng/ml) gave significant responses, although the minimum effective dose in other similar experiments was 0.10 ng/flask. Table II shows a typical result from a series of fragments or modified fragments, all of which were inactive at up to 100 ng/flask. This general lack of activity in fragments containing deletions in the N-terminal part of the LH-RH molecule confirms other work.[13,17,33] The tripeptide acid <Glu-His-Trp-OH (**22**) showed significant activity at two dose levels (100 and 10 ng/flask) in one out of four experiments (Table III). This is an intriguing result in view of other conflicting reports of activity or lack of activity in this tripeptide[18,31] and the corresponding amide.[25,30] The analog containing a Ser(Bu^t) group in place of Ser failed to show activity except at the highest dose level in one of four experiments (Table IV). This lack of activity indicates structural importance for the residue in position 4, but this is not associated with the hydroxyl group since [Ala^4]-LH-RH is reported to be active.[19,22,27] The lack of activity found in [Leu^4]-LH-RH (Table V) may indicate that steric constraints in position 4 are an important factor. The replacement of Ser by Ser(Bu^t) in position 6 of bradykinin also abolishes activity[45] although the glycine analog is fully active.

Experimental Section

Thin-layer chromatograms were run on silica gel GF_{254} (Merck). R_f values refer to the following systems: MeOH–$CHCl_3$ mixtures, R_f^{A1} 1:19, R_f^{A2} 1:9, R_f^{A3} 3:17, R_f^{A4} 1:4; *n*-BuOH–AcOH–H_2O mixtures, R_f^{B1} 10:1:3, R_f^{B2} 4:1:1, R_f^{B3} 3:1:1; R_f^{C} *n*-BuOH–AcOH–pyridine–H_2O (15:3:10:6); R_f^{D} cyclohexane–AcOEt–MeOH (1:1:1); $CHCl_3$–MeOH–AcOH mixtures, R_f^{E1} 10:2:1, R_f^{E2} 20:2:1; R_f^{F} *n*-BuOH–AcOH–H_2O–AcOEt (1:1:1:1); R_f^{G} *i*-PrOH–pyridine–AcOH–H_2O (10:5:4:4); $CHCl_3$–MeOH–AcOH–H_2O mixtures, R_f^{H1} 30:20:4:6, R_f^{H2} 30:20:2:3, R_f^{H3} 60:18:2:3, R_f^{H4} 45:30:4:6. Spots were detected by use of ninhydrin, chlorine and starch iodide, and uv illumination. Optical rotations were measured with a Perkin-Elmer 141 automatic polarimeter (1-dm cell). A Locarte high-voltage apparatus was used for electrophoresis (HVE) at 100–120 V cm^{-1} (Whatman 3MM paper). Melting points were determined with a Kofler hot-stage apparatus. Organic solutions were dried over sodium sulfate. Samples for amino acid analysis were hydrolyzed at 110° for 16–18 hr in constant boiling HCl with the addition of phenol. Analyses were carried out on a Jeol JLC 6AH machine. Countercurrent distribution (>20 transfers) was carried out using a Q and Q 100 tube steady-state apparatus.

Synthesis of LH-RH (15a) (Chart I). Z-Pro-Gly-NH$_2$ (1). Z-Pro-OTcp (18 g, 0.04 *M*) and Et_3N (4.9 ml) were added to a stirred mixture of Gly-NH_2, HCl (3.9 g, 0.035 *M*), and DMF (40 ml). The mixture was stirred for 2 hr, diluted with AcOEt (300 ml) and EtOH (50 ml), washed (H_2O, saturated $NaHCO_3$, H_2O; each saturated with NaCl), dried, and evaporated. The residue with Et_2O gave white crystalline **1**: 5.7 g (53%); mp 118–120° (lit.[32] mp 120°); R_f^{A2} 0.42, R_f^{E1} 0.86; attempted recrystallization gave gel-like material. A subsequent preparation starting from Z-Pro-ONp gave 58% of compound **1** as white needles: mp 144–145° (from MeOH–Et_2O) (lit.[12] mp 145–146°); R_f^{E2} 0.51.

Z-Arg(NO$_2$)-Pro-Gly-NH$_2$ (3). A solution of HBr in AcOH (45% w/v, 45 ml) was added to a stirred solution of compound **1** (6.0 g, 5 m*M*) in AcOH (15 ml). After 1.5 hr ether (450 ml) was added and the crystalline **Pro-Gly-NH$_2$·2HBr (2)** washed with ether: 5.9 g (88%); $[\alpha]^{22}D$ −14.4° (*c* 2, H_2O); R_f^{C} 0.27, R_f^{H1} 0.46. Anal. ($C_7H_{13}N_3O_2 \cdot 2HBr \cdot 0.5H_2O$) C, H, N, Br. A mixture of compound **2** (5.9 g, 17 m*M*), Et_3N (7 ml), and Z-Arg(NO_2)-OPcp (17.4 g, 29 m*M*) in DMF (35 ml) was stirred for 20 hr. The mixture was filtered, evaporated, triturated with Et_2O, and partitioned between the upper (1 vol) and lower (3 vol) layers of a mixture of AcOEt–AcOH–H_2O (3:1:5) for 20 transfers. Fractions containing product (TLC) were combined, evaporated, and repeatedly triturated with Et_2O giving tripeptide **3** as an amorphous powder: 7.2 g (82%); $[\alpha]^{25}D$ −22.7°; $[\alpha]^{25}_{546}$ −27.1° (*c* 1, DMF) [lit.[12] $[\alpha]^{23}D$ −25.4° (*c* 1, DMF)]; R_f^{B1} 0.36, R_f^{B3} 0.46, R_f^{C} 0.60, R_f^{E1} 0.30, R_f 0.63. Anal. ($C_{21}H_{30}N_8O_7$) C, H, N.

Z-Leu-Arg(NO$_2$)-Pro-Gly-NH$_2$ (5). Removal of the Z group from compound **3** (6.7 g, 13 m*M*) as described for compound **2** gave white solid **Arg(NO$_2$)-Pro-Gly-NH$_2$·2HBr (4):** $[\alpha]^{25}D$ −11.9° (*c* 1, DMF); R_f^{C} 0.26, R_f^{G} 0.49, R_f^{H1} 0.34. Anal. ($C_{13}H_{24}N_8O_5 \cdot 2HBr$) Br: calcd, 30.5; found, 29.9. A mixture of this dihydrobromide, Et_3N (5 ml), and Z-Leu-ONp (5.9 g, 15 m*M*) in DMF (30 ml) was stirred for 1 hr and kept at 0° for 16 hr. The mixture was filtered, evaporated, dissolved in $CHCl_3$ (800 ml) and EtOH (150 ml), washed (2 *N* HCl, H_2O, saturated $NaHCO_3$, H_2O; each saturated with NaCl), dried, and evaporated. Repeated trituration with Et_2O gave white solid tetrapeptide **5:** 5.6 g (68%); $[\alpha]^{26}D$ −41.1° (*c* 1, DMF) [lit.[13] $[\alpha]^{28}D$ −49.0° (*c* 1, MeOH)]; R_f^{B1} 0.42, R_f^{B3} 0.52, R_f 0.64, R_f^{E1} 0.39, R_f^{F} 0.68. Anal. ($C_{27}H_{41}N_9O_8$) C, H, N.

Z-Gly-Leu-Arg(NO$_2$)-Pro-Gly-NH$_2$ (7). Removal of the Z group from compound **5** (25 g, 0.04 *M*) as described for compound **2** gave white solid **Leu-Arg(NO$_2$)-Pro-Gly-NH$_2$·1.8HBr** (**6**): $[\alpha]^{20}D$ −16.2° (*c* 1, DMF); R_f^{C} 0.50. Anal. ($C_{19}H_{35}N_9O_6 \cdot 1.8HBr$) C, H, N, Br. This hydrobromide **6** was dissolved in DMF (100 ml) and Et_3N (12 ml) and Z-Gly-OTcp (18.7 g, 0.048 *M*) were added. The mixture was stirred for 2 hr and worked up as for compound **5** giving white solid **7:** 24.4 g (89%); $[\alpha]^{20}D$ −34.7° (*c* 1, DMF) [lit.

$[\alpha]^{26}D$ −51.0° (c 1, MeOH)]; R_f^{E1} 0.55, R_f^{H3} 0.61. Anal. ($C_{29}H_{44}N_{10}O_9$) C, H, N.

Z-Tyr-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (9). Removal of the Z group from compound 7 (22.6 g, 33 m*M*) as described for compound 2 gave white solid **Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2·2HBr (8):** R_f^C 0.51, R_f^G 0.76, R_f^{H1} 0.58. Anal. ($C_{21}H_{38}N_{10}O_7$·2HBr) C, N; H: calcd, 5.7; found, 5.2; Br: calcd, 22.7; found 23.6. A mixture of hydrobromide 8 (95% of the total prepared above), Et_3N (18 ml), and Z-Tyr-ONp (16.0 g, 37 m*M*) in DMF (100 ml) was stirred for 18 hr. The reaction mixture was worked up as for compound 5 giving hexapeptide 9 after precipitation with EtOH–Et_2O: 24.1 g (89%); $[\alpha]^{20}D$ −32.6° (c 1, EtOH); R_f^{B2} 0.45 with trace impurities. Anal. ($C_{38}H_{53}N_{12}O_{11}$) H; C: calcd, 53.45; found, 52.8; N: calcd, 19.7; found, 20.2.

Z-Ser-Tyr-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (11). Removal of the Z group from compound 9 (22 g, 26 m*M*) as described for compound 2 with the addition of phenol (5% w/v) gave the hydrobromide **10:** R_f^{H3} 0.32 with impurities. This hydrobromide was dissolved with stirring in DMF (100 ml), with the addition of excess Et_3N and Z-Ser-OPcp (13 g, 48 m*M*) and kept at 4° for 2 days. Work-up of the reaction mixture as for compound 5 gave a product showing one major spot with impurities. The crude product was purified by countercurrent distribution between the upper (18 ml) and lower (25 ml) layers of a mixture of AcOEt–EtOH–H_2O (10:2:5), 670 transfers, $K = 2.3$. Fractions containing product (TLC) were combined, evaporated, and precipitated with EtOH–Et_2O giving heptapeptide **11:** 11 g (45%); $[\alpha]^{20}D$ −44.0° (c 1, 10% AcOH); R_f^{B3} 0.55, R_f^C 0.69, R_f^G 0.90, R_f^{H3} 0.38. Anal. ($C_{41}H_{58}N_{13}O_{13}$ · H_2O) C, H, N.

Z-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 · AcOH (13). Compound 11 (7 g, 7.3 m*M*) was hydrogenated in 50% AcOH (100 ml) and 10 *N* HCl (1.4 ml) over 10% Pd/C (0.4 g) for 24 hr (room temperature, 1 atm). The mixture was filtered (Celite) and evaporated, EtOH (100 ml) was added and evaporated (twice), and the residue precipitated from EtOH with Et_2O to give **Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 · 2HCl (12)** as a white powder: 6.7 g; R_f^C 0.50; Tyr 0.97, Gly 2.05, Leu 0.98, Pro 1.04, Arg 0.96, Ser present. To a solution of compound 12 (5.8 g) and Et_3N (2.1 ml) in DMF (35 ml) was added Z-Trp-ONp (5.5 g, 12 m*M*). The mixture was kept at −15° for 66 hr, filtered, and evaporated. The crude product was purified by countercurrent distribution between upper (18 ml) and lower (25 ml) layers of a mixture of AcOEt–pyridine–AcOH–H_2O (20:1:1:8), 677 transfers, $K = 0.1$. Evaporation of the fraction containing product (TLC) and trituration with Et_2O gave white powdery octapeptide acetate **13:** 3.0 g (40% from compound 11); $[\alpha]^{20}D$ −44.0°; $[\alpha]^{20}{}_{546}$ −50.3° (c 1, 10% AcOH); R_f^{B3} 0.50, R_f^C 0.65, R_f^G 0.81, R_f^{H1} 0.87; Tyr 0.98, Leu 0.97, Gly 1.93, Pro 1.05, Arg 1.07, Ser, Trp present. Anal. ($C_{52}H_{69}N_{13}O_{12}$ · CH_3CO_2H · $3H_2O$) C, H, N.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LH-RH, 15a). Octapeptide 13 (2 g, 1.7 m*M*) was hydrogenated in 60% AcOH (24 ml) over 10% Pd/C (0.3 g) for 2 hr (room temperature, 1 atm). The mixture was filtered (Celite) and evaporated to give octapeptide acetate **14** as a gum: R_f^C 0.25, R_f^{H2} 0.24. A sample was dissolved in 10% AcOH, filtered, and lyophilized. Anal. ($C_{44}H_{63}N_{13}O_{10}$ · $3CH_3CO_2H$ · $7H_2O$) C, N; H: calcd, 7.2; found, 6.5. To a solution of the remaining compound 14 in DMF (12 ml) and Et_3N (slight excess) was added a solution of <Glu-His-N_3 prepared from the hydrazide (1.04 g, 3.7 m*M*) in DMF (21 ml) as described.[46] The mixture was stirred at −20° (1 hr), 0° (5.5 hr), kept at −15° overnight, and filtered, and the crude product precipitated by adding Et_2O. Cl^- ions were removed by filtering a solution in 1% AcOH (50 ml) through DEAE-Sephadex A25 resin (50 m*M*) and evaporating. The crude acetate was chromatographed (a) on a column (97 × 2.5 cm) of CM-Sephadex C-25 (pyridinium form) equilibrated with 1% AcOH and 1% pyridine (v/v) and eluted with a gradient to 5% AcOH and 5% pyridine (v/v) and then (b) by partition chromatography on a Sephadex LH 20 column (100 × 2.5 cm) with $CHCl_3$–AcOH–H_2O–EtOH (10:1:9:15). Fractions containing product were combined, evaporated, and triturated with Et_2O, and an aqueous solution was lyophilized, giving LH-RH-acetate (**15a**): 1.14 g (52%); $[\alpha]^{20}{}_{578}$ −49.8° (c 1, 10% AcOH); R_f^{B3} 0.1, R_f^C 0.43, R_f^G 0.76, R_f^{H1} 0.50; Tyr 1.00, Leu 0.97, Gly 1.99, Pro 1.03, Glu 1.00, His 1.00, Ser 0.84, Arg 1.01. Anal. ($C_{55}H_{75}N_{17}O_{13}$·$2CH_3CO_2H$ · H_2O) C, H, N.

Gly-Leu-Arg-Pro-Gly-NH_2-pivalate (16). Hydrogenation of compound 7 (1.2 g, 1.7 m*M*) in 70% AcOH (15 ml) over 10% Pd/C (0.1 g; room temperature, 1 atm) for 19 hr, followed by chromatography on a column (20 × 1.5 cm) of CM-Sephadex C-25 resin [eluting with HOAc–pyridine–H_2O from (v/v) 1:1:98 (pH 4.7) to 5:5:98 (pH 4.7)] gave pentapeptide acetate: one spot on TLC. A solution of the acetate in H_2O (20 ml) was filtered through DEAE-Sephadex A25 (pivalate form, 10 m*M*), and the filtrate was evaporated and triturated repeatedly with Et_2O giving pentapeptide pivalate (**16**): 0.89 g (77%); $[\alpha]^{25}D$ −37.5°: $[\alpha]^{25}{}_{546}$ −44.6° (c 1, DMF); R_f^{B3} 0.03, R_f^C 0.26, R_f^F 0.16; Arg 0.99, Pro 0.99, Gly 2.02, Leu 1.02. Anal. ($C_{21}H_{39}N_9O_5$ · $1.25(CH_3)_3CCO_2H$ · H_2O) C, H, N.

Simplified Isolation of Arginine Peptides Z-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2-acetate (17). A solution of pentapeptide 16 (0.53 g, 0.8 m*M*) and Z-Tyr-ONp (0.39 g, 0.9 m*M*) in DMF (4 ml) was kept overnight; no compound 16 could then be detected (TLC). The solution was evaporated, dissolved in DMF–H_2O (3:1 v/v; 10 ml), and filtered through CM-Sephadex C-25 resin [H^+ form; ca. 5 m*M*; equilibrated with DMF–H_2O (3:1)] in a 20-ml separating funnel plugged with cotton wool. The resin was washed (DMF–H_2O 3:1) and the filtrate and washings (containing HONp and Z-Tyr-ONp but not product, TLC) were rejected. Product was washed off the resin with pyridine–AcOH–DMF–H_2O (1:1:6:2); evaporation and trituration with ether gave hexapeptide acetate **17:** 0.69 g (97%); $[\alpha]^{25}D$ −39.7°; $[\alpha]^{25}{}_{546}$ −47.3°; R_f^{B3} 0.35, R_f^C 0.57, R_f^F 0.63; Tyr 1.00, Gly 2.02, Leu 1.02, Arg 0.97, Pro 1.00. Anal. ($C_{38}H_{54}N_{10}O_9$ · CH_3CO_2H · H_2O) C, H, N.

Synthesis of LH-RH (15b) (Chart II). Tyr-Gly-Leu-Arg-Pro-Gly-NH_2-pivalate (18). A solution of compound 9 (3.5 g, 4 m*M*) in 70% AcOH (60 ml) was hydrogenated over 10% Pd/C (0.6 g; room temperature, 1 atm) for 24 hr. The crude product was chromatographed on a CM-Sephadex C-25 column (85 × 2.5 cm) as described for compound 16 and on trituration with Et_2O gave 18 acetate (2.4 g): $[\alpha]^{25}D$ −40.5° (c 2, DMF); R_f^{B3} 0.30, R_f^C 0.45, R_f^G 0.70, R_f^{H1} 0.75. Treatment of the acetate (1.02 g) with DEAE-Sephadex (pivalate form) as for compound 16 gave hexapeptide pivalate (**18**): 0.91 g (63%); $[\alpha]^{25}D$ −45.0° (c 1, DMF); R_F^{B3} 0.30, R_f^C 0.45, R_f^G 0.70, R_f^F 0.47, R_f^{H1} 0.75. Anal. ($C_{30}H_{48}N_{10}O_7$·$1.5(CH_3)_3CCO_2H$ · H_2O) H, N; C: calcd, 54.1; found, 54.6.

Z-Ser(Bu^t)-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2-acetate (19). A solution of compound 18 (1.03 g, 1.2 m*M*), Et_3N (0.05 ml), and Z-Ser(Bu^t)-ONp (0.78 g, 1.9 m*M*) in DMF (7 ml) was kept at 0° for 16 hr. The mixture was worked up as for compound 17 [washing the product off the resin with pyridine–AcOH–DMF–H_2O (1:1:4:4)] giving heptapeptide **19:** 1.14 g (92%); $[\alpha]^{25}D$ −35.1°; $[\alpha]^{25}{}_{546}$ −42.3° (c 1, DMF); R_f^{B3} 0.45, R_f^C 0.60, R_f^F 0.67, R_f^G 0.87, R_f^{H1} 0.75 with only trace impurities. Anal. ($C_{45}H_{67}N_{11}O_{11}$ · CH_3CO_2H) C, H, N. Trace impurities were removed by chromatography on a column of CM-Sephadex C-25 as described for compound 16 (overall yield 65%).

<Glu-His-Trp-OMe (21). A solution of <Glu-His-N_3 in DMF (2.5 ml) prepared[46] from the hydrazide (0.18 g, 0.65 m*M*) was stirred at −40° and H-Trp-OMe · HCl (0.33 g, 1.3 m*M*) and excess Et_3N (0.2 ml) were added. The mixture was partitioned between upper (2 ml) and lower (2 ml) layers of pyridine–0.1% AcOH–$CHCl_3$–EtOH (3:5:4:4) for 19 transfers and fractions containing product were combined, evaporated, and dissolved in EtOH (10 ml). The solution, on standing, deposited crystalline **21:** yield (after recrystallization from EtOH–Et_2O) 81 mg (27%); mp 237–240° dec; $[\alpha]^{20}D$ −52.0°; $[\alpha]^{20}{}_{546}$ −72.0° (c 1, 10% AcOH); R_f^C 0.57, R_f^G 0.74, R_f^{H1} 0.69, R_f^{H3} 0.24. Anal. ($C_{23}H_{26}N_6O_5$) C, H, N. A preparation via Z-His-Trp-OMe gave a similar product.

<Glu-His-Trp-OH (22). A mixture of compound 21 (45 mg, 0.1 m*M*) and 2 *M* NaOH (2 ml) was stirred for 30 min, AcOH (1 ml) and H_2O (10 ml) were added, and the solution was filtered through SP-Sephadex C-25 resin (12 m*M*) and washed off with pyridine–H_2O (1:19) (no Cl^- ions detected). Evaporation and washing the resulting solid with EtOH gave **22:** 45 mg (94%); $[\alpha]^{20}{}_{578}$ −19.0°; $[\alpha]^{20}{}_{546}$ −21.6° (c 0.5, 50% EtOH); R_f^C 0.38, R_f^G 0.53, R_f^{H1} 0.48. Anal. ($C_{22}H_{24}N_6O_5$ · $2.5H_2O$) C, H; N: calcd, 16.9; found, 16.45.

Ser(Bu^t)-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2-pivalate (20). Hydrogenation of compound 19 (0.87 g, 0.87 m*M*) in 60% AcOH (15 ml) over 10% Pd/C (75 mg; room temperature, 4 hr) and conversion to the pivalate as described for compound 16 gave heptapeptide pivalate **20** as a white power: 0.63 g (67%); $[\alpha]^{25}D$ −34.1° (c 1.3, DMF); R_f^C 0.53, R_f^F 0.60 (streaking), R_f^G 0.84, R_f^{H1} 0.72; Arg 0.97, Ser 0.99 (corrected), Pro 0.98, Gly 2.05, Leu 1.01, Tyr 0.98. Anal. ($C_{37}H_{61}N_{11}O_9$ · $(CH_3)_3CCO_2H$ · H_2O) C, H, N.

<Glu-His-Trp-Ser(Bu^t)-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2·AcOH ([Ser(Bu^t)^4]-LH-RH, 23a). A solution of compound 20 (0.60 g, 0.65 m*M*), compound 22 (0.35 g, 0.70 m*M*), and triethylamine (10 mg) in DMF (3 ml) was cooled to −15°. DCCI (0.32 g, 1.55 m*M*) and HONSu (0.18 g, 1.55 m*M*) were added and the mixture was kept at −15° for 5 days. The mixture was filtered, evaporated, and triturated with Et_2O and the crude product chromato-

graphed on a CM-Sephadex C-25 column (31 × 1.5 cm) as described for compound **16** giving white, powdery decapeptide **23a:** 0.23 g (26%); $[\alpha]^{25}D$ −29.4° (*c* 0.8, DMF); R_f^C 0.34, R_f^{H1} 0.80, R_f^{H2} 0.40. Anal. ($C_{59}H_{83}N_{17}O_{13} \cdot 2CH_3CO_2H$) C, H, N.

LH-RH (15b). A solution of decapeptide **23a** (0.20 g, 0.15 m*M*) in TFA–H_2O (85:15, 6.5 ml) was kept at −10° under N_2 for 30 min and at room temperature for 3 hr. Addition of Et_2O (90 ml) precipitated a white solid which was washed with Et_2O, dissolved in H_2O, filtered through DEAE-Sephadex A-25 (AcO^- form, 5 m*M*), evaporated, and triturated with Et_2O to give crude **15:** 0.17 g (84%); R_f^C 0.25, R_f^G 0.78 with only trace impurities. Chromatography on a column (35 × 2 cm) of CM-Sephadex C-25 resin as for compound **16** gave white powdery LH-RH acetate **15b** (after trituration with Et_2O): 0.14 g (70%); $[\alpha]^{20}D$ −53.6°; $[\alpha]^{20}_{546}$ −60.0° (*c* 0.5, H_2O); R_f^C, R_f^G, R_f^{H1} identical with **15a;** Arg 0.99, Glu 1.04, Gly 1.94, His 0.99, Leu 1.00, Pro 1.04, Ser 0.83, Tyr 1.01, NH_3 1.33; HVE, R_{His} 0.60 (pH 5.3), Pauly and Sakaguchi reagents. Anal. ($C_{55}H_{75}N_{17}O_{13} \cdot 2.5CH_3CO_2H$) C, H, N.

Synthesis of [Ser(Bu^t)^4]-LH-RH (23b) (Chart III). Z-Trp-Ser(Bu^t)-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$-acetate (24). A solution of compound **20** (0.67 g, 0.7 m*M*) and Z-Trp-ONp (0.40 g, 0.9 m*M*) in DMF (3 ml) was kept at 0° overnight. Work-up as described for compound **19** gave **24:** 0.87 g (97%); TLC, one major spot with only trace impurities. Chromatography as described for compound **16** gave (after trituration with Et_2O) octapeptide **24:** 0.62 g (69%); $[\alpha]^{25}D$ −33.0°; $[\alpha]^{25}_{546}$ −39.5° (*c* 0.8, DMF); R_f^{B3} 0.43, R_f^C 0.68, R_f^F 0.73, R_f^{H3} 0.50, R_f^{H2} 0.60. Anal. ($C_{56}H_{77}N_{13}O_{12} \cdot CH_3CO_2H \cdot H_2O$) C, H, N.

Trp-Ser(Bu^t)-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$-acetate (25). Octapeptide **24** (0.60 g, 0.5 m*M*) was hydrogenated in 60% AcOH (15 ml) over 10% Pd/C (0.1 g; room temperature, 1 atm) for 4 hr, and the mixture was filtered, evaporated, and triturated with Et_2O giving acetate **25:** 0.56 g (98%); R_f^{B3} 0.26, R_f^C 0.59, R_f^{H2} 0.39; HVE, 1 spot (Sakaguchi), pH 6.5. Anal. ($C_{48}H_{71}N_{13}O_{10} \cdot 2CH_3CO_2H \cdot 2H_2O$) C, H, N.

[Ser(Bu^t)^4]-LH-RH (23b). To a stirred solution of compound **25** (0.51 g, 0.44 m*M*) with a trace of Et_3N in DMF (2.5 ml) at −25° was added a solution of <Glu-His-N_3 prepared[32] from the hydrazide (0.15 g, 0.55 m*M*) in DMF (2 ml). The mixture was kept at −15° for several days and worked up as for compound **19,** washing with pyridine–DMF–H_2O (1:5:5) to remove all coproducts. Trituration with Et_2O gave decapeptide **23b** as an off-white solid: 0.46 g (76%); R_f^C, R_f^{H2} identical with **23a;** HVE, 1 spot (Pauly, Sakaguchi), pH 6.5.

[Leu^4]-LH-RH (28) (Chart IV). Z-Leu-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$-acetate (26). A solution of compound **18** (0.06 g, 0.07 m*M*), Z-Leu-ONp (0.06 g, 0.15 m*M*), and $(CH_3)_3CCO_2H$ (0.1 g, 1 m*M*) in DMF (2.5 ml) was kept at 0° overnight. Work-up as for compound **19** gave heptapeptide acetate **26** (after trituration with Et_2O): 0.06 g (76%); R_f^C 0.65, R_f^F 0.57, R_f^{H1} 0.81; Arg 1.02, Leu 1.98, Tyr 0.99, Gly 2.01, Pro present. Anal. ($C_{44}H_{65}N_{11}O_{16} \cdot 2CH_3CO_2H \cdot H_2O$) C, H, N.

Leu-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$ · 2HCl (27). A solution of compound **26** (60 mg, 0.06 m*M*) in MeOH (3.5 ml) and 0.1 *M* HCl (1.8 ml) was hydrogenated over 10% Pd/C (10 mg) for 1.5 hr. The mixture was filtered (Celite), evaporated, dissolved in H_2O (4 ml), and lyophilized to give **27:** 52 mg (89%); R_f^{B3} 0.40, R_f^C 0.58, R_f^{H1} 0.65; Tyr 0.99, Gly 2.01, Pro 0.99, Arg present. Anal. ($C_{36}H_{59}N_{11}O_8 \cdot 2HCl \cdot 10H_2O$) H, N; C: calcd, 42.5; found, 43.0.

<Glu-His-Trp-Leu-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$ · AcOH ([Leu^4]-LH-RH, 28). To a solution of heptapeptide **27** (39 mg, 0.05 m*M*), tripeptide **22** (42 mg, 0.09 m*M*), and Et_3N (10 μl) in DMF at −15° (0.2 ml) was added HONSu (10.5 mg, 0.09 m*M*) and DCCI (18.8 mg, 0.09 m*M*). The mixture was kept at 0–5° overnight, diluted with buffer (pyridine–AcOH–H_2O 1:1:98), and chromatographed as for compound **16** on CM-Sephadex C-25 (50 × 1.3 cm column). The product (36 mg) with trace impurities on TLC was purified by preparative TLC (solvent H1) and the product (29 mg) rechromatographed on CM-Sephadex giving, after lyophilization, decapeptide acetate **28:** 20 mg (41%); $[\alpha]^{20}D$ −41.2°; $[\alpha]^{20}_{546}$ −48.8° (*c* 0.7, 10% AcOH); R_f^{B3} 0.38, R_f^C 0.51, R_f^{H4} 0.45; His 0.96, Glu 1.04, Pro 1.04, Gly 2.00, Leu 1.96, Tyr 1.00.

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EFFECTS OF CERTAIN ANALOGUES OF SYNTHETIC LUTEINIZING HORMONE RELEASING HORMONE ON THE RELEASE OF LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE IN THE ANOESTROUS EWE

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SUMMARY

The ability of eight nonapeptide analogues of synthetic luteinizing hormone releasing hormone (LH-RH) to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anoestrous ewe was studied and compared with the activity of synthetic LH-RH. In all the analogues, the glycineamide residue in position 10 was replaced with ethylamine; seven of them also had various substituents in positions 6 and/or 7. In a screening experiment using a single i.v. dose, the majority of the analogues were more potent than synthetic LH-RH in terms of the total amounts of LH and FSH released. The most potent overall was [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide which produced LH and FSH responses in terms of peak height, duration and area within the physiological range of the natural preovulatory peak. The dose–response relationships of this analogue and synthetic LH-RH were also compared. Deviations from parallelism were observed when all the aspects of the LH and FSH responses were considered, and with the FSH response deviations from linearity were also seen. These facts precluded the computation of a potency estimate using conventional statistical methods for the analysis of six-point biological assays, although it was clear that the analogue was substantially more potent than synthetic LH-RH. It was calculated that the administration of a single i.v. dose of between 6 and 30 μg of the analogue to anoestrous ewes would result in peaks in the concentrations of LH and FSH similar in all respects to those occurring naturally at oestrus, a situation previously found impossible to mimic with single injections of synthetic LH-RH.

INTRODUCTION

Since the characterization of porcine and ovine hypothalamic luteinizing hormone releasing hormone (LH-RH) (Matsuo, Baba, Nair, Arimura & Schally, 1971; Burgus, Butcher, Amoss, Ling, Monahan, Rivier, Fellows, Blackwell, Vale & Guillemin, 1972) and the synthesis of its decapeptide molecule (Geiger, König, Wissmann, Geisen & Enzmann, 1971), there have been many reports on the synthesis and biological activity of analogues of synthetic LH-RH (e.g. Fujino, Shinagawa, Yamazaki, Kobayashi, Obayashi, Fukuda, Nakayama, White & Rippel, 1973; Coy, Coy, Schally, Vilchez-Martinez, Hirotsu & Arimura, 1974). Coy *et al.* (1974) suggested that their [D-Ala^6] Des Gly-NH_2^{10} LH-RH ethylamide analogue could have important clinical and veterinary applications in situations

where prolonged releases of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are desirable.

We have shown previously (Foster & Crighton, 1974; Crighton, Foster, Haresign & Scott, 1975) that a single i.v. injection of synthetic LH-RH in anoestrous ewes resulted in a release of LH which was much smaller in height, duration and area than the natural preovulatory surge. In order to achieve an induced release of LH closer in form to the natural preovulatory surge the same total dose (150 μg) was used as a series of i.v. injections. In the present work, anoestrous ewes were given single i.v. injections of eight nonapeptide analogues of synthetic LH-RH based on the ethylamide substitution of Fujino *et al.* (1973) and with various other modifications in positions 6 and/or 7. The responses were assessed by determining the concentrations of LH and FSH in the plasma and comparing them with the concentrations observed after a single injection of synthetic LH-RH and with the releases of LH and FSH seen immediately before ovulation in the normal oestrous cycle.

MATERIALS AND METHODS

The amount of LH in samples of plasma was assessed by the specific homologous double antibody radioimmunoassay of Foster & Crighton (1974), and a heterologous double antibody radioimmunoassay was developed to estimate the concentration of FSH in plasma samples.

Preparation of ^{125}I-labelled FSH

The FSH preparation for labelling with ^{125}I was purified at the Department of Biochemistry, University of Cambridge from an abattoir collection of ovine pituitary tissue using a modification of the method described for the purification of human FSH by Shownkeen, Stockell Hartree, Stewart, Mashiter & Stevens (1976). The FSH activity and LH contamination were determined at Sutton Bonington by biological assay at each stage of purification (Steelman & Pohley, 1953; Parlow, 1958). The relative potency (with fiducial limits of error at $P = 0{\cdot}95$ in parentheses) by biological assay of the FSH preparation (sheep FSH, Seph. II, 17-6-74) was 41·37 (25·86–69·14) × NIH-FSH-S9 ($\lambda = 0{\cdot}22$). Contamination with LH was undetectable. The method of iodination was based on that described by Greenwood, Hunter & Glover (1963), except that 0·5 mM-sodium hypochlorite was used in place of chloramine T (Redshaw & Lynch, 1974). Four micrograms of the FSH preparation were added to a tube containing 1 mCi ^{125}I (Radiochemical Centre, Amersham, Bucks.), the procedure being otherwise identical to that of Redshaw & Lynch (1974); specific activities of 80–110 μCi/μg were regularly obtained. The labelled protein was stored at 4 °C. Before addition to an assay, a portion of the labelled material in 0·01 M-phosphate buffer was passed through a column of cellulose CF11 to remove any damaged protein. Egg albumin (3%) in 0·01 M-phosphate buffer was then added to the top of the column to elute the undamaged protein, which was then diluted and used as labelled antigen in the assay.

Antisera

Rabbits were immunized with multiple-site injections of ovine FSH, and antisera from 20 rabbits were tested with labelled ovine FSH (sheep FSH, Seph. II, 17-6-74) and in some cases labelled human FSH (human pituitary FSH: 110 mg NIH-FSH-S8/mg by biological assay; Dr Anne Stockell Hartree, University of Cambridge). Since these antisera proved to be non-specific for FSH, further antisera were then obtained from Dr W. Butt (Birmingham & Midland Hospital for Women). These had been raised in rabbits against human pituitary FSH (CPDS/6) by the multiple-site injection technique of Lynch & Shirley (1975).

Dose–response curves were prepared for each antiserum with NIH-FSH-S11 and preparations of other ovine adenohypophysial hormones (LH, NIH-LH-S18; prolactin, NIH-P-S8; growth hormone, NIH-GH-S7). No suitable preparation of thyroid-stimulating hormone (TSH) was available so antisera were tested for cross-reaction with TSH by assaying plasma samples believed to contain varying amounts of TSH but a constant concentration of FSH. Samples obtained from sheep every 10 min for 1 h before and every 5 min for 3 h after injection of 300 μg synthetic TSH releasing hormone (TRH) were used.

While the majority of antisera proved to be non-specific for FSH, negligible cross-reaction was seen with hormones other than FSH up to a concentration of 500 ng/tube (≡ 10 μg/ml) with antiserum M91. When this antiserum was used, no significant change in the level of FSH was observed when synthetic TRH was injected. It was, therefore, used in all further work. When 50 μl hypophysectomized ewe plasma (kindly supplied by Dr H. Buttle, N.I.R.D., Reading) were added to the standard curve, a negligible effect on parallelism was observed. It was noticed however, that the formation of the precipitates was clearer in the presence of sheep plasma and so 50 μl hypophysectomized ewe plasma were routinely added to each tube in the standard curve.

Sensitivity

Sensitivity was taken as the quantity of unlabelled hormone required to inhibit binding of the tracer by an amount equal to two standard deviations compared with binding in the absence of unlabelled hormone. In the early stages of development of the assay, a total incubation volume of 1·0 ml was used. It was, however, found that reduction of this to 250 μl led to an approximately fourfold increase in sensitivity and this volume was used routinely thereafter. Incubation times were varied in order to find which gave the highest percentage binding and sensitivity and these were finally set as described under Assay, giving a sensitivity of 10 ng NIH-FSH-S11/ml.

Precision

Intra-assay and interassay variations (coefficients of variation) were 6% ($n = 12$) and 6% ($n = 8$) respectively.

Comparison of biological and radioimmunological assays

Five ovine pituitary gonadotrophin preparations of widely varying FSH potency were assayed for FSH using both a biological assay (Steelman & Pohley, 1953) and the new radioimmunological assay. The mean index of discrimination (biological assay/radio-immunological assay) was 1·03 (Table 1).

Assay

All solutions were made up in a diluent of 0·01 M-phosphate buffer containing 0·9 g sodium chloride and 0·01 g sodium merthiolate/100 ml to which 0·25% egg albumin and 0·2% gelatin were added. Fifty microlitres of a solution of antiserum (giving a final dilution of 1:64000) containing 1:600 normal rabbit serum in diluent were added to each assay tube. A standard curve was established for concentrations of NIH-FSH-S11 ranging from 6·25 to 400 ng/ml. Four tubes, each containing 75 μl were set up for each concentration, and 50 μl hypophysectomized sheep plasma were added to each tube. Plasma samples were assayed in duplicate. After incubation at 4 °C for 48 h, 50 μl labelled FSH (diluted to give 10000 counts/4 min) were added, and the preparation was incubated for a further 72 h at 4 °C. Twenty-five microlitres of a 1:15 dilution of anti-rabbit gamma-globulin (Wellcome Reagents Ltd, Beckenham, Kent) were then added and the tubes incubated for a further 48 h at 4 °C.

Table 1. *FSH content of five ovine gonadotrophin preparations as measured by biological and radioimmunological assays*

Preparation	FSH content (mg NIH-FSH-S9/mg) Biological assay	Index of precision (λ)	Radio-immuno-logical assay	Index of discrimination (biological assay: radio-immuno-logical assay)
Sheep FSH; Seph. I (28-3-72)	1·58 (0·91–2·39)	0·16	1·93	0·82
Sheep FSH; SH 8·0820 Schering	2·47 (1·41–4·27)	0·24	2·03	1·22
Sheep FSH; Seph. II (28-3-72)	7·61 (5·41–11·94)	0·15	—	—
	6·62 (3·21–10·46)	0·23	—	—
Mean of two determinations	7·12		14·35	0·50
Sheep FSH; Seph. II (2-12-74)	12·67 (4·54–22·96)	0·23	—	—
	14·66 (5·93–23·55)	0·18	—	—
Mean of two determinations	13·67	—	10·65	1·28
Sheep FSH; Seph. II (17-6-74)	41·37 (25·86–69·14)	0·22	31·08	1·33
				1·03*

All values are single determinations unless otherwise stated.
Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.
* Mean index of discrimination

After addition of 1 ml diluent, each tube was centrifuged at 3000 g for 30 min, the supernatant fluids were aspirated and the radioactivity in the precipitates was counted in an ICN Gammaset 500 spectrometer.

Under these conditions, 20–35% of the counts added were bound by the antiserum. The concentration of FSH in unknown samples in terms of the standard was calculated by interpolating the mean percentage binding obtained on the standard curve.

Animals

Clun Forest ewes from the School of Agriculture flock were used during mid-seasonal anoestrus (June–July). All had shown reproductive cycles in the previous breeding season but had not been mated.

Analogues

The structures of synthetic LH-RH and the eight analogues administered to anoestrous ewes are shown in Table 2.

Table 2. *Structure of synthetic luteinizing hormone-releasing hormone (LH-RH) and the eight analogues administered to anoestrous ewes*

LH-RH	pyroGlu-His-Trp-Ser-Tyr-Gly - Leu-	Arg-Pro-Gly-NH_2
1	pyroGlu-His-Trp-Ser-Tyr-Gly - Leu-	Arg-Pro-NH-C_2H_5
2	pyroGlu-His-Trp-Ser-Tyr-D-Ser-Leu	Arg-Pro-NH-C_2H_5
3	pyroGlu-His-Trp-Ser-Tyr-D-Ser(Bu^t)*-Leu-	Arg-Pro-NH-C_2H_5
4	pyroGlu-His-Trp-Ser-Tyr-D-Ala-Leu-	Arg-Pro-NH-C_2H_5
5	pyroGlu-His-Trp-Ser-Tyr-Gly-Ser(Bu^t)-	Arg-Pro-NH-C_2H_5
6	pyroGlu-His-Trp-Ser-Tyr-Gly-Lys(Boc)†-	Arg-Pro-NH-C_2H_5
7	pyroGlu-His-Trp-Ser-Tyr-D-Ala-Lys(Boc)-	Arg-Pro-NH-C_2H_5
8	pyroGlu-His-Trp-Ser-Tyr-D-Leu-Ser(Bu^t)-	Arg-Pro-NH-C_2H_5

* (Bu^t): The hydroxyl group of D-serine was protected as its tertiary butyl ether.
† (Boc): The side-chain amino group of lysine was protected as the tertiary butoxycarbonyl derivative.

Blood samples

Blood samples were collected through indwelling jugular venous cannulae. Each sample was centrifuged at 3000 ***g*** for 10 min within 1 h of collection and the plasma removed and stored at −20 °C. Samples were later assayed for LH and FSH.

In the screening experiment, 18 ewes were divided into nine groups of two; two animals were used for each analogue and two for LH-RH. Blood samples were taken at 15 min intervals for 1 h before and 11 h after the i.v. administration of 30 μg of each preparation through the cannula. In the experiment to compare the dose–response curves for analogue 3, [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide (the hydroxyl group of D-serine protected as its tertiary butyl ether), and LH-RH, 17 ewes were divided into five groups of three and one of two. Sampling was as in the screening experiment. Three groups were injected i.v. with the analogue and three groups with LH-RH. The doses of analogue and LH-RH administered were 1·2, 6·0 and 30·0 μg, and 6·0, 30·0 and 150·0 μg respectively. In addition, two ewes were sampled at 15 min intervals for 26 h from the onset of oestrus to determine values for the natural preovulatory release of LH and FSH.

RESULTS

Screening experiment

The results of the screening experiment are shown in Table 3. The mean height of the LH peak produced in response to each of the analogues with the exception of no. 6,

Table 3. *Responses of plasma LH and FSH in a screening experiment involving the i.v. administration of synthetic luteinizing hormone releasing hormone (LH-RH) and eight of its analogues to anoestrous ewes*

	Peak height (ng hormone/ml)				Duration of peak (h)				Area under peak (arbitrary units)			
	LH		FSH		LH		FSH		LH		FSH	
LH-RH	71 60	66	108 194	151	3·50 4·50	4·00	4·00 3·50	3·75	1946 1536	1741	3 584 4 086	3835
1	66 122	94	153 97	125	5·00 6·00	5·50	4·75 1·75	3·25	2112 4146	3129	5812 1211	3 512
2	129 144	137	180 255	218	6·00 5·00	5·50	3·75 3·50	3·75	4811 3604	4208	4818 6714	5766
3	248 203	226	418 324	371	9·50 9·00	9·25	7·50 5·25	6·50	11 841 10311	11 076	20896 15 328	18112
4	210 56	133	474 61	268	8·00 6·00	7·00	9·50 5·50	7·50	9272 2363	5818	34402 3126	18764
5	142 72	107	93 45	69	5·50 4·50	5·00	2·25 1·00	1·75	3825 1287	2556	1628 160	894
6	64 —		60 —		5·00 —		2·75 —		2082 —		780 —	
7	63 195	129	50 318	184	3·75 5·75	4·75	3·00 4·25	3·75	2613 7554	5083	1070 10498	5784
8	162 195	178	182 251	217	4·50 6·50	5·50	2·50 5·75	4·25	4471 6599	5535	3534 8564	6049
Natural preovulatory release	183 166	175	324 91	208	8·25 10·00	9·25	8·00 8·50	8·25	12615 15797	14206	25108 7304	16206

See Table 2 for details of analogues used.
The value outside each parenthesis is the mean of the values inside the parenthesis.

[Lys(Boc)[7]] Des Gly-NH_2[10] LH-RH ethylamide (the side-chain amino group of lysine protected as the tertiary butoxycarbonyl derivative), for which only one value was available because no clear peak was distinguishable in the second animal, was greater than that produced in response to synthetic LH-RH. Judged by this parameter, the most potent analogue was no. 3 ([D-Ser(Bu[t])[6]] Des Gly-NH_2[10] LH-RH ethylamide: see Table 3).

The mean height of the FSH peak was greater after administration of five of the analogues than after synthetic LH-RH, and was lower after administration of analogues 1 and 5. For analogue 6, data were available from one animal only since in the other, the level of FSH failed to rise above the sensitivity of the assay throughout the sampling period. Judged by this parameter the most potent of the analogues was no. 3 (Table 3). As for LH, there was considerable variation in response within the pairs of animals injected with several of the analogues.

In terms of the mean duration of the LH response, all of the analogues appeared more potent than LH-RH itself. Because of the difficulty of measuring duration accurately, the evidence for this must be treated with some caution in the marginal cases, but analogue 3 was clearly the most potent in this respect (Table 3).

The situation regarding the duration of the response was less clear for FSH than for LH. Only two of the analogues (nos 3 and 4) produced unequivocally longer responses than LH-RH. Some animals injected with the other analogues showed very transitory FSH responses, in several cases substantially shorter than the corresponding LH response.

In terms of the total amount of LH released (the mean areas of the LH peaks) all of the analogues, with the exception of no. 6, in which it was impossible to judge for reasons already referred to, produced greater responses than synthetic LH-RH. The mean response to analogue 3 was clearly the greatest. Five of the analogues produced mean total FSH responses greater than synthetic LH-RH; these were nos 2, 3, 4, 7 and 8.

When these responses obtained in anoestrous ewes were compared with mean values obtained from two ewes for the natural preovulatory release of LH, it was apparent that while, as expected, single injections of synthetic LH-RH failed to mimic the natural release of LH in any of the parameters examined, LH peaks of a height similar to the natural preovulatory peak in the level of LH were produced in response to several of the analogues. Only analogue 3 produced a response of similar duration to that of the natural release of LH, and this analogue was also the only one to produce a mean total release of LH comparable to the natural release. Whereas single injections of synthetic LH-RH failed to mimic the natural preovulatory release of FSH in duration or area, FSH peaks of height similar to the natural FSH peak were produced by LH-RH and several of the analogues. Only analogues 3 and 4 produced responses with a duration and a mean total area similar to those of the natural preovulatory release of FSH.

When all three parameters were considered together, it was clear that analogue 3 ([D-Ser(Bu[t])[6]] Des Gly-NH_2[10] LH-RH ethylamide) produced an LH response within the physiological range of the natural preovulatory release for all three parameters examined. Analogues 3 and 4 produced FSH responses within the physiological range of the natural preovulatory surge for all three parameters examined. It was therefore decided to compare the potency of analogue 3 with that of synthetic LH-RH at several dose levels.

Dose–response experiment

The results for the release of FSH and LH in the dose–response experiment are shown in Table 4. The LH responses to synthetic LH-RH and to analogue 3 showed clearly different patterns. In terms of both peak height and area, the dose–response lines for LH-RH failed to show a significant regression of response on dose, whereas with the analogue, steep linear regressions of response on dose were apparent ($P < 0{\cdot}01$). In terms of duration of

Table 4. *Responses (mean* ± S.E.M.) *of plasma LH and FSH in a dose–response curve experiment involving i.v. administration of synthetic luteinizing hormone releasing hormone (LH-RH) and analogue 3,* [D-*Ser*(Bu^t)^6] *Des Gly-*NH_2^{10}*LH-RH ethylamide, to anoestrous ewes*

Dose (μg)	Peak height (ng hormone /ml)		Duration of peak (h)		Area under peak (arbitrary units)	
	LH	FSH	LH	FSH	LH	FSH
LH-RH						
6·0	42±5	99±30	3·00±0·14	2·75±0·00	781±76	1786±92
30·0	46±15	176±21	3·17±0·17	4·50±0·00	1514±395	4976±538
150·0	53±13	94±26	4·50±0·75	4·00±0·00	1667±254	2777±953
Analogue 3						
1·2	110±17	159±4	3·91±0·22	2·91±0·30	2885±741	3209±398
6·0	177±18	343±10	6·00±0·14	5·25±0·14	7050±563	15215±386
30·0	248±26	329±38	9·08±0·30	6·75±0·75	13378±2344	19206±1944

(Bu^t): The hydroxyl group of D-serine was protected as its tertiary butyl ether.

response, however, the dose–response line for LH-RH showed a significant regression when the dose was increased from 30 to 150 μg. The regression line for the analogue was again steep and linear ($P < 0{\cdot}01$).

The patterns of the FSH responses to synthetic LH-RH and analogue 3 were different from one another and also different from the LH responses. Significant curvature of the dose–response lines was apparent ($P < 0{\cdot}01$) when all three parameters were considered. In terms of peak height and area, the responses to synthetic LH-RH were not significantly different from one another at the various doses used. Doses of 6·0 and 30·0 μg analogue also produced peaks not significantly higher than one another, but both were higher ($P < 0{\cdot}01$) than that produced in response to 1·2 μg analogue. Each increment in dose, however, produced a significant increase in response in terms of peak area (1·2 and 6·0 μg: $P < 0{\cdot}01$; 6·0 and 30·0 μg: $P < 0{\cdot}05$). With regard to the duration of the response, the curvatures of the dose–response lines for LH-RH and analogue 3 were opposed ($P < 0{\cdot}01$). With analogue 3, each increment in dose produced a significant increase in the response (1·2 and 6·0 μg: $P < 0{\cdot}01$: 6·0 and 30·0 μg: $P < 0{\cdot}05$). With LH-RH only the responses to 6·0 and 30·0 μg were different ($P < 0{\cdot}05$); the response to 150 μg did not differ significantly from those to either 6·0 or 30·0 μg.

DISCUSSION

The finding that an homologous system involving the iodination of ovine FSH and antisera raised against ovine FSH failed to be specific for ovine FSH confirms the work of several groups (e.g. L'Hermite, Niswender, Reichert & Midgley, 1972). The heterologous system developed eventually proved highly specific and sufficiently sensitive for routine use in anoestrous ewes. In only two animals were the basal levels of FSH below the working sensitivity of the assay. A similarly based assay has recently been described by McNeilly, McNeilly, Walton & Cunningham (1976), and applied to the measurement of serum levels of ovine FSH during the oestrous cycle.

It is impossible to draw any detailed conclusions from the present work about structure–activity relationships of LH-RH and its analogues because of the limited number of analogues used. The LH-RH analogues tested all contained the ethylamide substitution in position 10 found by Fujino *et al.* (1973) to increase potency of the molecule. In the present work, analogue 1 which contained this substitution alone, was more potent than synthetic LH-RH in terms of LH release but not in terms of FSH release. Where the

ethylamide modification was accompanied by substitution by various amino acids in positions 6 and/or 7, the effect was usually to increase the potency further but this was not always true (analogue 5). Where D-amino acids were substituted in position 6 in addition to the ethylamide substitution (analogues 2, 3 and 4), the effect was to increase the potency for both LH and FSH release compared with synthetic LH-RH.

In the present work, the main object was to examine a group of potentially potent analogues of synthetic LH-RH in the sheep with a view to finding one which could mimic, in the anoestrous ewe after i.v. administration of a single low dose, the natural preovulatory release of LH and FSH characteristic of the oestrous cycle. Accordingly, seven of the analogues, although most were more potent than synthetic LH-RH, were examined only in the screening experiment. The eighth (analogue 3, [D-Ser(Bu^t)^6] Des $GlyNH_2^{10}$ LH-RH ethylamide) appeared from the screening experiment to most closely approach this requirement.

In the dose–response experiment, the dose–response lines for LH-RH and analogue 3 deviated significantly from parallelism in all cases and in several cases from linearity. While the former can be explained by different rates of degradation (Coy, Labrie, Savary, Coy & Schally, 1975) and characteristics of attachment at receptor sites (Fujino, Fukuda, Shinagawa, Kobayashi, Yamazaki, Nakayama, Seely, White & Rippel, 1974) of LH-RH and analogue 3, the reason for the latter is not clear and requires further investigation. These findings dictated that comparison of the potencies of the two peptides was not possible using conventional statistical methods for the analysis of six-point biological assays. It was, however, obvious that analogue 3 was substantially more potent than LH-RH when each of the parameters examined was considered. For LH, the mean peak height, duration and area produced in response to 30·0 μg analogue 3 were respectively 5·4, 2·8 and 8·8 times those produced in response to the same dose of LH-RH. When the same comparison was made for FSH the corresponding values were 1·9, 1·5 and 6·9 respectively.

As expected, in view of the results of Foster & Crighton (1974) and Crighton *et al.* (1975), single i.v. doses of LH-RH of 6·0, 30·0 or 150·0 μg failed to produce a release of LH or FSH approaching that seen in the oestrous cycle for any of the parameters examined. On the other hand, depending upon the parameter examined, a single i.v. dose of 6·0 or 30·0 μg analogue 3 resulted in values for induced release of LH and FSH within the range observed for the natural preovulatory surge. This has previously been found impossible to achieve with single injections of LH-RH, but could be achieved with a schedule of multiple injections (Crighton *et al.* 1975).

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RELATIVE ACTIVITY, PLASMA ELIMINATION AND TISSUE DEGRADATION OF SYNTHETIC LUTEINIZING HORMONE RELEASING HORMONE AND CERTAIN OF ITS ANALOGUES

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SUMMARY

The abilities of three nonapeptide analogues of synthetic luteinizing hormone releasing hormone (LH-RH) to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in anoestrous and cyclic ewes were examined, as were their elimination from the plasma *in vivo* and degradation by extracts of the hypothalamus, anterior pituitary gland, lung, kidney, liver and plasma *in vitro*. In all cases, comparisons were made with synthetic LH-RH.

When injected i.v. into mature ewes as a single dose, the potencies of the analogues were graded and Des Gly-NH_2^{10} LH-RH ethylamide was found to be the least potent. It was not possible to demonstrate any significant increase in the potency of this analogue over LH-RH, although a trend was apparent with each parameter examined. [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide had the greatest potency. There were no differences between the responses of anoestrous ewes and those of ewes treated on day 10 of the oestrous cycle. None of the analogues had a rate of elimination from the plasma different from that of LH-RH during either the first or the second components of the biphasic disappearance curve.

The incubation of LH-RH with tissue extracts showed that extracts of the hypothalamus and anterior pituitary gland degraded LH-RH to a similar extent. Both the hypothalamic and anterior pituitary gland extracts degraded more LH-RH than did lung extract, which in turn destroyed more LH-RH than did extracts of kidney or liver tissue. The degradative abilities of kidney and liver extracts did not differ from each other. Plasma failed to degrade LH-RH or the analogues. Although LH-RH was rapidly destroyed by hypothalamic extract *in vitro*, of the analogues, only Des Gly-NH_2^{10} LH-RH ethylamide was degraded. The anterior pituitary gland and kidney extracts failed to degrade [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide as rapidly as LH-RH. Extracts of liver and lung were incapable of catabolizing any of the analogues.

There was an inverse correlation between the LH- and FSH-releasing potency of an analogue and its rate of degradation by anterior pituitary gland extract. The slower rates of catabolism of certain analogues of LH-RH by the anterior pituitary gland may explain their increased LH- and FSH-releasing potency.

INTRODUCTION

The elucidation of the structure of luteinizing hormone releasing hormone (LH-RH; Matsuo, Baba, Nair, Arimura & Schally, 1971; Burgus, Butcher, Amoss, Ling, Monahan, Rivier, Fellows, Blackwell, Vale & Guillemin, 1972) and the synthesis of its decapeptide molecule (Geiger, König, Wissmann, Geisen & Enzmann, 1971) have led to a search for analogues of LH-RH with greater biological activity. The first success was achieved by Fujino, Shinagawa, Yamazaki, Kobayashi, Obayashi, Fukuda, Nakayama, White & Rippel

(1973), who found that substituting ethylamine for the glycineamide residue in position 10 increased the luteinizing hormone (LH)-releasing activity of the molecule. Placing D-alanine in position 6 also increased the potency of the molecule (Monahan, Amoss, Anderson & Vale, 1973).

Coy, Coy, Schally, Vilchez-Martinez, Hirotsu & Arimura (1974) produced a nonapeptide ethylamide analogue with D-alanine at position 6. This analogue had a very high potency and long-acting properties and it was suggested that it could have important clinical and veterinary applications in situations where prolonged release of LH and follicle-stimulating hormone (FSH) is desirable.

A group of potentially potent nonapeptide analogues of LH-RH were examined for LH- and FSH-releasing activity in the ewe (Crighton, 1976; Siddall & Crighton, 1977). Of these, Des Gly-NH_2^{10} LH-RH ethylamide appeared more potent than synthetic LH-RH in terms of LH release, but not in terms of FSH release. In terms of both LH- and FSH-releasing activity, [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide appeared to be of intermediate potency between Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ser$(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide, which was substantially more potent than synthetic LH-RH. The apparent graded potency of these three analogues offered the possibility of studying the structure–activity relationships of the molecules.

In the present study, the three analogues and synthetic LH-RH have been compared more comprehensively with regard to their ability to release LH and FSH. Their rates of elimination and degradation in various tissues have been examined to investigate the mechanisms by which potency, relative to LH-RH, is determined.

MATERIALS AND METHODS

Plasma concentrations of LH and FSH were determined as described by Foster & Crighton (1974) and Siddall & Crighton (1977) respectively. The concentrations of LH-RH in plasma and incubation medium were determined by the method of Nett, Akbar, Niswender, Hedlund & White (1973) with minor modifications. Original double-antibody radioimmunoassays were developed to measure the concentrations of the analogues in the plasma and incubation medium.

Iodination of LH-RH and the analogues

Synthetic LH-RH and the analogues used for iodination and as standards were donated by Dr J. Sandow, Farbwerke Hoechst AG, Frankfurt. The iodination method was based on that described by Greenwood, Hunter & Glover (1963), but 0·5 mM-sodium hypochlorite was used in place of chloramine T for iodination of the analogues (Redshaw & Lynch, 1974). The material to be iodinated (5 μg) was added to a tube containing ^{125}I (1mCi, The Radiochemical Centre, Amersham, Bucks.). ^{125}I-Labelled polypeptide was separated by elution on a cellulose CF11 column with 0·01 M-phosphate buffer (pH 7·2) to remove the free iodine and damaged polypeptide and the undamaged labelled material was eluted with 0·01 M-phosphate buffer containing 1% egg albumin. This eluate was then diluted and used as labelled antigen in the assay. By this method, specific activities of approximately 100 Ci/g were obtained, as determined by electrophoresis.

Antisera

For the assay of LH-RH, antisera were donated by Dr S. L. Jeffcoate, St Thomas's Hospital, London and Dr T. M. Nett, Colorado State University, U.S.A.

Analogues were conjugated to bovine serum albumin by the method of Jeffcoate, Fraser, Gunn & Holland (1973). The conjugate (100 μg in 0·5 ml) was emulsified with Freund's complete adjuvant (0·5 ml). Antisera were raised in Dutch rabbits (body weight 1·5 kg) by administration of multiple-site (ten sites) subcutaneous injections of the emulsion. Blood samples were obtained 6 weeks later, titres of antisera were determined and booster injections

of freshly conjugated analogue were given, using the same procedure as before. This booster treatment was repeated 15 weeks after primary immunization and a final blood sample was taken at 21 weeks. An antiserum raised against [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide was donated by Dr J. Sandow, Farbwerke Hoechst AG, Frankfurt and was used for all subsequent work on this analogue since the titre of the equivalent antiserum raised at Sutton Bonington was low.

Sensitivity and specificity

Sensitivity was taken as the quantity of unlabelled hormone required to inhibit the binding of the tracer by an amount equal to two standard deviations, compared with binding in the absence of unlabelled hormone. The sensitivities of the two LH-RH antisera were as quoted elsewhere (4·5 pg/ml plasma, Nett *et al.* 1973; 20 pg/ml plasma, Jeffcoate & Holland, 1975). The sensitivities of the antisera raised against Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide were 2 and 5 pg/ml plasma respectively; the sensitivity of the antiserum against [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide was 8 pg/ml plasma. Dose–response curves were prepared for antisera selected for high titre and prepared against Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide (using the respective analogue), the two other analogues of the three tested, various other analogues modified in the 6 and/or 7 positions (e.g. [D-Ala^6] Des Gly-NH_2^{10} LH-RH ethylamide, [D-Ala^6 Lys^7] Des Gly-NH_2^{10} LH-RH ethylamide, [D-Leu^6 Ser(Bu^t)^7] Des Gly-NH_2^{10} LH-RH ethylamide, [Ser(Bu^t)^7] Des Gly-NH_2^{10} LH-RH ethylamide and [Lys(Boc)^7] Des Gly-NH_2^{10} LH-RH ethylamide) and synthetic LH-RH, Des amido LH-RH, 7–10 LH-RH, 6–10 LH-RH, 4–10 LH-RH and 1–6 LH-RH.

The antiserum raised against Des Gly-NH_2^{10} LH-RH ethylamide bound 22·9% of the labelled analogue when used at a final dilution of 1 : 25 000. At this dilution there was 100% cross-reaction with [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide, [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide, [D-Ala^6 Lys^7] Des Gly-NH_2^{10} LH-RH ethylamide, 50% cross-reaction with 7–10 LH-RH, 4–10 LH-RH, 10% cross-reaction with [D-Ala^6] Des Gly-NH_2^{10} LH-RH ethylamide and no detectable cross-reaction with the remaining peptides tested. The antiserum raised against [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide bound 23·9% of the labelled analogue when used at a final dilution of 1 : 20 000. At this dilution, it failed to cross-react with any of the other peptides tested. The antiserum to [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide donated by Dr Sandow bound 10% of the labelled analogue at a final dilution of 1 : 16 000. At a dilution of 1 : 20 000, this antiserum showed a high degree of specificity; it failed to cross-react with LH-RH, 2–10 LH-RH, Des amido LH-RH and cross-reacted by only 1% with Des Gly-NH_2^{10} LH-RH ethylamide (Sandow, von Rechenberg, König, Hahn, Jerzabek & Fraser, 1978).

Plasma samples obtained from ewes before the administration of LH-RH or its analogues gave values for the levels of LH-RH (both Jeffcoate and Nett antisera) and Des Gly-NH_2^{10} LH-RH ethylamide below the sensitivities of the assays and indicated values of 33 and 50 pg/ml for [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide respectively. When 5·0 ng analogue were added to 1·0 ml plasma from a hypophysectomized ewe (donated by Dr H. Buttle, NIRD, Reading), the recoveries were 82, 86 and 84% for Des Gly-NH_2^{10} LH-RH ethylamide, [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide respectively. When 100 μl plasma from a hypophysectomized ewe were extracted and added to the standard curves of the assays for LH-RH and analogues, no significant deviation from parallelism was observed.

Precision

The intra-assay coefficients of variation were 2·4% ($n= 8$) for the determination of Des Gly-NH_2^{10} LH-RH ethylamide, 3·5% ($n= 8$) for [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide

and 2·4% ($n = 8$) for [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide. The interassay coefficients of variation were 9·2% ($n = 5$), 7·8% ($n = 6$) and 4·6% ($n = 10$) respectively.

Assay of LH-RH and analogues

Sample or standard (0·1 ml; undiluted extracted plasma or incubation medium diluted 1 : 50) plus 0·2 ml of the appropriate antiserum at the appropriate dilution (Dr Jeffcoate's antiserum to LH-RH, final dilution 1 : 80 000; Dr Nett's antiserum to LH-RH, final dilution 1 : 100 000; antiserum to Des Gly-NH_2^{10} LH-RH ethylamide, final dilution 1 : 25 000; antiserum to [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide, final dilution 1 : 20 000; antiserum to [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide, final dilution 1 : 16 000) and containing normal rabbit serum at a dilution of 1 : 600 were incubated overnight at 4 °C. The next day the appropriate tracer (15 000–20 000 counts/min) was added and the mixture incubated again overnight at 4 °C. On the third day, 0·1 ml precipitating antibody (RD 17, Wellcome Laboratories, diluted 1 : 12) was added. On the final day, 0·6 ml assay diluent (0·01 M-phosphate buffer containing 0·9 g sodium chloride and 0·01 g sodium merthiolate/100 ml and 0·25% albumen) was added to each tube and the tubes were centrifuged at 5000 ***g*** for 25 min at 4 °C. The supernatant fractions were aspirated and the precipitates counted.

Animals and treatment

Mature Clun Forest ewes from the School of Agriculture flock were used. Sixteen ewes which had regular oestrous cycles, as detected by a colour-marked vasectomized ram, were removed from the flock on day 9 of the cycle and jugular venous cannulae were inserted. On the day after cannulation (day 10), the ewes were bled (2 ml) at 15 min intervals for 1 h preceding and for up to 12 h after a 30 μg injection, through the cannula, of either LH-RH or one of the analogues (four animals per group). Blood samples were centrifuged at 3000 ***g*** for 10 min at 4 °C within 1 h of collection and the plasma was removed and stored at −20 °C until required for assay. During the next anoestrous season, 16 mature Clun Forest ewes were fitted with jugular venous cannulae. On the day after cannulation the ewes were treated and bled as before (four animals/group), except that from the time of administration of LH-RH or analogue the ewes were bled every 3 min for 1 h and then every 15 min for the remaining period. The 3 min sampling period was designed to allow determination, by radioimmunoassay, of the rate of elimination of the peptides from the plasma. The blood samples were treated as described previously.

Extraction of LH-RH and analogues from plasma samples

A sample (0·25 ml) of plasma was precipitated with 3 vol. methanol at room temperature for 10 min. The sample was then centrifuged at 3000 ***g*** for 20 min again at room temperature. The supernatant fraction was decanted and evaporated to dryness in a water bath at 50 °C under a constant stream of dry air. The residue was taken up in 0·25 ml assay diluent. Portions (0·1 ml) of this were used for the assay. Recoveries of ^{125}I-labelled peptide by this method were 83% for LH-RH, 89% for Des Gly-NH_2^{10} LH-RH ethylamide, 87% for [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and 85% for [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide.

Extraction of hypothalamic, anterior pituitary, lung, kidney, liver and plasma peptidases

Hypothalamic tissue, the anterior pituitary gland and sections of lung, kidney and liver were removed from mature ewes during the anoestrous season after stunning by electric shock and exsanguination at a local abattoir.These tissues were placed in chilled Medium 199 (Difco Laboratories). The method of extraction of the enzymes was basically that which Griffiths & Hooper (1974*a*) used in other species. Briefly, in the laboratory, the tissues were blotted, weighed and homogenized in 5 vol. 0·25 M-sucrose with a glass/Teflon homogenizer. A nuclear cell debris fraction was collected by centrifugation at 500 ***g*** for 10 min at 4 °C. The

tissue supernatant fraction or blood plasma prepared by centrifugation at 3000 **g** for 10 min at 4 °C was then centrifuged at 25 000 **g** for 1 h at 4 °C. The supernatant fraction was dialysed against distilled water for 24 h at 4 °C. The protein content of the diffusate was then determined by a microbiuret method using bovine serum albumin as standard (Sigma Chemicals, fraction V; 15% N_2) and corrected to the experimental concentration by the addition of assay diluent and phosphate buffer (0·05 mol/l, pH 7·25) in the ratio 3 : 1. The protein concentration of the extract was 300 μg non-diffusable protein/ml.

Incubation of LH-RH and analogues

Incubations (30 min) were performed in the 3 : 1 mixture of assay diluent and phosphate buffer in a total volume of 1·0 ml. Controls were arranged in three ways: phosphate buffer and 500 ng LH-RH or analogue; extract and buffer; preboiled (15 min) extract and 500 ng LH-RH or analogue. The extract and 500 ng LH-RH or analogue were incubated in the experimental tubes. Each incubation included four replicates and was carried out on two separate occasions. Incubations were terminated by boiling (10 min) in a water bath. The tubes were sealed and stored at −20 °C until required for radioimmunoassay of the residual LH-RH or analogue.

Statistical analyses

The results were analysed by Duncan's multiple range test or Student's *t*-test as appropriate.

RESULTS

The mean LH and FSH responses of the anoestrous ewes to the dose of 30 μg LH-RH or analogue together with the statistical significance of the differences are shown in Table 1. The mean responses of the cyclic animals together with the statistical significance of differences are shown in Table 2. No significant differences were observed between the responses of ewes treated during anoestrus and on day 10 of the cycle.

Elimination of LH-RH and analogues from plasma

The elimination of LH-RH from the plasma could be regarded as biphasic. The initial components of the half-life (means ± S.E.M., four animals in each group) measured between 6 and 12 min after administration were 3·59 ± 0·11 min (Jeffcoate antiserum) and 3·29 ± 0·69 min (Nett antiserum). There was no significant difference between these values. These results compare with 3·73 ± 0·60 min for Des Gly-NH_2^{10} LH-RH ethylamide, 3·50 ± 0·10 min for [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and 3·30 ± 0·10 min for [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide.

The second components of the curves (means ± S.E.M., four animals in each group) measured between 18 and 30 min after administration were 20·68 ± 5·50 (Jeffcoate antiserum to LH-RH), 22·16 ± 3·21 min (Nett antiserum to LH-RH; no significant difference between the two values), 24·59 ± 6·40 min (Des Gly-NH_2^{10} LH-RH ethylamide), 23·90 ± 2·18 min ([D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide) and 22·25 ± 4·90 min ([D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide). None of the analogues had a rate of elimination from the plasma significantly different from that of LH-RH during either the first or the second components of the disappearance curve.

Tissue degradation of LH-RH and analogues

The amounts of LH-RH or analogue remaining after incubation of 500 ng with extracts of various tissues on one out of two occasions are shown in Table 3. On the second occasion the results were virtually identical with those obtained initially. Incubation of phosphate buffer or pre-boiled tissue extract with 500 ng LH-RH or analogue resulted in insignificant

Table 1. *Responses (means ± S.E.M.) of plasma LH and FSH after i.v. administration of 30 μg synthetic luteinizing hormone releasing hormone (LH-RH) or three of its analogues to anoestrous ewes*

	Height of peak (ng hormone/ml)		Duration of peak (h)		Area under peak (arbitrary units)	
	LH	FSH	LH	FSH	LH	FSH
1. LH-RH	57·00 ± 4·43	73·00 ± 11·21	4·06 ± 0·34	3·25 ± 0·10	1498 ± 59	823 ± 11
2. Des Gly-NH_2^{10} LH-RH ethylamide	64·50 ± 5·32	128·75 ± 12·31	4·50 ± 0·10	4·31 ± 0·12	2556 ± 83	1412 ± 35
3. [D-Ser[6]] Des Gly-NH_2^{10} LH-RH ethylamide	151·25 ± 38·37	190·00 ± 30·27	6·50 ± 0·51	5·38 ± 0·22	5595 ± 1354	3446 ± 214
4. [D-Ser(Bu^t)[6]] Des Gly-NH_2^{10} LH-RH ethylamide	217·00 ± 13·99	248·50 ± 38·55	11·36 ± 0·24	10·13 ± 0·90	14 560 ± 1773	12 553 ± 605
Significance of differences (*P*) between:						
1 and 2	NS	NS	NS	NS	NS	NS
2 and 3	< 0·05	NS	< 0·05	NS	NS	< 0·001
3 and 4	< 0·05	NS	< 0·001	< 0·001	< 0·001	< 0·001
1 and 3	< 0·001	< 0·01	< 0·001	< 0·01	< 0·01	< 0·001
1 and 4	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001

NS, Not significant.

Table 2. *Responses* (*mean* ± S.E.M.) *of plasma LH and FSH after i.v. administration of 30 μg synthetic luteinizing hormone releasing hormone (LH-RH) or three of its analogues to cyclic ewes*

	Height of peak (ng hormone/ml)		Duration of peak (h)		Area under peak (arbitrary units)	
	LH	FSH	LH	FSH	LH	FSH
1. LH-RH	35·50 ± 5·72	51·25 ± 14·90	3·25 ± 0·39	2·88 ± 0·74	833 ± 101	772 ± 251
2. Des Gly-NH_2^{10} LH-RH ethylamide	68·25 ± 19·10	65·00 ± 7·14	4·19 ± 0·70	5·19 ± 1·19	1902 ± 533	2100 ± 438
3. [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide	155·75 ± 8·35	201·25 ± 3·25	9·63 ± 0·66	7·06 ± 1·14	7488 ± 874	6284 ± 901
4. [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide	217·50 ± 22·41	226·75 ± 27·21	11·50 ± 0·29	10·88 ± 0·24	13 066 ± 2025	8128 ± 1210
Significance of differences (*P*) between:						
1 and 2	NS	NS	NS	NS	NS	NS
2 and 3	< 0·01	< 0·001	< 0·001	NS	< 0·01	< 0·01
3 and 4	< 0·05	NS	< 0·05	< 0·01	< 0·05	NS
1 and 3	< 0·001	< 0·001	< 0·001	< 0·001	< 0·01	< 0·001
1 and 4	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001

NS, Not significant.

Table 3. *Mean* (± S.E.M.) *residual amount (ng) of synthetic luteinizing hormone releasing hormone (LH-RH) and three of its analogues after incubation of 500 ng hormone or analogue with extracts of hypothalamus, anterior pituitary gland, lung, kidney, liver and plasma*

	Hypothalamus	Anterior pituitary gland	Lung	Kidney	Liver	Plasma
1. LH-RH	15·83 ± 1·82	43·25 ± 5·22	187·50 ± 10·90	277·50 ± 57·20	322·50 ± 12·33	461·25 ± 12·14
2. Des Gly-NH_2^{10} LH-RH ethylamide	401·25 ± 10·28	53·75 ± 11·61	475·00 ± 25·00	293·50 ± 13·30	475·00 ± 10·20	455·00 ± 8·29
3. [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide	470·00 ± 24·23	93·75 ± 12·48	495·00 ± 8·42	373·25 ± 14·97	468·75 ± 20·85	471·75 ± 18·83
4. [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide	475·00 ± 15·00	373·25 ± 14·97	517·50 ± 5·95	439·00 ± 6·12	490·00 ± 8·50	502·50 ± 10·51
Significance of differences (*P*) between:						
1 and 2	< 0·001	NS	< 0·001	NS	< 0·001	NS
2 and 3	< 0·01	< 0·05	NS	NS	NS	NS
3 and 4	NS	< 0·001	NS	NS	NS	NS
1 and 3	< 0·001	< 0·01	< 0·001	< 0·05	< 0·001	NS
1 and 4	< 0·001	< 0·001	< 0·001	< 0·01	< 0·001	NS

NS, Not significant.

degradation of peptide in each case. Incubation of tissue extract with buffer resulted in a value for LH-RH of <2 ng/ml medium and values for the analogues were below the sensitivities of the assays.

When the degradative abilities of the various tissues with respect to LH-RH were compared, the extracts of hypothalamus and anterior pituitary gland degraded LH-RH to similar extents, but both degraded LH-RH significantly more than did lung extract ($P<0.001$ in each case). Lung extract degraded LH-RH significantly more than did kidney or liver extracts ($P<0.05$, $P<0.001$ respectively). The degradative abilities of kidney and liver extracts were not significantly different from each other. The hypothalamic extract was found to be highly degradative with respect to LH-RH, but of the analogues only Des Gly-NH_2^{10} LH-RH ethylamide was catabolized. The anterior pituitary extract was similarly effective at removing LH-RH from the system. Des Gly-NH_2^{10} LH-RH ethylamide was degraded to a similar extent to LH-RH, but [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide were degraded less than LH-RH. A graded resistance to degradation with increasing complexity of the analogues was observed. Whereas both the lung and liver extracts degraded LH-RH, neither extract degraded any of the analogues. Kidney extract degraded LH-RH and all three analogues. Des Gly-NH_2^{10} LH-RH ethylamide was degraded to a similar extent to LH-RH by kidney extract, but [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide were degraded less than LH-RH. Plasma failed to degrade LH-RH or any of the analogues.

DISCUSSION

The finding that the LH and FSH responses to LH-RH and the three analogues in ewes during the anoestrous season were not significantly different in any respect (peak height, duration and area beneath the curve) from the responses to the peptides on day 10 of the oestrous cycle confirms the results of Crighton, Foster, Haresign & Scott (1975) with respect to LH-RH and extends those findings to include the three analogues. As suspected from previous work (Siddall & Crighton, 1977), the activities of the three analogues were graded; Des Gly-NH_2^{10} LH-RH ethylamide was the least potent (not significantly different from LH-RH in any of the parameters examined, although a trend was apparent with each parameter) and [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide had the greatest potency at both reproductive stages. The remaining experiments were designed to examine the reasons for this gradation in activity.

The plasma half-life of LH-RH has been measured in the rat (Gordon & Reichlin, 1973; Ben-Jonathan, Mical & Porter, 1974), sheep (Nett *et al.* 1973) and man (Keye, Kelch, Niswender & Jaffe, 1973; Redding, Kastin, Gonzalez-Barcena, Coy, Coy, Schalch & Schally, 1973; Jeffcoate, Greenwood & Holland, 1974; Pimstone, Epstein, Hamilton, Le Roith & Hendricks, 1977). The composite disappearance curve could be regarded as biphasic, with an initial fast component and a second slow component. The present study agrees well with these findings. The elimination of analogues of LH-RH from the plasma has been little described previously. Sandow, Eckert, Stoll & von Rechenberg (1977) showed that the half-life of ^{125}I-labelled [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide in the plasma of the rat does not differ from that of LH-RH. Reeves, Tarnavsky, Becker, Coy & Schally (1977) compared the disappearance of iodinated [D-Ala^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-Leu^6] Des Gly-NH_2^{10} LH-RH ethylamide with that of LH-RH in ovariectomized rats and found that the half-lives of the analogues in the serum did not differ from that of the synthetic LH-RH. The present study using new radioimmunoassays shows that the rates of elimination from the plasma of LH-RH and the three chosen analogues did not differ in either the primary or the secondary phase in spite of clear differences in the LH- and FSH-releasing potencies of the molecules.

The ability of hypothalamic homogenates to inactivate physiologically active polypeptides was reported by Hooper (1962). Griffiths & Hooper (1974*a*, *b*) and Griffiths, Hooper & Hopkinson (1975) showed that hypothalamic extracts are able to destroy the activities of oxytocin and LH-RH very rapidly. Sandow, Heptner & Vogel (1973) demonstrated the degradative ability of other tissues with respect to LH-RH, but little information exists on the fate of LH-RH or its analogues once they have entered the tissues. Sandow *et al.* (1977) showed that the anterior pituitary gland, kidney and liver accumulate iodinated [D-Ser(Bu^t)^6] Des Gly-NH_2^{10} LH-RH ethylamide and retain it when iodinated LH-RH is no longer detectable, while Dupont, Labrie, Pelletier, Puviani, Coy, Coy & Schally (1974) demonstrated that tritiated LH-RH is accumulated mainly in the pituitary gland, kidney and bladder with minor accumulations in the liver, lungs and heart. Reeves *et al.* (1977) measured the disappearance and fate of ^{125}I-labelled [D-Leu^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-Ala^6] Des Gly-NH_2^{10} LH-RH ethylamide and found that these analogues are taken up to a greater degree than ^{125}I-labelled LH-RH by the anterior pituitary gland and remain there longer. Resistance to anterior pituitary and hypothalamic degradation of analogues altered in the 6 and/or 7 positions was reported by Koch, Baram, Hazum & Fridkin (1977), who suggested that resistance to degradation is inversely related to biological activity. Data to support this have been presented here. It is generally thought that one point of cleavage of the LH-RH molecule is the Gly^6–Leu^7 bond (Griffiths & Hooper, 1974*a*; Koch, Baram, Chobsieng & Fridkin, 1974) and indirect evidence has been provided for this with respect to hypothalamic, pituitary, lung, kidney and liver degradation, since modification in position 6 resulted in resistance to degradation.

Although lung, kidney and liver extracts, at the protein concentrations used in the present work, were less effective than anterior pituitary extract in degrading synthetic LH-RH and the analogues, the total degradative capacity of these tissues would be considerable. Pimstone *et al.* (1977) showed that in human patients with kidney failure, the half-life of synthetic LH-RH is considerably extended and thus the kidney may be a major organ for removal of injected LH-RH. The present results *in vitro* support this. There was a trend towards graded resistance to degradation of the three analogues, the pattern being similar to that demonstrated for the anterior pituitary gland. Pimstone *et al.* (1977) found that moderate liver dysfunction did not alter the half-life of synthetic LH-RH, although in the present work *in vitro*, liver extract was active in degrading LH-RH. This activity appeared to be highly specific and the same was true for lung extract, since none of the analogues were degraded by either of these tissue extracts. The hypothalamic extract, however, was able to degrade Des Gly-NH_2^{10} LH-RH ethylamide, but not the two analogues modified at position 6 in addition to the ethylamide substitution. Plasma appears to contain no enzymes capable of degrading synthetic LH-RH or the analogues.

The relative extents to which tissues are active *in vivo* in the catabolism of synthetic LH-RH and its analogues are difficult to assess. Plasma is presumably not active at all. The absence of degradative activity with respect to the analogues in the lung and liver and the slower rates of degradation with respect to LH-RH in the hypothalamus and kidney suggested by the present work cannot be important factors in the increased release of LH and FSH by the analogues, unless it is postulated that a sustained low level of analogue in the bloodstream (below the sensitivities of the assays used here) causes repeated stimulation of the pituitary receptors. Whether such low levels could be sufficient to have this effect is unknown. It is more likely that slower rates of catabolism of certain LH-RH analogues by the anterior pituitary gland explains their increased LH- and FSH-releasing potency. The inverse correlation found in the present work between the potency in releasing LH and FSH and rate of degradation by anterior pituitary extract supports this hypothesis.

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THE EFFECTS OF CERTAIN STEROID HORMONES ON THE ACTIVITY OF OVINE HYPOTHALAMIC LUTEINIZING HORMONE-RELEASING HORMONE (LH-RH) – DEGRADING ENZYMES

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1. Introduction

Peptidases capable of degrading luteinizing hormone-releasing hormone (LH-RH) have been shown to exist in the hypothalamus of the rat [1], rabbit [2] and sheep [3,4]. The activity of these peptidases has also been reported to be stimulated in the rat by repeated injection of oestradiol and progesterone [5]. We wish to describe here the effects on the activity of ovine hypothalamic LH-RH-degrading enzymes of low doses of 17β-oestradiol, progesterone and corticosterone applied directly to enzyme-containing extracts in vitro in the presence of synthetic LH-RH.

2. Materials and methods

2.1. *Extraction of hypothalamic peptidases*

Hypothalamic tissue was removed from seasonally anoestrous ewes slaughtered at a local abattoir. The tissue was placed in chilled medium 199 (Difco Laboratories) for transport to the laboratory. The method of enzyme extraction was basically that in [6] with minor modifications [4]. The protein content of the dialysed extract was determined by a microbiuret method using bovine serum albumin (Sigma Chemicals, fraction V; 15% N_2) as standard and was corrected to the experimental concentration by the addition of a 3:1 mixture of 0.01 M phosphate buffer (containing 0.9% NaCl, 0.01% sodium merthiolate and 0.25% egg albumin (pH 7.2)) and 0.05 M phosphate buffer (pH 7.25). The dialysed extract was adjusted to either 100 μg or 300 μg non-diffusable protein/ml.

2.2. *Incubation of synthetic LH-RH with hypothalamic peptidases*

Incubations (either 15 or 30 min) were performed at 37°C in the 3:1 mixture of phosphate buffers in 1 ml total vol. Controls were arranged in three ways: extract and buffer; buffer and 500 ng synthetic LH-RH, preboiled (15 min) extract and 500 ng synthetic LH-RH. The extract and 500 ng synthetic LH-RH were incubated in the experimental tubes either alone or in the presence of steroid hormones. The latter were added to the extract by resuspending the dried residues from aliquots of the steroids in ethanol, in phosphate buffer mixture to give correct concentrations in the incubation media (1 pg, 10 pg and 100 pg 17β-oestradiol/ml; 0.1 ng, 1 ng and 10 ng progesterone/ml; 0.1 ng, 1 ng and 10 ng corticosterone/ml). Incubations were terminated by boiling (10 min) in a water bath. The tubes were sealed and stored at –20°C until required for assay of residual LH-RH. Each incubation was performed as 4 replicates and on 2 occasions with different hypothalamic preparations.

2.3. *Assay of LH-RH*

The incubation media were diluted 1:50 before

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radioimmunoassay for LH-RH. Either an antiserum donated by Dr S. L. Jeffcoate, St Thomas's Hospital, London (final dilution 1:80 000) or an antiserum (Lot R42) donated by Dr T. M. Nett, Colorado State University (final dilution 1:100 000) was used. The details of the assay method are in [4].

3. Results

The incubation of 500 ng synthetic LH-RH with hypothalamic extract at a 300 μg non-diffusable protein/ml for 30 min resulted in a 95% degradation of LH-RH, consistent with [3]. Preboiled hypothalamic extract did not degrade LH-RH significantly. There were no statistically significant differences between the LH-RH contents of the media when they were assayed using the Jeffcoate or Nett antisera. The presence of 17β-oestradiol in the medium greatly enhanced the enzymic activity of the hypothalamic extract and when 10 pg/ml oestradiol were added no residual LH-RH was detected when either antiserum was used. The reduction both of the protein content of the medium (to 100 μg non-diffusable protein/ml) and of the duration of the incubation (to 15 min) resulted in less degradation of LH-RH (40% degradation). Under these conditions, the addition of 10 pg/ml and 100 pg/ml (but not 1 pg/ml) 17β-oestradiol significantly increased the degradation of LH-RH ($P < 0.01$ and $P < 0.001$, respectively). The addition of 10 ng/ml progesterone and 10 ng/ml corticosterone also increased LH-RH degradation significantly ($P < 0.01$ in each case) while lower concentrations of both were ineffective. The results of one 15 min incubation at 100 μg non-diffusable protein/ml when assayed with the Jeffcoate antiserum are shown in fig.1.

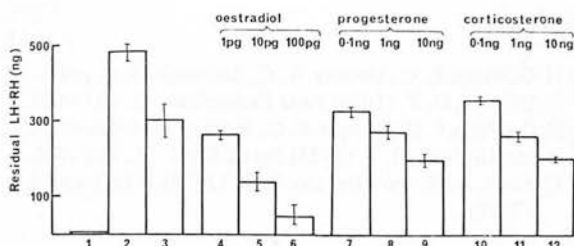

Fig.1. Effects of 17β-oestradiol, progesterone and corticosterone on the degradation of synthetic LH-RH (500 ng) when incubated with hypothalamic tissue extract at 100 μg non-diffusable protein/ml for 15 min. Each column is the mean of 4 incubations. Vertical bars represent ± SEM. Column (1) endogenous LH-RH present in the extract; (2) residual LH-RH when synthetic LH-RH was incubated with pre-boiled extract; (3) residual LH-RH when synthetic LH-RH was incubated with extract; (4–6) residual LH-RH when synthetic LH-RH was incubated with extract containing 1, 10 or 100 pg/ml of 17β-oestradiol, respectively; (7–9) residual LH-RH when synthetic LH-RH was incubated with extract containing 0.1, 1 or 10 ng/ml of progesterone, respectively; (10–12) residual LH-RH when synthetic LH-RH was incubated with extract containing 0.1, 1 or 10 ng/ml of corticosterone, respectively. The statistical significance of differences (P) between columns: 1 and 2, < 0.001; 1 and 3, < 0.001; 2 and 3, < 0.05; 3 and 4, NS; 3 and 5, < 0.01; 3 and 6, < 0.001; 3 and 7, NS; 3 and 8, NS; 3 and 9, < 0.01; 3 and 10, NS; 3 and 11, NS; 3 and 12, < 0.01; NS, not significant.

4. Discussion

The stimulation of hypothalamic peptidase activity obtained by treating animals with steroid hormones has been imitated in this study by adding steroids directly to enzyme-containing extracts. That steroids can act directly on these enzymes has not, to our knowledge, been reported previously. New enzyme molecules could not have been formed under the conditions of these incubations since there was no nuclear material present. The rapidity with which stimulation of activity occurred suggests that the steroids may either remove an inhibitor from the enzyme molecules or stimulate the enzyme activity allosterically. Work is in progress using a greater range of steroid molecules in order to examine further the specificity of this effect which may play a role in the feedback of steroid hormones on gonadotrophin secretion.

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Localization of luteinizing hormone-releasing factor in the pre-optic area. D. B. CRIGHTON and H. P. G. SCHNEIDER, *Department of Physiology, University of Texas Southwestern Medical School, Dallas, Texas.*

There is accumulated evidence which supports the hypothesis that pituitary gonadotrophic function has a dual neural control, a tonic level of control being responsible for stimulation and maintenance of basal gonadotrophin secretion and a cyclic control of the luteinizing hormone (LH)-release apparatus that may be located in or operate through the septal pre-optic area. Hypothalamic LH-releasing factor (LRF) concentrations were estimated by any assay which employs as the end point the LH released into the medium by anterior pituitaries incubated *in vitro*. The hypothalamic LRF concentration in rats with supra-chiasmatic lesions which induced hypothalamic-constant oestrus was compared to that in intact oestrous rats. There was a significant decrease of LRF activity in the rats with lesions, suggesting a localization of the LRF-secreting neurons as far rostrally as the optic chiasma. Since the animal with hypothalamic-constant oestrus is continually subjected to high levels of endogenously secreted oestrogen, the different hypothalamic LRF concentrations might have been caused by endogenous oestrogen feeding back to reduce LRF levels rather than by a destruction of supra-optic located LRF-secreting neurons in the rats with lesions. Consequently, similar lesions to those which produce constant oestrus in intact rats were produced in spayed rats. Again, hypothalamic LRF concentrations were significantly lower than in spayed control rats without lesions. Finally, a small cube was dissected from the supra-optic area of frozen brains from intact oestrous rats and tested for LRF activity. The crude acid extract of the suprachiasmatic cubes caused a marked rise in release of LH. LRF-secreting neurons may be located in the supra-optic area and be responsible for the discharge of ovulatory amounts of LH.

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The effects of a synthetic decapeptide on the release of luteinizing hormone and follicle-stimulating hormone from ovine pituitary tissue *in vitro*

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The ability of a synthetic decapeptide to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the ovine pituitary gland was examined. The structure of the decapeptide was that proposed by Matsuo, Baba, Nair, Arimura & Schally (1971) for a gonadotrophin-releasing factor. The experimental technique involved an *in vitro* pituitary tissue incubation linked with specific biological assays of the incubation medium for LH using the ovarian ascorbic acid depletion method (Parlow, 1958) and for FSH using the augmentation method (Steelman & Pohley, 1953). The specificity of the technique and its use for detecting changes in the LH-releasing activity of ovine hypothalami during the oestrous cycle have been described previously (Crighton, Hartley & Lamming, 1972*a*, *b*).

The activity of the synthetic decapeptide was examined in dose–response experiments in which minimal effective doses were established for the stimulation of both LH and FSH release into the incubation medium. The results were compared with those from similar experiments using other synthetic peptides (e.g. synthetic thyrotrophin-releasing factor).

The synthetic decapeptide was effective in provoking both LH and FSH release from ovine pituitary tissue *in vitro*. In the case of LH and FSH respectively the minimal effective doses of decapeptide were 0·5 and 0·25 ng per incubation flask in 2·5 ml. medium. The other synthetic peptides tested failed to influence the release of either LH or FSH even when included in the incubation medium at a dose of 10·0 nanograms per incubation flask (4·0 nanograms per ml. incubation medium). These results suggest that the decapeptide may be the physiological releasing factor for both LH and FSH in the sheep.

The effects of the synthetic decapeptide on the release of pituitary gonadotrophins *in vivo* in the sheep are at present being investigated.

The author is grateful to Hoechst Pharmaceuticals for providing the synthetic decapeptide.

[P.T.O.

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The effects of a synthetic preparation of gonadotrophin releasing factor on gonadotrophin release from the ovine pituitary *in vitro* and *in vivo*. By
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Recently, a decapeptide structure has been determined for luteinizing hormone releasing factor (LH-RF) by Matsuo, Baba, Nair, Arimura & Schally (1971) who claimed that the material was also follicle-stimulating hormone releasing factor (FSH-RF). The availability of a synthetic preparation of this decapeptide made possible an examination of its effects on the ovine pituitary *in vitro* and *in vivo*. (The synthetic peptides were kindly provided by Mr J. Best, Hoechst Pharmaceuticals.)

In the case of the in-vitro studies, the incubation and assay system previously described by Crighton, Hartley & Lamming (1972) for the detection of LH-releasing activity was employed, with the addition that, in some experiments, media were assayed for FSH using the assay of Steelman & Pohley (1953). The effects of the synthetic decapeptide on LH and FSH release were compared with the effects of other synthetic peptides at doses equivalent to those used for the synthetic decapeptide.

The synthetic decapeptide consistently provoked significant increases in the LH content of the incubation medium when added at doses equal to or in excess of 0·5 ng/flask (0·2 ng/ml medium). It also provoked significant increases in the FSH content of the incubation medium when added at doses equal to or in excess of 0·25 ng/flask (0·1 ng/ml medium).

Thus it was possible to demonstrate for sheep pituitary tissue that the decapeptide releases both LH and FSH *in vitro*. Other synthetic peptides, including the tripeptide thyrotrophin releasing factor (TRF) (Pyroglu-His-Pro-NH_2) and the tripeptide Pyroglu-Val-Ser-NH_2, failed to influence either LH or FSH release *in vitro* when included in the medium at similar doses to those used for the decapeptide. Previous experiments had shown that the synthetic octapeptide vasopressin failed to influence LH release even when included in the medium in μg quantities (Crighton *et al*. 1972).

The decapeptide or the other synthetic peptides already tested *in vitro* were administered to Clun Forest ewes on day 11 or 12 of the approximately 17-day oestrous cycle. Twenty-four hours prior to administration a catheter was placed in the jugular vein. Blood samples were obtained from the catheter every 10 min for 1 h before and every 5 min for 3 h after administration of each peptide via the catheter. The LH content of these samples was measured by a double antibody radioimmunoassay developed by the authors. The specificity of this assay has been studied carefully and cross-reactions with other hormones are minimal or absent. Administration of 50 μg of the decapeptide caused an increase in plasma LH from 1·0–2·0 ng NIH-LH-S17 equiv./ml to 10–30 ng NIH-LH-S17 equiv./ml. Administration of 150 μg of the decapeptide caused the plasma LH level to increase to 65–75 ng NIH-LH-S17 equiv./ml. Administration of 300 μg of each of the tripeptides TRF and Pyroglu-Val-Ser-NH_2 caused no significant increases in LH release.

[P.T.O.

Both ewes treated with 150 μg of the decapeptide showed recent ovulations when examined 2 days after treatment. This effect was not observed in any of the other ewes examined.

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Review of Releasing Hormones in Domestic Animals

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SINCE THE discovery of hypothalamic luteinizing hormone (LH) releasing activity and follicle stimulating hormone (FSH) releasing activity by McCann, *et al.* (1961) and Igarashi & McCann (1964) respectively, many attempts at separation and purification have been carried out. Matsuo, *et al.* (1971) determined the structure of a decapeptide which they claimed to be an LH/FSH releasing hormone. This decapeptide was synthesised by Geiger, *et al.* (1971) and became available for experimental use in the domestic species. Interest was aroused in the possible use of the decapeptide to alter the normal reproductive pattern of the domestic species for production purposes.

In the sheep, using incubation of pituitary tissue, it was confirmed that the decapeptide releases both LH and FSH (Crighton, 1972). Work was then carried out in both the sheep and the domestic fowl to evaluate the effects of the synthetic decapeptide *in Vivo.*

In the Clun Forest ewe, the normal pre-ovulatory peak of LH has been characterised by blood sampling from a jugular intravenous cannula at 15 min. intervals from the onset of oestrus (detected by a vasectomised ram) showing a peak height of approximately 250 ng/ml. reached about 200 mins. after the start of the rise and with a duration of raised LH of about 650 mins.

Injection of 150 μg of the decapeptide intravenously in Clun Forest ewes on Day 12 of the oestrous cycle or in mid-anoestrus resulted in the production of LH peaks and ovulation (Foster & Crighton, 1973). Ewes were sampled at 10 min. intervals for 50 mins. before injection, and at five min. intervals for three hours after injection. Peaks of LH in cycling ewes (79 ± 12 ng/ml.) and anoestrous ewes (109 ± 20 ng/ml.) (means $\pm$ S.E.M.) were not significantly different. They were, however, substantially lower than the natural pre-ovulatory peak. The duration of the increased LH level (approximately 200-240 mins.) also appeared much less than in the pre-ovulatory LH peak. The injection of 300 μg of synthetic thyrotrophin releasing factor (TRF) failed to raise LH levels above baseline.

Ovulation was induced in over 80 per cent. of ewes treated with the decapeptide, but no correlation was apparent between the height of the peak and the occurrence of ovulation. Ewes injected with synthetic TRF failed to ovulate.

Although follicle growth and ovulation undoubtedly occurred in response to decapeptide injection, further studies have shown that luteal function after ovulation was not normal in these ewes (Crighton, *et al.*, 1973). Low peaks or baseline levels of progesterone were apparent when daily sampling was carried out after ovulation and levels did not, in any case, approach those characteristic of the luteal phase of the oestrous cycle. Clearly, the treatment in the form employed was not suitable for establishing pregnancy in the sheep.

In the domestic fowl, similar experiments have been carried out (Falconer & Crighton, unpublished observations). In the adult female during the egg-laying cycle, sampling at two-hourly intervals showed a rise in plasma LH concentration shortly before oviposition and the subsequent ovulation. The rise was from a baseline of approximately 3 ng LH/ml. to a peak of approximately 8 ng/ml. In the immature female, baseline levels were maintained throughout the 24-hour-cycle. Injection of immature female birds with 5, 10 or 20 μg of the synthetic decapeptide resulted in increased plasma LH concentrations from baseline values of 3-10 ng/ml. to peaks of 20-50 ng/ml. Levels remained above baseline for approximately 35 mins. after injection. When mature laying females were injected with 10 or 20 μg of the synthetic decapeptide no LH increases were observed, levels remaining at baseline during the sampling period. Injection of immature or laying females with 40 μg synthetic TRF failed to raise LH levels. The reasons for the pattern of responses seen are not clear.

More physiological knowledge is required on the hypothalmo-pituitary-gonadal axis before the use of releasing factors can become soundly based, and a substantial amount of experimental work is required in all commercially important species.

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NOTICES

It would be of great help to the editorial staff if Notices intended to be printed in the later pages of each issue could be received three weeks before the desired date of publication. To ensure smooth production, the size and general outline of each week's "Record" has to be planned well in advance, and late Notices may be squeezed out through lack of space. The pressure on space is especially acute at the present time.

May we also remind those who send in Notices that the first insertion is free for B.V.A. Divisions, but that each repetition is charged for at the normal classified advertisement rates.

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Effects of various synthetic fragments of the decapeptide molecule of gonadotrophin releasing factor on the release of luteinizing hormone from ovine pituitary tissue *in vitro*. By D. B. CRIGHTON and D. J. SCHAFER.* *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire, and *Reckitt and Colman, Pharmaceutical Division, Dansom Lane, Hull*

The ability of synthetic preparations of fragments of the decapeptide molecule described as gonadotrophin releasing factor by Matsuo, Baba, Nair, Arimura & Schally (1971) to stimulate the release of luteinizing hormone (LH) from ovine pituitary tissue was examined *in vitro*. The technique, involving pituitary tissue incubation linked with a biological assay specific for LH has been described previously (Hartley, Crighton & Lamming, 1973).

In this system a preparation of the decapeptide molecule caused a significant increase in the LH content of the incubation medium when added to it at doses equal to, or in excess of 0·1 ng/flask (0·04 ng/ml medium). Various fragments of the decapeptide molecule were tested for LH-releasing activity at doses of 0·1, 1·0, 10·0 and 100·0 ng/flask (0·04, 0·4, 4·0 and 40·0 ng/ml medium). Of these fragments, only one, the tripeptide Pyroglu-His-Trp-OH, possessed any LH-releasing activity at the doses used. This material caused significant increases in the LH content of the medium at doses of 10·0 and 100·0 ng/flask and thus possessed approximately 1 % of the activity of the synthetic decapeptide.

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Relative activity, plasma elimination and hypothalamic and pituitary degradation of synthetic luteinizing hormone releasing hormone and certain of its analogues. By A. D. Swift and D. B. Crighton. *Department of Physiology and Environmental Studies, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, LE12 5RD*

Synthetic luteinizing hormone releasing hormone (LH-RH) and three of its nonapeptide analogues (Des Gly-NH_2^{10} LH-RH ethylamide, [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide) were injected intravenously into both anoestrous and cyclic (day 10) Clun Forest ewes in single dose (30 μg) experiments to determine their relative ability to release LH (radioimmunoassay of Foster & Crighton, 1974) and follicle-stimulating hormone (FSH) (radioimmunoassay of Siddall & Crighton, 1977). In the cyclic ewes, blood sampling was carried out at 15 min intervals. In the anoestrous ewes the blood sampling interval was reduced to 3 min to allow the determination of plasma elimination of the injected peptides. For the latter purpose, antisera to each peptide were raised in rabbits and were used as the bases of radioimmunoassays. In addition, in-vitro degradation studies were performed on the peptides using peptidase preparations for hypothalamic and pituitary tissues as described by Griffiths & Hooper (1974) for hypothalamic tissue.

In the anoestrous ewes, the response to Des Gly-NH_2^{10} LH-RH ethylamide was not significantly different from that to synthetic LH-RH, in terms of peak height and duration, but responses to [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide were significantly greater ($P<0{\cdot}05$ and $P<0{\cdot}001$ respectively). The areas under the response curves of all three analogues were significantly greater than that of LH-RH ($P<0{\cdot}001$ in each case). The FSH responses showed a similar pattern. There were no significant differences between treated anoestrous ewes and ewes treated identically on day 10 of the cycle.

The half-life of LH-RH in plasma was $3{\cdot}59 \pm 0{\cdot}11$ (S.E.M.) min. None of the analogues had a plasma half-life which was significantly different from that of synthetic LH-RH.

In the hypothalamic incubation system 97·3% of added LH-RH was degraded within 30 min compared with 4·8% of Des Gly-NH_2^{10} LH-RH ethylamide, 4·1% of [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and 6·8% of [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide. The pituitary incubation system degraded 89·6% of added LH-RH within 30 min, 88·1% of Des Gly-NH_2^{10} LH-RH ethylamide, 79·0% of [D-Ser^6] Des Gly-NH_2^{10} LH-RH ethylamide and 52·0% of [D-$Ser(Bu^t)^6$] Des Gly-NH_2^{10} LH-RH ethylamide.

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Contributions to books

Reprint

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EFFECTS OF CERTAIN ANALOGUES OF SYNTHETIC LUTEINIZING HORMONE-RELEASING HORMONE ON LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE RELEASE IN THE ANESTROUS EWE

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ABSTRACT

The ability of eight nonapeptide analogues of synthetic luteinizing hormone-releasing hormone (LH-RH) to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the anestrous ewe was studied and compared with the activity of synthetic LH-RH itself. In all the analogues the glycinamide residue in position 10 was replaced with ethylamine. Seven of the analogues also had various substituents in position 6 and/or 7. In a screening experiment using a single i.v. dose of 30 µg most of the analogues proved more potent than LH-RH in producing LH and FSH release. The most promising analogue was [Des-Gly-NH_2^{10}, L-Pro-ethylamide^9, D-Ser $(Bu^t)^6$] LH-RH, which produced LH and FSH peaks similar in area to those of the natural pre-ovulatory peaks of the cycle. This analogue was further compared with synthetic LH-RH in a dose-response experiment employing single i.v. doses of 1.2, 6.0 and 30.0 µg of the analogue and 6.0, 30.0 and 150.0 µg synthetic LH-RH. In the case of LH release the dose-response lines for the analogue were almost linear and very steep. The response lines for LH-RH were much flatter and did not approach the same response range as those for the analogue. In the case of FSH release the responses tended to level off or decline at the highest dose in each case, although the maxima for the analogue were greater than those for LH-RH. A single i.v. dose of the analogue between 6.0 and 30.0 µg could produce in the anestrous ewe LH and FSH peaks very similar in height, duration and area to those of the cycle, a situation previously found impossible to achieve with single injections of synthetic LH-RH.

Introduction

Since the characterization of the LH-RH of the porcine and ovine hypothalamus [1,2] and the synthesis of its decapeptide molecule [3] there have been many reports on the synthesis and biological activity of analogues of synthetic LH-RH. While many of the analogues synthesized have had minimal biological activity some have shown higher gonadotropin-releasing activity than the native molecule. The first example of this was [Des-Gly-NH_2^{10}, L-Pro-ethylamide^9)]-LH-RH by Fujino and colleagues [4]. It has also been shown that the incorporation of a D-amino acid in position 6 increases the biological activity of the molecule considerably [5]. Combination of these two modifications [6] resulted in a very high potency analogue with long-acting properties when tested in the rat in vivo.

We have shown previously in the anestrous ewe that single i.v. injections of synthetic LH-RH result in an LH peak which is much smaller in height, duration and area than the natural pre-ovulatory surge [7,8]. In order to achieve an induced LH peak closer in form to the natural surge a multiple i.v. injection schedule was used [8].

In the present work, anestrous ewes were given single injections i.v. of eight nonapeptide analogues of synthetic LH-RH based on the Fujino modification [4] and with various other modifications in positions 6 and/or 7. The plasma LH and FSH responses were assessed by radioimmunoligical assay and compared with the responses to single injections of synthetic LH-RH and with the natural pre-ovulatory LH and FSH plasma surges.

Materials and Methods

Clun Forest ewes from the School of Agriculture flock were used.

The structures of synthetic LH-RH and the eight analogues are shown in Fig. 1.

Blood sampling was carried out at 15 min intervals for one hour before and 11 hours after administration of LH-RH or analogue i.v. In addition two ewes were sampled at 15 min intervals for 26 h from onset of estrus to determine values for

the natural pre-ovulatory LH and FSH peaks.

In the screening experiment 18 ewes were divided equally into nine groups, two animals per analogue and two animals for LH-RH. All animals received a dose of 30 μg i.v.

LH-RH	⌐Glu-His-Trp-Ser-Tyr- Gly	- Leu	-	Arg-Pro-Gly-NH_2
1	⌐Glu-His-Trp-Ser-Tyr- Gly	- Leu	-	Arg-Pro-NH-C_2H_5
2	⌐Glu-His-Trp-Ser-Tyr- D-Ser	- Leu	-	Arg-Pro-NH-C_2H_5
3	⌐Glu-His-Trp-Ser-Tyr-D-Ser(Bu^t)	- Leu	-	Arg-Pro-NH-C_2H_5
4	⌐Glu-His-Trp-Ser-Tyr-D-Ala	- Leu	-	Arg-Pro-NH-C_2H_5
5	⌐Glu-His-Trp-Ser-Tyr- Gly	- Ser (Bu^t)	-	Arg-Pro-NH-C_2H_5
6	⌐Glu-His-Trp-Ser-Tyr- Gly	- Lys (Boc)	-	Arg-Pro-NH-C_2H_5
7	⌐Glu-His-Trp-Ser-Tyr-D-Ala	- Lys (Boc)	-	Arg-Pro-NH-C_2H_5
8	⌐Glu-His-Trp-Ser-Tyr-D-Leu	- Ser (Bu^t)	-	Arg-Pro-NH-C_2H_5

Fig. 1. The structures of synthetic LH-RH and the eight analogues administered to anestrous ewes.

In the dose-response experiment for analogue 3 [Des-Gly-NH_2^{10}, L-Pro-ethylamide, D-Ser $(Bu^t)^6$)]-LH-RH and LH-RH, 17 ewes were divided into 6 groups, five of three and one of two. Three groups were injected with the analogue i.v. and three groups with LH-RH i.v.. Doses of the analogue of 1.2, 6.0 and 30.0 μg and of the LH-RH of 6.0, 30.0 and 150.0 ug were used.

Results

The LH and FSH results for the screening experiment are shown in Tables I and II. When these mean responses obtained in anestrous ewes were compared with mean values obtained from two ewes for the natural pre-ovulatory peaks of the cycle it was clear that analogue 3 produced an LH response within the physiological range of the natural pre-ovulatory LH peak in all three parameters examined. Analogues 3 and 4 produced FSH responses within the physiological range of the natural pre-ovulatory

peak in all three parameters examined. It was therefore decided to examine the potency of analogue 3 [Des-Gly-NH_2^{10}, L-Pro-ethylamide, D-Ser $(Bu^t)^6$)]-LH-RH in a dose-response experiment comparing the analogue with synthetic LH-RH at several dose levels.

Table I. Screening of analogues: LH peaks (means).

	Height (ng/ml)	Duration (h)	Area (arbitary units)
Natural pre-ovulatory	175	9.25	14206
LH-RH	66	4.00	1741
1	94	5.50	3129
2	137	5.50	4208
3	226	9.25	11076
4	133	7.00	5818
5	107	5.00	2556
6	64	5.00	2082
7	129	4.75	5083
8	178	5.50	5535

Table II. Screening of analogues: FSH peaks (means).

	Height (ng/ml)	Duration (h)	Area (arbitary units)
Natural pre-ovulatory	208	8.25	16206
LH-RH	151	3.75	3835
1	125	3.25	3512
2	218	3.75	5766
3	371	6.50	18112
4	268	7.50	18764
5	69	1.75	894
6	60	2.75	780
7	184	3.75	5784
8	217	4.25	6049

The LH and FSH results for the dose-response experiment are shown in Tables III and IV.

Table III. Comparison of analogue 3 with LH-RH: LH peaks (means).

	Height (ng/ml)	Duration (h)	Area (arbitary units)
Natural pre-ovulatory	175	9.25	14206
LH-RH			
6.0 μg	42	3.00	781
30.0 μg	46	3.25	1514
150.0 μg	53	4.50	1667
Analogue 3			
1.2 μg	111	4.00	2885
6.0 μg	177	6.00	7050
30.0 μg	248	9.00	13378

Table IV. Comparison of analogue 3 with LH-RH: FSH peaks (means).

	Height (ng/ml)	Duration (h)	Area (arbitary units)
Natural pre-ovulatory	208	8.25	16206
LH-RH			
6.0 μg	99	2.75	1786
30.0 μg	176	4.50	4976
150.0 μg	94	4.00	2777
Analogue 3			
1.2 μg	159	3.00	3210
6.0 μg	343	5.25	15216
30.0 μg	330	6.75	19206

The LH responses to synthetic LH-RH and the analogue showed a clearly different pattern from one another. In terms of peak height, the response to synthetic LH-RH was flat with increasing dose while it was much steeper with the analogue. The mean peak height produced in response to 1.2 μg analogue was more than twice that produced in response to 150 μg synthetic LH-RH and 30 μg analogue

produced a peak height approximately 4.7 times that produced by 150 µg synthetic LH-RH. The same situation in terms of slopes of the dose-response lines was apparent when duration of the LH peak was considered. In this case the mean duration of response produced by 1.2 µg analogue was similar to that produced by 150 µg synthetic LH-RH, and 30.0 µg analogue produced a mean LH response exactly twice as long as that produced by 150 µg synthetic LH-RH. The mean total LH release produced in response to 1.2 µg analogue was approximately 1.7 times that produced in response to 150 µg synthetic LH-RH and 30.0 µg analogue produced a mean total LH response approximately eight times that produced by 150 µg synthetic LH-RH.

The FSH responses to synthetic LH-RH and the analogue again showed a different pattern from one another. In terms of peak height, the response to synthetic LH-RH was maximal at the 30 µg dose and fell at the 150 µg dose. This effect was seen, although less clearly, with the analogue where the peak responses to the 6.0 and 30.0 µg doses were similar. However, the mean peak height produced in response to 6.0 µg analogue was approximately twice that produced in response to 30.0 µg synthetic LH-RH. While the FSH responses to 30 µg and 150 µg synthetic LH-RH were of similar duration the responses to the analogue rose in almost straight line fashion. The mean response to 30.0 µg analogue was approximately 1.5 times that produced by 30 µg synthetic LH-RH. Thus the responses to the analogue not only showed differences from those to synthetic LH-RH, but the LH and FSH responses were different from one another in the cases of both peptides. The general tendency was thus for the slope of the FSH responses to be flatter than that of the corresponding LH responses and where the LH responses themselves were flat to reach a peak at the middle dose and decline at the highest dose.

Discussion

In the present work, the main object was to examine the group of potentially potent analogues of synthetic LH-RH with a view to finding one with which the natural pre-ovulatory LH and FSH peaks of the cycle could be reproduced in the anestrous ewe by single low dose i.v. administration. Accordingly, seven of the analogues, although they were in most cases more potent than synthetic LH-RH were only examined in the screening experiment. The eighth analogue (analogue 3, [Des-Gly-NH_2^{10}, L-Pro-ethylamide, D-Ser $(Bu^t)^6$)]-LH-RH) appeared from the screening experiment to most closely approach this requirement. In the dose-response experiment its high potency was confirmed and examination of the results showed

that a dose of the analogue between 6.0 and 30.0 μg could produce in the anestrous ewe LH and FSH peaks very similar in height, duration and area to those of the cycle, a situation previously found impossible to achieve with single injections of synthetic LH-RH [7.8].

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CHAPTER 2. THE HYPOTHALAMO-PITUITARY-OVARIAN SYSTEM IN DOMESTIC ANIMALS

Interrelationships during the oestrous cycle, seasonal and lactational anoestrus.

Papers in scientific journals (refereed)

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THE LACTATIONAL ANOESTRUS OF THE SOW: THE STATUS OF THE ANTERIOR PITUITARY-OVARIAN SYSTEM DURING LACTATION AND AFTER WEANING

D. B. CRIGHTON AND G. E. LAMMING
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(*Received* 18 *May* 1968)

SUMMARY

The status of the anterior pituitary-ovarian system of the sow was examined during lactational anoestrus and after weaning up to the occurrence of the first oestrus after weaning. The ovaries and uteri were examined and the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) contents of the anterior pituitary glands were measured.

Suppression of follicular growth and uterine development was apparent during lactation. This was relieved by weaning, when follicle size increased and oestrus and ovulation occurred within 6 days.

A high level of pituitary FSH was found during lactation which was maintained after weaning until oestrus occurred, when the level fell. The level of LH was low during lactation, rose after weaning and fell when oestrus occurred.

Ovariectomy of sows during lactation did not affect the growth of the young. While ovariectomy during the oestrous cycle produced the expected uterine atrophy, ovariectomy during lactation did not result in any further decline in uterine weight or length, suggesting that oestrogen secretion is at a very low level during late lactation. Pituitary LH content rose as a result of ovariectomy performed during the oestrous cycle but failed to do so as a result of ovariectomy performed during lactation, thus demonstrating inability of the lactating sow to increase LH synthesis under circumstances when the cycling sow is capable of doing so.

From these results it is postulated that (*a*) a considerable release of both FSH and LH occurs at about the time of the after-weaning oestrus, similar to that which is believed to occur at cyclic oestrus, (*b*) failure primarily of release of FSH and of synthesis of LH occurs during lactation which accounts for suppression of follicle growth, anoestrus and anovulation during lactation in the sow.

INTRODUCTION

Oestrous cycles in the pig are interrupted by the establishment of pregnancy and the inhibition of ovulatory oestrus is continued throughout lactation if the latter is

not extended (Marshall & Hammond, 1937; Burger, 1952; Heitman & Cole, 1956). A post-partum oestrus occurs in some pigs within 2 or 3 days of parturition, but this is not accompanied by ovulation (Warnick, Casida & Grummer, 1950; Burger, 1952). Accordingly, the period of lactation is one in which conception does not occur except in exceptional circumstances. In normal husbandry, the inhibition of oestrus and ovulation is terminated by the separation of the sow from her young 6–8 weeks after parturition, oestrus recurring usually within 4–7 days (Marshall & Hammond, 1937; Burger, 1952; Self & Grummer, 1958; Smidt, Scheven & Steinbach, 1965). The first oestrus after weaning is accompanied by ovulation and a further pregnancy is almost invariably established by mating at this time.

Few reports dealing specifically with the lactational anoestrus of the sow have been published so far. In the rat, the endocrinology of lactational anoestrus has been subjected to detailed examination (Stotsenburg, 1923; Desclin, 1936; Desclin & Grégoire, 1937; McKeown & Zuckerman, 1938; Desclin, 1947; Rothchild & Parlow, 1960; McCann, Graves & Taleisnik, 1961). The present study attempts to clarify the reproductive and endocrine status of the sow during lactational anoestrus. The work was conducted in two parts. The first was designed to establish the patterns of ovarian and pituitary activity between late lactation and after-weaning ovulation. The second was concerned with the effects of ovariectomy during lactation.

In the first experiment, sows were examined in late lactation (52 or 53 days *post partum*) and during the period after weaning at 56 days of lactation up to the occurrence of the first oestrus after weaning. The ovaries and uteri were examined and the pituitary contents of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) of individual sows were measured at each stage to obtain evidence on the nature of any changes.

In the second experiment, ovariectomy was used to examine the ability of the lactating sow to synthesize large amounts of LH. Ovariectomy is known to result in hypersecretion of LH as judged by increased pituitary levels in the rat (Greep & Chester Jones, 1950; Cozens & Nelson, 1961; Taleisnik & McCann, 1961; Ramirez & McCann, 1963; Parlow, 1964) and has been shown to raise the LH content of the blood of the rat to levels detectable by biological assay (Gans, 1959; Taleisnik & McCann, 1961; Ramirez & McCann, 1963; Parlow, 1964). Parlow, Anderson & Melampy (1964) found increased levels of pituitary LH after ovariectomy in the pig, showing that the same effect occurs in this species. It was hoped by carrying out concurrent assays of anterior pituitary and serum LH to demonstrate whether there was a different effect of ovariectomy on the synthesis and release of LH in lactating as compared with cycling sows. The results of the unsuccessful attempts to measure serum LH have been presented elsewhere (Crighton, 1968). Those of the measurements of pituitary LH are presented here.

MATERIALS AND METHODS

Animals. A total of 27 Large White sows from the School of Agriculture herd was used. In the first experiment 13 sows were used after approximately 45 days of lactation, at which time they were nursing litters ranging from four to 11 young. They were tested daily for oestrus with a boar from approximately 45 days of lactation until slaughter. The final determination of sexual receptivity took place not more

than 5 hr. before slaughter. The details are given in Table 1. In the second experiment, seven of the sows were ovariectomized and seven laparotomized to act as sham-operated controls. Six of the sows were placed on experiment after 10 days of lactation and the remaining eight at various stages of oestrous cycles initiated as a result of weaning. The lactating sows were nursing litters ranging from six to 11 piglets at the start of the experiment. Laparotomy or ovariectomy was carried out on day 20 or 21 of lactation. From 22 until 42 days of age each piglet was weighed every day. The piglets received no supplementary feeding during this period and thus the

Table 1. *Allocation of sows to groups in first experiment*

No. of sows	Reproductive status at slaughter	Time of slaughter
4	Lactating, anoestrous	52nd or 53rd day of lactation
3	After-weaning, anoestrous	3 or 4 days after weaning after 56 days of lactation
6	After-weaning, oestrous	4–6 days after weaning after 56 days of lactation

Table 2. *Allocation of sows to groups in second experiment*

No. of sows	Reproductive status at operation	Operation	Time of slaughter
3	Lactating, 20 or 21 days, anoestrous	Laparotomy	25 days after operation
3	Lactating, 20 or 21 days, anoestrous	Ovariectomy	25 days after operation
4	Cycling, 5th day of cycle	Laparotomy	5th day of cycle subsequent to that of operation
4	Cycling, 5th day of cycle	Ovariectomy	25 days after operation

mean growth curve of each litter reflected the milk production of the sow. Each cycling sow was observed through two successive oestrous periods before the operation, which was carried out on the 5th day of the oestrous cycle (the first day of oestrus being designated the first day of the cycle). All sows were tested for oestrus daily with a boar throughout the experiment. The lactating sows and the sows ovariectomized during the cycle were slaughtered 25 days after operation. The animals which had been laparotomized during the cycle were slaughtered on the 5th day of the subsequent cycle, the mean interval from operation to slaughter being 22 days. Details are given in Table 2.

Surgical procedures. Each sow was injected in an ear vein with sufficient sodium pentobarbitone (Veterinary Nembutal Solution, Abbott Laboratories Ltd.) to produce light anaesthesia. Anaesthesia was maintained with halothane (Fluothane, Imperial Chemical Industries Ltd.). The operations were carried out using aseptic techniques and from a flank approach in order to avoid the mammary gland area. The litters of lactating sows were allowed to suckle as soon as the latter recovered sufficiently to obviate any danger of crushing the young. This usually occurred within 2 hr. after the operation.

Slaughtering arrangements. Each sow was stunned with a captive bolt pistol and then killed by exsanguination from the jugular vein. The pituitary gland was removed within 20 min. after death and the reproductive tract was obtained when the carcass was eviscerated.

Treatment of tissues. Immediately after removal, the pituitary gland was dissected free of extraneous tissue, the posterior lobe removed and the anterior lobe crushed on a glass slide and dried for 24–36 hr. *in vacuo* over calcium chloride at room temperature. The dry anterior pituitary tissue was then scraped from the slide, weighed, ground into powder and stored in a small bottle *in vacuo* over calcium chloride at -20° until required for assay.

The ovaries were collected individually into 0·9 % NaCl solution and were examined for recent ovulation before being fixed in formol-saline. After fixation, the ovaries were cut serially at approximately 1 mm. It was thus possible to follow and measure the diameters of individual follicles throughout each ovary. The number and diameters of all follicles of 3·0 mm. and over were recorded.

In the second experiment the uterine horns were dissected out by sectioning at the utero-tubal junction and at their junction with the uterine body. The supporting ligaments and blood vessels were removed, the two horns were weighed together and their lengths were noted. In both experiments, samples were taken from the mid-section of each uterine horn and fixed in formol-saline. After fixation, the samples were dehydrated, mounted in wax, sectioned at 5 μ and stained with Ehrlich's haematoxylin-eosin.

Assay procedures. The anterior pituitary glands were assayed individually by the following methods.

Assay of FSH. The assay method used was the 'augmentation assay' described originally by Steelman & Pohley (1953) and modified for use in mice by Brown (1955) with minor modifications. Intact, immature female F_1 crossbred ($C_3H \times A$) mice were used. They received five s.c. injections, each in 0·5 ml. distilled water, over a 3-day period. Injections were made twice daily on the first 2 days and once on the third. Each mouse received an augmenting dose of 40 i.u. HCG (Lutormone, Burroughs Wellcome and Company) during the 3 days. Autopsy was carried out 24 hr. after the last injection. The combined weight of the two ovaries was expressed as mg./10·0 g. body weight.

Assay of LH. The assay method used was the ovarian ascorbic acid depletion (OAAD) assay of Parlow (1958, 1961) used as modified by Schmidt-Elmendorff & Loraine (1962) with further modifications. Intact immature rats from an inbred colony of Wistar origin were used. Each animal was pretreated with an s.c. injection of 50 i.u. pregnant mare serum gonadotrophin (PMSG) (Boots Pure Drug Co. Ltd. or Organon Laboratories Ltd.) and 72 hr. later an s.c. injection of 25 i.u. HCG (Lutormone, Burroughs Wellcome and Co.). The standard and the unknown materials were dissolved in 2·0 ml. 0·9 % NaCl solution and were injected i.p. (Mukerji, Bell & Loraine, 1962) instead of i.v. The animals were killed with ether 4 hr. $\pm$ 3 min. after the i.p. injection and the two ovaries were removed. The ascorbic acid content of each ovary was determined using the 2,6-dichlorophenolindophenol reaction as described by Dekanski & Harvie (1960) with minor modifications only. The ascorbic acid content of each ovary was expressed as μg. ascorbic acid/100 mg. ovary.

Design of assays. After obtaining preliminary information on dose–response relationships, quantitative assays were performed for FSH and LH on individual anterior pituitary glands. In almost all instances the design was a symmetrical four-point assay with two dose levels of the standard (S) and unknown (U) respectively

and with equal spacing of the log doses. In the FSH assays the log dose interval was $\log_{10} 3$ (0·4771) except in one assay where it was $\log_{10} 2$ (0·3010) and in the LH assays $\log_{10} 5$ (0·6990) except in four assays where it was $\log_{10} 3$. In all assays six or seven animals were used at each dose level of S and U. The standard preparation of FSH was NIH-FSH-S1 (doses usually 25·0 and 75·0 μg.) and the LH standards were NIH-LH-S3 and NIH-LH-S8 (doses usually 0·6 and 3·0 μg.) in the first and the second experiments respectively.

Statistical analysis. A validity test was performed on each four-point assay and this and the calculation of the index of precision (λ), relative potency and fiducial limits of error on all assays were carried out as recommended by Gaddum (1953). When replicate assays were carried out, they were combined to provide a weighted mean after the method of Sheps & Moore (1960) and this value was used in arriving at the group mean. The significance of differences between group means was assessed by analysis of variance.

RESULTS

Late lactation and the period after weaning

Ovarian follicles. Since three of the sows in the after-weaning, oestrous group had freshly ovulated follicles while three had not ovulated, this group was divided for the purpose of analysing the data on ovarian follicles, thus making four groups in all. The changes in the ovarian follicles between late lactation and the after-weaning ovulation are shown in Table 3.

Table 3. *Changes in the ovarian follicles of sows between late lactation and the after-weaning ovulation* (*means* ± S.E.)

Reproductive status at slaughter	Diameter (mm.) of largest follicle	No. of follicles of over 5·0 mm.
Lactating, anoestrous	6·4 ± 0·4	6·8 ± 2·1
After-weaning, anoestrous	7·8 ± 0·5	23·7 ± 1·5
After-weaning, oestrous, not ovulated	8·3 ± 1·0	20·3 ± 3·7
After-weaning, oestrous, ovulated	5·3 ± 1·2	1·3 ± 1·2

The diameter of the largest follicle in either ovary showed an increase after weaning, reaching a maximum at oestrus and followed by a decrease at ovulation. These changes approached significance at $P = 0.05$. The total number of large follicles (diameter over 5·0 mm.) present in both ovaries showed an increase ($P < 0.001$) after weaning but no further significant change until ovulation, when a decrease ($P < 0.001$) occurred.

Ovulation and corpora lutea. None of the ovaries of sows slaughtered before the occurrence of the after-weaning ovulation showed recent corpora lutea. Old corpora were represented by dark brown streaks in the ovarian tissue. The ovaries of sows slaughtered after the after-weaning ovulation showed freshly ovulated follicles with ovulation points clearly apparent. The mean ovulation rate (± S.E.) of these sows as judged by counting the freshly ovulated follicles on the surface of the ovaries was 19·0 ± 2·3.

Uteri. The uterine diameter of sows after weaning was greater than that of

lactating sows and in the former the endometrium was thicker and the uterine glands more numerous than during lactation, particularly in the basal region close to the myometrium. These changes were greatest at the after-weaning oestrus.

Table 4. *Follicle-stimulating hormone (FSH) content of pituitaries from lactating and after-weaning sows*

Individual results and group means

Sow no.	Reproductive status at slaughter	λ	Relative potency (μg. NIH-FSH-S1)	Fiducial limits ($P = 0{\cdot}95$)	FSH concentration/mg. dry gland (μg. NIH-FSH-S1 ± S.E.)	FSH content/gland (μg. NIH-FSH-S1 ± S.E.)
1	Lactating, anoestrous	0·25	19·20	8·98–37·64	13·78 ± 3·02	2650 ± 568
2		0·23	18·74	10·93–30·02		
3		0·41	9·42	0·50–17·26		
4		0·46	7·75	0·03–19·40		
5	After-weaning, anoestrous	0·30	18·83	8·31–34·38	15·92* ± 2·91	2417* ± 251
6		0·16	13·01	9·01–18·80		
7		—	—	—		
8	After-weaning, oestrous	0·20	6·35	1·88–11·41	< 4·53 ± 1·32	< 775 ± 254
9		0·28	6·49	3·21–15·37		
10		0·36	0·55	0·00–34·10		
11		—	< 2·80†	—		
12		0·26	2·00	0·52–3·51		
13		0·49	8·97	0·00–25·69		

* Mean results from two anterior pituitary glands only.

† The highest dose used failed to produce a response as great as that produced by the lower dose of the standard. The relative potency was calculated on the basis that it had produced such a response and this value was used in arriving at the group means.

Table 5. *Luteinizing hormone (LH) content of pituitaries from lactating and after-weaning sows.*

Individual results and group means

Sow no.	Reproductive status at slaughter	λ	Relative potency (μg. NIH-LH-S3)	Fiducial limits ($P = 0{\cdot}95$)	LH concentration/mg. dry gland (μg. NIH-LH-S3 ± S.E.)	LH content/gland (μg. NIH-LH-S3 ± S.E.)
1	Lactating, anoestrous	0·20	5·77	4·40–7·64	3·96 ± 0·65	750 ± 55
2		0·56	3·01	1·19–6·55		
3		0·43	3·01	1·37–6·70		
4		0·57	4·06	1·46–8·95		
5	After-weaning, anoestrous	0·27	12·17	9·00–16·47	9·68 ± 1·38	1595 ± 206
6		0·34	6·73	4·28–10·59		
7		0·43	10·14	5·55–21·94		
8	After-weaning, oestrous	0·52	1·26	0·12–2·69	3·43 ± 1·65	585 ± 276
9		0·52	6·32	2·96–30·03		
10		0·31	0·66	0·19–1·24		
11		0·53	0·16	0·03–0·38		
12		0·47	1·85	0·36–3·60		
13		0·43	10·32	5·34–19·03		

Pituitary gonadotrophin content

FSH. The individual results and group means are shown in Table 4. Neither FSH content/mg. pituitary nor the total content of the gland was significantly changed

after weaning and before the onset of oestrus, but there was a decrease in both content/mg. and the total glandular content with the onset of oestrus ($P < 0{\cdot}01$ and $P < 0{\cdot}05$ respectively).

LH. The individual results and group means are shown in Table 5. Both LH content/mg. pituitary and total content of the gland showed an increase ($P < 0{\cdot}05$) after weaning and before the onset of oestrus and a decrease ($P < 0{\cdot}05$) with the onset of oestrus.

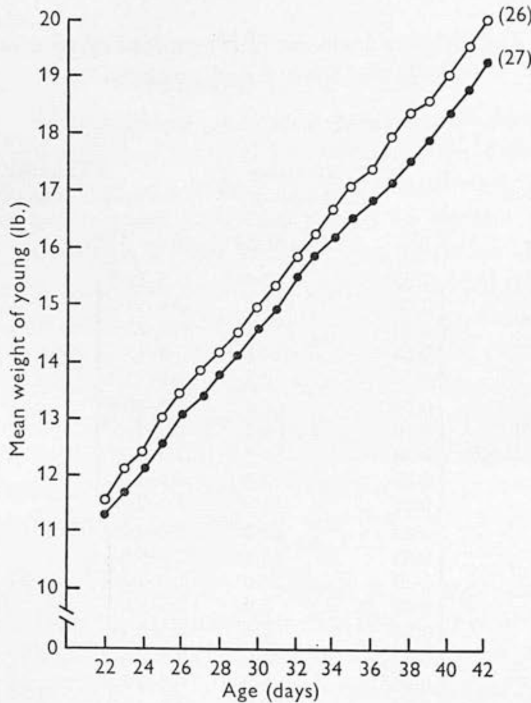

Fig. 1. Growth of young of intact (○——○) and ovariectomized (●——●) lactating sows. There was no significant difference between the growth rates of the two groups. Numbers of young in each group in parentheses.

Table 6. *Weights and lengths of uterine horns of lactating and cycling sows (means* ± S.E.)

Reproductive status at slaughter	Weight of uterine horns (g.)	Length of uterine horns (mm.)
Lactating 45 or 46 days, anoestrous	204 ± 44	139 ± 4
Lactating 45 or 46 days, ovariectomized 25 days previously	201 ± 41	132 ± 22
Cycling, 5th day of cycle	713 ± 85	204 ± 12
Ovariectomized 25 days previously on 5th day of cycle	206 ± 32	103 ± 9

Effects of ovariectomy

Growth of the young of lactating sows. There was no significant difference between the young of intact and ovariectomized lactating sows from 22 days of age to 42 days of age (Fig. 1).

Ovulation and corpora lutea. None of the ovaries obtained from lactating sows showed recently formed corpora lutea. All ovaries obtained from sows on the 5th day of the oestrous cycle contained recently formed corpora lutea, the ovulation points being visible in almost all cases. The mean ovulation rates (± s.e.) of the two groups of cycling sows as judged by counting the corpora lutea on the surface of the ovaries were 17·3 ± 3·9 for the sows examined at slaughter and 17·3 ± 3·5 for the sows examined at ovariectomy.

Table 7. *Luteinizing hormone (LH) content of pituitaries from lactating and cycling sows*

Individual results and group means

Sow no.	Reproductive status at slaughter	λ	Relative potency (μg. NIH-LH-S8)		Fiducial limits (P = 0·95)	LH concentration/ mg. dry gland (μg. NIH-LH-S8 ± s.e.)	LH content/ gland (μg. NIH-LH-S8 ± s.e.)
14	Lactating 45 or 46 days, anoestrous	0·29	9·3	7·6	5·2–11·1	9·0 ± 0·7	1209 ± 71
		0·28		11·5	8·8–16·9		
15		0·50	10·0	13·1	6·8–35·0		
		0·28		9·5	6·6–13·7		
16		0·35		7·6	4·9–11·8		
17	Lactating 45 or 46 days, ovariectomized 25 days previously	0·64	4·3	4·5	1·5–10·8	9·1 ± 2·8	1568 ± 395
		0·40		4·2	2·3–7·1		
		0·36		4·4	2·8–6·9		
18		0·33		9·0	5·4–15·2		
19		0·32		14·0	8·8–22·2		
20	Cycling, 5th day of cycle	0·41	4·2	5·2	2·9–8·9	4·1 ± 0·3	515 ± 41
		0·39		3·4	2·0–6·2		
21		0·47		4·0	1·9–7·3		
22		0·51		4·6	2·1–9·0		
23		0·25		3·4	2·4–4·7		
24	Ovariectomized 25 days previously on 5th day of cycle	0·44		31·9	17·8–58·1	22·8 ± 4·9	2284 ± 319
25		0·45		9·9	5·6–20·2		
26		0·39		20·7	11·0–48·2		
27		0·33		28·5	19·4–44·3		

Uteri. The results obtained using two criteria for uterine development are shown in Table 6. The weights and lengths of the uterine horns of the cycling group slaughtered on the 5th day of the cycle were greater than the weights and lengths for the other three groups ($P < 0{\cdot}01$ to $P < 0{\cdot}001$). There was no significant difference between the values for the two lactating groups, nor between the values for the two ovariectomized groups. The histological appearance of the uteri of the intact lactating sows, ovariectomized lactating sows and sows ovariectomized on the 5th day of the cycle was similar. The uteri on the 5th day of the cycle had a thicker endometrium and the uterine glands were more developed.

LH content of pituitary glands. The individual results and group means are shown in Table 7. When the LH content was expressed/mg. dry anterior pituitary tissue there was no significant difference between the two groups of lactating sows. The value for the sows ovariectomized 25 days previously on the 5th day of the cycle was higher ($P < 0{\cdot}01$) than that for the group slaughtered on the 5th day of the cycle. The value for the group ovariectomized 25 days previously on the 5th day of the cycle was also higher than the values for both lactating groups ($P < 0{\cdot}05$ in each

case). When the LH content was expressed as the total content of the gland, there was no significant difference between the two groups of lactating sows. The value for the group ovariectomized 25 days previously on the 5th day of the cycle was, however, higher ($P < 0{\cdot}001$) than that for the group slaughtered on the 5th day of the cycle. The difference between the value for the group ovariectomized during the cycle and the lactating, laparotomized group was significant ($P < 0{\cdot}05$) while the difference between the former and the lactating, ovariectomized group approached significance at $P = 0{\cdot}05$.

DISCUSSION

Examination of the ovaries of sows showed a considerable inhibition of follicular growth during lactation which was relieved by weaning. This was shown most clearly by a three- to fourfold increase after weaning in the mean numbers of large follicles present. These observations agree well with those of Palmer, Teague & Venzke (1965*a*) which were made over a similar period using a similar criterion of follicular development. Comparable evidence on the effects of removal of the young pigs on follicular growth has been obtained for the early stages of lactation by Warnick *et al.* (1950) and Lauderdale, Kirkpatrick, First, Hauser & Casida (1965).

The finding of an absence of corpora lutea from the ovaries of lactating sows is in agreement with other observations made at various stages of lactation (Corner, 1919; Warnick *et al.* 1950; Burger, 1952; Palmer *et al.* 1965*a*). The presence of dark brown scars in the ovarian tissue in the 8th week of lactation has also been noted by Palmer *et al.* (1965*a*). These presumably represent the degenerate corpora lutea of the preceding pregnancy.

The histological picture of the uteri observed is in agreement with the more detailed observations of Palmer, Teague & Venzke (1965*b*). The structure of the uterus of the lactating sow appears somewhat similar to that of the cycling sow during late dioestrus as described by Corner (1921). The increased epithelial height and glandular development after weaning and during after-weaning oestrus were similar to the description given by Corner (1921) for the changes occurring before and during cyclic oestrus. The data on the weights and lengths of the uterine horns of cycling sows in the second experiment illustrate the well-known effect of ovariectomy in causing atrophy of the uterus. There was a decrease in the weights and lengths of the uterine horns 25 days after ovariectomy, presumably due to the removal of the source of oestrogen. In the lactating sows, however, uterine weights and lengths were not significantly different 25 days after ovariectomy from those in intact sows at the same stage of lactation. This suggests that the uterus of the sow is in a state of atrophy during lactation due to a low level of circulating oestrogen.

The decrease in pituitary FSH and LH content at the after-weaning oestrus to approximately one-third of the levels in the after-weaning period before oestrus agrees with measurements made by others during the follicular phase of the cycle and cyclic oestrus in a number of species. Decreases in the pituitary content of FSH during cyclic oestrus or immediately preceding it have been reported in the ewe (Santalucito, Clegg & Cole, 1960; Robertson & Hutchinson, 1962) and sow (Day, Anderson, Hazel & Melampy, 1959; Parlow *et al.* 1964) and decreases in LH have been observed in the rat (Mills & Schwartz, 1961; Gorski & Barraclough, 1962;

Schwartz & Bartosik, 1962), ewe (Santalucito *et al.* 1960; Robertson & Hutchinson, 1962) and sow (Parlow *et al.* 1964). These decreases were considered to represent release of the hormones to produce ovulation and the follicular growth immediately preceding it. This interpretation is supported by evidence of increased levels of LH in the blood at or just before oestrus in the rat (McCann & Ramirez, 1964; Anderson & McShan, 1966), cow and sow (Anderson & McShan, 1966). The same interpretation is placed on the FSH and LH results from after-weaning sows in the present study.

Interpretation of the results of pituitary FSH and LH assays when comparing late lactation with the after-weaning period before oestrus is more difficult. The results suggest a difference in the ways in which the synthesis and release of the two hormones are affected by lactation.

With regard to FSH, the pituitary levels did not alter significantly between late lactation and the period after weaning before oestrus. During lactation follicular growth was suppressed but after weaning considerable growth was evident. The fact that follicular growth occurred in the presence of a high level of FSH in the anterior pituitary gland and was followed by oestrus associated with a considerably decreased pituitary FSH level, suggests that the after-weaning period before oestrus is analogous to the follicular phase of the oestrous cycle. The pituitary FSH content is highest at this stage of the cycle in the sow (Parlow *et al.* 1964). Thus it appears that in spite of a high level of pituitary FSH during late lactation, release of the hormone is inhibited. The finding of a high level of FSH in the pituitary during lactation in the sow is in agreement with the results of Lauderdale *et al.* (1965) and Melampy, Henricks, Anderson, Chen & Schultz (1966), although both these studies were carried out in early lactation. These workers found the FSH content to be high towards the end of pregnancy and their results indicated that the level was maintained to the 16th day of lactation. This high level was associated with decreased follicular activity. These results support the hypothesis of inhibition primarily of release rather than inhibition of synthesis of FSH during lactation.

With regard to LH, a different pattern is apparent from that shown by FSH between late lactation and the after-weaning period before oestrus, there being an increase in the pituitary content. A low level of pituitary LH in the sow during early lactation was found by Melampy *et al.* (1966). The level on the 14th day of lactation was lower than that at any stage of pregnancy, during which a steady decline in the LH level took place. The level at this stage of lactation was approximately the same as that on the 10th day of the oestrous cycle as observed by Parlow *et al.* (1964).

Two possible situations were suggested by the LH results of the first experiment when they were considered without reference to the ovarian and uterine evidence. First, the results might represent pituitary LH release during lactation which was inhibited by weaning while synthesis continued, thereby causing the anterior pituitary gland content to rise. Secondly, the results might represent inhibition primarily of synthesis (and secondarily of release) of LH during lactation, this inhibition being relieved by weaning, causing the pituitary gland content to rise.

When these two interpretations were considered in relation to the evidence available on ovarian and uterine activity before and after weaning, it was apparent that the second interpretation was a better explanation of the facts. First, there were no luteinized follicles or other evidence suggesting LH release in late lactation.

Secondly, the rapid follicular growth in the after-weaning period before oestrus was accompanied by uterine changes which suggested oestrogen secretion by the follicles. There is good evidence that the presence of LH is required for oestrogen secretion (Greep, van Dyke & Chow, 1942; Simpson, 1961). The close relationship between the circulating LH level and oestrogen secretion as detected by uterine weight changes during the oestrous cycle of the rat has been pointed out by McCann & Ramirez (1964). That synthesis and release of LH are inhibited during lactation in the rat has been shown by Rothchild and Parlow (1960) and by McCann *et al.* (1961). It seemed from the results of the first experiment that a similar situation might exist in the sow and the second experiment was designed to test this hypothesis.

Although no reports have been found on lactation after ovariectomy in the sow, ovariectomy is known not to affect lactation in the rat (Long & Evans, 1922; Folley & Kon, 1938; Barsantini & Masson, 1947; Flux, 1955). The present results showed no significant difference between the growth rates of the young of ovariectomized lactating sows and the young of intact lactating sows from the 22nd to 42nd day of lactation, demonstrating that lactation was unaffected by ovariectomy.

In the second experiment the mean level of pituitary LH in the lactating sows was higher than that in the first experiment. Part of this difference arose because the standard preparation used in the second experiment was less potent than that used in the first experiment. In addition, the experiments were not concurrent. The results in the cycling sows confirmed the observation that ovariectomy in the cycling animal results in increased levels of pituitary LH. The finding that the anterior pituitary LH level characteristic of late lactation was not significantly different from that at the same stage of lactation but 25 days after ovariectomy demonstrates inability of the pituitary gland of the lactating sow to increase synthesis of LH in circumstances when the gland of the cycling sow is capable of doing so. It also shows that the ovaries play no part in the maintenance of the low level of anterior pituitary LH found in late lactation in the sow. The factor concerned in suppressing LH synthesis is probably the suckling stimulus, as is apparently the case in the rat (Desclin, 1947; Rothchild, 1960). The results of the second experiment thus support the hypothesis that the ability of the sow to synthesize LH is inhibited during lactation.

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GONADOTROPHIN CONTENT OF THE PITUITARIES OF SHEEP OF DIFFERING FERTILITY AT THREE STAGES OF THE OESTROUS CYCLE

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Summary. The pituitaries of Merino × Blackface ewes were found to contain more LH at mid-cycle (Day 10) than Finn × Blackface ewes. The content in purebred Blackface ewes was intermediate. No differences were observed in the relative activities of FSH or LH in the pituitaries of the three breed types around the time of oestrus, or of FSH on Day 10 of the cycle. It is postulated that more LH may be released during mid-cycle by the more highly fertile breed type.

The Finnish Landrace breed is recognized as having a greater prolificity than British breeds of sheep (Maijala, 1966; Donald & Read, 1967). Land (1971) has suggested that a high level of endogenous gonadotrophin stimulation may be a common factor underlying the various aspects of increased reproductive activity in Finnish Landrace sheep.

The gonadotrophin activity in the pituitaries of ewes of differing prolificity was examined in the present study. Three breed types were chosen: purebred Scottish Blackface (Blackface), Finnish Landrace × Blackface (Finn × Blackface) and Tasmanian Merino × Blackface (Merino × Blackface). The characteristics of the two crossbred types have been described by Donald, Read & Russel (1968, 1970) and Land (1970); the mean litter sizes at birth of 3-year-old Finn × Blackface and Merino × Blackface ewes were observed to be 2·30 and 1·16 lambs, respectively. The mean litter size of Blackface ewes under similar conditions was observed to be 1·75 (Wiener, 1967).

A group of ten ewes of each of the three breed types was established at the Animal Breeding Research Organisation Field Laboratory, Roslin, Midlothian. All ewes were kept in the same building and allocated at random to one of three similar pens. The ewes were between 4½ and 6½ years of age at the time of the experiment.

The experimental ewes were tested once a day with a vasectomized ram until all had shown at least one oestrus. Subsequently, testing was carried out thrice daily at 08.00, 14.00 and 23.00 hours; ewes which returned to oestrus 16 to 18 days after their previous oestrus were slaughtered 4 hr, 36 hr or 10 days

after the return to oestrus. For each breed, with two exceptions (Finn × Blackface—two ewes at 4 hr; Merino × Blackface—two ewes at 36 hr), three ewes were killed at each of the three time intervals. In this way, nine groups were established.

All ewes were slaughtered between 6th December 1968 and 17th January 1969. The pituitaries were removed, cleaned and frozen on dry ice within 10 min of slaughter. Freeze-drying was commenced within 6 hr of collection and the glands were then stored individually in a vacuum desiccator at $-15°$ C until they were assayed.

Before assay, each gland was weighed and then ground to a fine powder. Assays were performed on pooled material from each group, material from each gland being allocated to the pool in proportion to its dry weight.

The assay used for FSH was the Augmentation Assay (Steelman & Pohley, 1953) and that for LH was the ovarian ascorbic acid depletion assay (Parlow, 1958). Both assays were employed with minor modifications only. In the case of FSH, a three-point design with one dose of unknown and two doses of standard (NIH-FSH-S7, National Institutes of Health, U.S.A.) was employed. In the case of LH, six assays were of a four-point design employing two doses of unknown and two of standard (NIH-LH-S14, National Institutes of Health, U.S.A.), and three were three-point assays.

The validity of the four-point assays and the relative potency, index of precision (λ) and fiducial limits of error at $P = 0{\cdot}95$ of all assays were calculated as recommended by Sakiz & Guillemin (1963), employing a KDF9 computer and a programme developed by Dr E. Sakiz.

The weights of the freeze-dried pituitaries did not differ significantly between the breed types or between the stages of the oestrous cycle. The mean values for the Finn and Merino crosses and Blackfaces were 175, 178 and 177 mg, respectively. At the 4-hr, 26-hr and 10-day stages of the oestrous cycle, the mean weights were 190, 180 and 161 mg, respectively.

The FSH and LH concentrations for each of the three breed types are shown in Tables 1 and 2, respectively. The concentration of FSH in the pituitaries of the three breed types can be seen to be similar when each of the stages of the oestrous cycle studied is considered separately. The concentration showed an apparent decline between 4 and 36 hr after the onset of oestrus and remained low 10 days after onset in each case. In no case were these changes significant.

The pituitary LH concentration of the Finn × Blackface ewes was significantly less than that of the Merino × Blackface ewes on Day 10 of the oestrous cycle, that of the Blackface group being intermediate. During oestrus, however, there were no significant differences between the breed types when either of the stages was considered separately. The concentration showed an apparent decline from 4 hr to 36 hr in each case, although this was only significant in the case of the Blackface group.

The present results show that the known difference in the fertility of Finn × Blackface and Merino × Blackface ewes is associated with a difference in the concentration of LH in their pituitaries at Day 10 of the oestrous cycle. The Finn × Blackface ewes, the litter size of which was almost twice that of the Merino × Blackface ewes, had an LH concentration less than half that of the

latter at this stage of the cycle. This difference could be attributed either to a greater release of LH from the pituitaries of the Finn × Blackface ewes before Day 10 or to a lower rate of synthesis after pituitary depletion at oestrus. The observation that the LH activity in the urine of Finn × Blackface ewes was over three times ($P<0{\cdot}05$) that of Merino × Blackface ewes during Days 9, 10

TABLE 1

THE FSH CONTENT OF THE PITUITARIES OF EXPERIMENTAL EWES

Time after onset of oestrus	*Breed type*	*Pituitary FSH content** (*µg NIH-FSH-S7/mg dry weight*)	λ
4 hr	Finn × Blackface	11·49 (5·14 to 18·20)	0·18
	Blackface	13·44 (0·37 to 33·49)	0·31
	Merino × Blackface	12·69 (5·87 to 20·74)	0·18
36 hr	Finn × Blackface	6·10 (2·77 to 9·61)	0·19
	Blackface	7·95 (2·68 to 13·97)	0·20
	Merino × Blackface	6·67 (3·22 to 10·56)	0·19
10 days	Finn × Blackface	6·28 (2·92 to 9·92)	0·19
	Blackface	8·79 (4·90 to 14·38)	0·19
	Merino × Blackface	6·08 (2·73 to 9·56)	0·19

* Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.

TABLE 2

THE LH CONTENT OF THE PITUITARIES OF EXPERIMENTAL EWES

Time after onset of oestrus	*Breed type*	*Pituitary LH content** (*µg NIH-LH-S14/mg dry weight*)	λ
4 hr	Finn × Blackface	30·64 (25·09 to 38·12)	0·08
	Blackface	26·34 (21·56 to 32·56)	0·08
	Merino × Blackface	29·01 (23·61 to 36·16)	0·08
36 hr	Finn × Blackface	19·32 (11·46 to 36·08)	0·26
	Blackface	14·21 (9·29 to 21·52)	0·21
	Merino × Blackface	17·13 (10·24 to 31·00)	0·26
10 days	Finn × Blackface	21·16 (15·26 to 29·34)	0·15
	Blackface	27·39 (20·34 to 36·90)	0·15
	Merino × Blackface	44·37 (34·85 to 56·48)	0·12

* Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.

and 11 of the oestrous cycle (R. B. Land & H. A. Robertson, unpublished data) indicates that the differences in pituitary concentration observed reflect different rates of release. The urinary LH estimations are also compatible with the present data in that no differences were observed between breed types in urinary LH activity at oestrus.

It appears, therefore, that Finn × Blackface ewes which have a greater litter size when compared with Merino × Blackface ewes exhibit a greater LH release during part of the oestrous cycle but not during the oestrous period itself. Evidence has thus been obtained for a higher level of gonadotrophin stimulation in Finn × Blackface ewes at one stage of the cycle. The significance of these observations in terms of the control of the development of ovarian follicles and ovulation rate merits further investigation.

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CHANGES IN THE LUTEINIZING HORMONE RELEASING ACTIVITY OF THE HYPOTHALAMUS, AND IN PITUITARY GLAND AND PLASMA LUTEINIZING HORMONE DURING THE OESTROUS CYCLE OF THE SHEEP

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SUMMARY

Thirty ewes with regular oestrous cycles were divided into six groups for slaughter relative to the time of first acceptance of the ram. The slaughter times were: 0 h (in practice within 40 min of onset of oestrus), 6, 12 and 36 h after onset and days 10 and 16 of the cycle. The hypothalamus was removed and the luteinizing hormone (LH) releasing factor activity extracted with 0·1 M-HCl. The extracts were tested for LH releasing activity by adding them to the medium in which anterior pituitary tissue from castrated male sheep was incubated. The LH content of the medium was measured by the ovarian ascorbic acid depletion method (Parlow, 1958).

The activity of the extract from the group slaughtered on day 16 of the cycle was high (minimal effective dose (MED) = 0·00625 hypothalamic equivalents (HE)). The potency declined with the onset of oestrus and remained low at 6 and 36 h after onset (MED in each case 0·025 HE) with intermediate potencies at 12 h and 10 days after onset (MED in each case 0·0125 HE). These changes are compared with changes in the LH content of the pituitary gland (bioassay) and of the plasma (radioimmunoassay) and with parameters of the ovarian activity of the animals.

INTRODUCTION

In the present study an ovine pituitary incubation system linked with biological assay of the incubation medium for luteinizing hormone (LH) was used to assess the LH releasing activity of extracts of sheep hypothalami obtained at different stages of the oestrous cycle. The details of this technique and its specificity have been reported previously (Hartley, Crighton & Lamming, 1973). The changes in the LH releasing activity in the hypothalamus were compared with changes in the LH content of the pituitary gland measured by biological assay and in plasma LH measured by radioimmunoassay, and with parameters of the ovarian activity of the animals.

MATERIALS AND METHODS

Incubation procedure

The method was that described by Hartley *et al.* (1973). Hypothalamic extracts dissolved in 0·5 ml 0·1 M-HCl were added to 'treated' flasks and the LH content of the medium compared with that in 'control' flasks to which 0·5 ml 0·1 M-HCl had been added.

Assay methods

Incubation media were assayed for LH by the ovarian ascorbic acid (OAAD) method (Parlow, 1958) with minor modifications. A $(2+1)$ design was used with two doses of standard (NIH-LH-S16; National Institutes of Health, U.S.A.) and one dose of medium designed to produce a response between the responses to the two doses of standard. The results were calculated as described by Gaddum (1953) and the hormone content of the medium was expressed as μg NIH-LH/mg anterior pituitary tissue incubated.

Pituitary glands from experimental ewes were also assayed for LH by the OAAD assay. A symmetrical $(2+2)$ design was used with two doses of standard (NIH-LH-S16) and two doses of unknown. The validity, relative potency, fiducial limits of error at $P = 0·95$ and index of precision (λ) were calculated as recommended by Sakiz & Guillemin (1963) using a computer programme devised by Dr E. Sakiz. The results of valid assays were expressed as μg NIH-LH equivalents/mg of dry anterior pituitary material and as the total content of LH/dry anterior pituitary gland.

Plasma samples from experimental ewes were assayed for LH using a specific double antibody radioimmunoassay method developed in this Department (J. P. Foster & D. B. Crighton, unpublished results). Ovine LH was first purified chemically by Dr Anne Stockell Hartree, Department of Biochemistry, University of Cambridge, the LH and follicle-stimulating hormone (FSH) activities of each fraction being assessed by the OAAD and augmentation (Steelman & Pohley, 1953) assays respectively. Antisera were developed in the rabbit against partly purified LH and a very pure preparation with LH activity approximately $2 \times$ NIH-LH-S_1 was labelled with ^{125}I using the chloramine-T method (Greenwood, Hunter & Glover, 1963). The initial dilution of antiserum used in the assay was 1:16000. Plasma samples were assayed at a dilution of 1:10 against a standard (NIH-LH-S17; National Institutes of Health). The results were expressed as ng NIH-LH equiv./ml plasma.

Experimental ewes

A group of 30 cyclic cross-bred ewes was kept indoors. The animals were approximately five to six years of age and weighed about 70 kg. Two vasectomized rams were smeared daily with a coloured marker and were used to detect the onset of oestrus. The ewes were checked daily for oestrous activity and at least one cycle length was established. The oestrous behaviour was then monitored more closely and the animals were observed continuously from 07.00 h to 18.00 h each day.

The precise onset of oestrus was recorded as the time at which the ewe first accepted the ram. Ewes were slaughtered at one of six set intervals from the onset of oestrus, 0 h (in practice within 40 min of first standing for the ram), 6 h, 12 h and

36 h after the onset of oestrus and on days 10 and 16 of the cycle. The animals were slaughtered by stunning and exsanguination.

Two ewes with very regular cycles were selected for blood sampling for the determination of plasma LH. Two-millilitre blood samples were withdrawn into heparinized syringes from the jugular vein at 8-h intervals in the period immediately before the expected onset of oestrus, at onset and at 8-h intervals thereafter until 32 h after onset. The plasma was obtained from these samples and stored at −20 °C until required for assay.

Table 1. *Changes in ovarian follicles, corpora lutea and plasma progesterone levels during the oestrous cycle in sheep.* (*Means* ± S.E.M.)

Stage of cycle	No. of follicles > 2 mm	No. of follicles > 5 mm	No. of recent corpora lutea	Plasma progesterone (ng/ml)
Day 16	5·4 ± 0·7	0·4 ± 0·2	2·2 ± 0·4	1·29 ± 0·21
Oestrus:				
0 h	5·2 ± 0·7	1·2 ± 0·4	0	0·67 ± 0·39
6 h	5·6 ± 0·7	2·0 ± 0·3	0	0·35 ± 0·09
12 h	4·4 ± 0·4	1·6 ± 0·4	0	0·44 ± 0·20
36 h	6·6 ± 0·7	0·4 ± 0·2	Freshly ovulated follicles	0·48 ± 0·17
Day 10	5·4 ± 0·7	0·4 ± 0·2	1·8 ± 0·2	2·12 ± 0·36

At slaughter the ovaries of all ewes were collected in 0·9 % NaCl solution and were examined for recent ovulations and corpora lutea before being placed in formol–saline. Subsequently, the ovaries were sliced serially at approximately 2-mm intervals and the number and diameters of follicles greater than 2 mm diameter were recorded.

A sample of blood was collected into a heparinized vessel from the jugular venous effluent at slaughter and the resulting plasma was assayed for progesterone by Dr I. J. Shearer of this Department using a protein-binding assay.

The hypothalamus and pituitary gland were removed and placed on ice. The hypothalamus was extracted in 1·0 ml 0·1 M-HCl and the extract stored at −20 °C. Equal aliquots of the supernatants obtained after centrifuging the five individual extracts in each group were pooled. The anterior pituitary gland after dissection was lyophilized at 0 °C for 24 h. The final dry weight was noted and the dry tissue was ground finely and stored in a dessicator over calcium chloride at −20 °C until required for assay.

RESULTS

Ovarian status

The changes in the ovarian follicles, the number of corpora lutea and plasma progesterone levels are shown in Table 1. There were more follicles greater than 5 mm diameter at 0 h, 6 h and 12 h after onset of oestrus than at the other stages of the cycle. Four of the five sheep slaughtered 36 h after the onset of oestrus had freshly ovulated follicles. Corpora lutea were present in all the animals of both the day 10 and day 16 groups although those of the latter group showed signs of regression as judged by their gross morphology. Plasma progesterone levels were highest on day 10, falling on day 16 and were low during oestrus up to and including the 36-h stage.

Hypothalamic LH releasing activity

A summary of the effects of hypothalamus extracts at the various stages of the oestrous cycle is shown in Table 2 and Fig. 1. Table 2 shows graded responses to increasing doses of hypothalamus extracts as observed previously in this system (Hartley *et al.* 1973). The LH releasing activity of the extract of the group slaughtered on day 16 of the cycle was high (MED = 0·00625 equivalents). The potency declined with the onset of oestrus and remained low at 6 h and 36 h after onset (MED = 0·025 equivalents in each case). Intermediate potencies were recorded at 12 h after the onset of oestrus and on day 10 of the cycle (MED = 0·0125 equivalents in each case).

Table 2. *Summary of the effects on luteinizing hormone (LH) release* in vitro *of hypothalamus extracts obtained at various stages of the oestrous cycle in sheep*

	Mean LH content of medium $\left(\frac{\text{treated}}{\text{control}}\times 100\right)$ at the following doses of extract (hypothalamus equivalents):				Minimal effective dose (hypothalamus equivalents)
Stage of cycle	0·025	0·0125	0·00625	0·00313	
Day 16	196·0 (2/2)	186·3 (3/3)	159·9 (2/3)	78·5 (0/1)	0·00625
Oestrus:					
0 h	232·5 (2/2)	153·9 (1/3)	74·5 (0/1)	—	0·025
6 h	278·1 (2/2)	158·5 (1/3)	119·7 (0/1)	—	0·025
12 h	248·4 (2/2)	215·8 (2/3)	91·3 (0/1)	—	0·0125
36 h	205·4 (2/2)	117·0 (0/2)	—	—	0·025
Day 10	216·8 (3/3)	228·2 (3/3)	126·7 (0/1)	—	0·0125

Figures in parentheses represent number of significant effects/number of experiments.

Pituitary LH content

The group mean results for the LH levels in the anterior pituitaries of experimental ewes are shown in Table 3 and Fig. 1. Pituitary LH content was greatest at 0 h, slightly lower at 6 h and had declined markedly by 12 h and 36 h after the onset of oestrus. By day 10 of the cycle the LH content had increased and this high level was maintained at day 16.

Plasma LH content

The mean LH content of the plasma of the two experimental ewes is shown in Fig. 1. Luteinizing hormone content of the plasma was low before and at the onset of oestrus. A marked peak was observed between the onset of oestrus and 16 h after onset, by which time the level had again returned to baseline. Levels remained low up to the final sample taken 32 h after onset of oestrus.

Table 3. *Changes in pituitary luteinizing hormone (LH) content during the oestrous cycle in sheep (Means* ± S.E.M.)

Stage of cycle	Anterior pituitary LH content (µg NIH-LH equiv./mg anterior pituitary)	Anterior pituitary LH content (mg NIH-LH equiv./gland)	Mean λ of assays
Day 16	15·3 ± 1·9	5·7 ± 1·0	0·19
Oestrus:			
0 h	24·7 ± 6·9	8·7 ± 2·1	0·16
6 h	19·6 ± 7·3	6·9 ± 2·4	0·15
12 h	4·8 ± 1·3	1·8 ± 0·4	0·17
36 h	6·7 ± 1·8	2·7 ± 0·8	0·15
Day 10	18·9 ± 2·7	7·2 ± 1·6	0·20

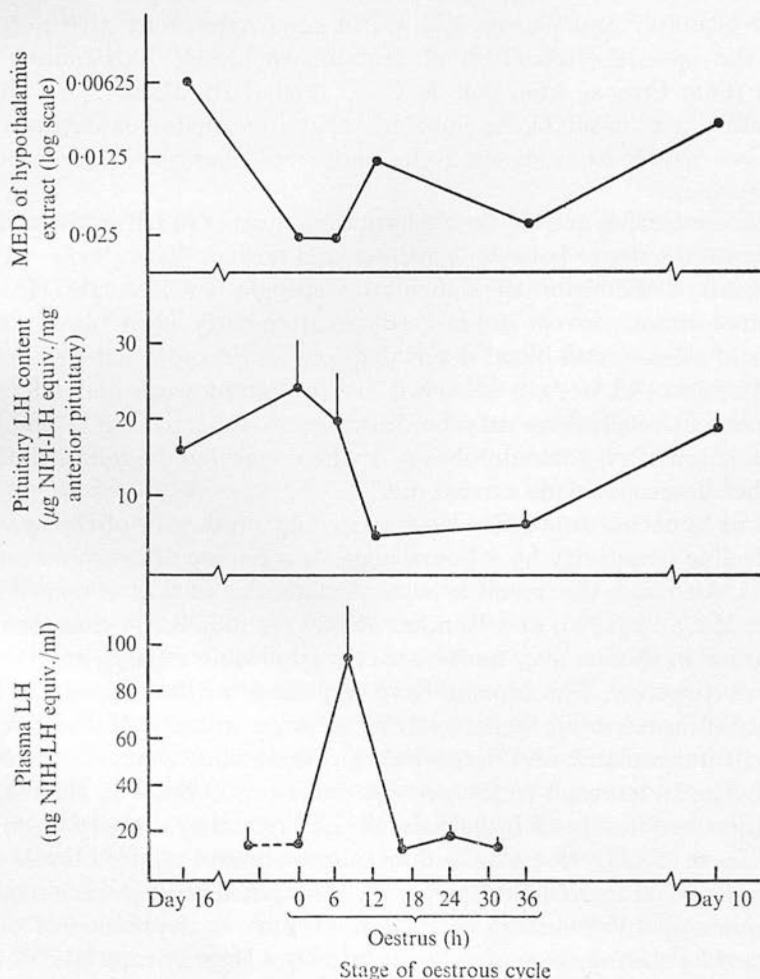

Fig. 1. Changes in the luteinizing hormone (LH) releasing activity of the hypothalamus and in LH in the pituitary gland and plasma during the oestrous cycle of sheep. Means + S.E.M. are shown. MED = minimal effective dose.

DISCUSSION

The variation in pituitary LH among individual animals was large, but a marked reduction in pituitary LH was apparent in the 12-h and 36-h groups of sheep. The decline in pituitary LH which occurred after 6 h correlates with the findings of Santolucito, Clegg & Cole (1960) and Robertson & Hutchinson (1962). Dierschke & Clegg (1968) produced evidence from pituitary assays for a maximal release of pituitary LH 8–16 h after the onset of oestrus. The peak of LH in the blood recorded by radioimmunoassay occurred within the first 16 h of oestrus, and this finding is in agreement with previous work (Niswender, Roche, Foster & Midgley, 1968; Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969). The rapid fall seen in pituitary LH coincides with the occurrence of this peak.

The results for oestrous cycle lengths, ovarian follicles and corpora lutea, plasma progesterone and pituitary and plasma LH are in good agreement with published information for the species (Robertson & Hutchinson, 1962; Nalbandov, 1964; Niswender *et al.* 1968; Cupps, Anderson & Cole, 1969; Erb, Randel & Callahan, 1971). It is therefore felt justifiable to conclude that hypothalamus extracts were obtained from ewes which were showing the endocrine changes characteristic of typical oestrous cycles.

The amount of LH releasing activity in the hypothalamus, as of LH in the pituitary gland, must represent a balance between synthesis and release. The interpretation of results for gland content of hormones is difficult and speculatory in nature. However, short-term falls in hormone levels in glands have frequently been interpreted as evidence of peaks of release, and blood determinations have confirmed this belief as in the case of LH release at oestrus observed in the present work and referred to earlier. Thus, tentative conclusions may be drawn as to the meaning of the hypothalamic LH releasing activity results obtained when considered against the background of the other determinations carried out.

The peak level of hypothalamic LH releasing activity on day 16 of the cycle and the subsequent decline in activity by 0 hours suggests a release of neurohumor from the hypothalamus. Although this result is somewhat similar to that observed in the rat by Chowers & McCann (1965) and Ramirez & Sawyer (1965), conclusions drawn from results obtained in the rat may not be strictly applicable to large animals such as the sheep. Unfortunately, few reports have appeared on fluctuations in hypothalamic gonadotrophin releasing factor activity in large animals: Hackett & Hafs (1969) studied pituitary gland and hypothalamic endocrine changes during the bovine oestrous cycle. In contrast to the present findings and those in the rat, they concluded that increased levels of hypothalamic LH releasing activity were associated with the release of LH, ovulation and luteal growth and reduced levels of LH releasing activity were characteristic of the period of maximal progesterone secretion. The method of assessing LH releasing activity used, namely depression of ovarian ascorbic acid caused by the injection of extracts into OAAD assay rats, is of doubtful specificity and this was not examined in detail. In view of these circumstances and the lack of other published information on hypothalamic gonadotrophin releasing activity in the cow, it is difficult to assess the significance of this work.

In the sheep, Jackson, Roche, Foster & Dziuk (1971) have obtained information

which became available only after the conclusion of the experiments described here. These workers used a rat pituitary incubation system to assess levels of LH releasing activity in ewes killed on days 4, 6, 10, 12, 14 and 16 of the cycle and during 'pro-oestrus'. Levels of hypothalamic LH releasing activity were low early in the cycle and rose gradually and significantly by days 10 and 12 and were lowest at 'pro-oestrus'. These workers did not study the oestrous period itself. In agreement with present findings for the 10-day group, high levels of hypothalamic LH releasing activity were recorded in mid-cycle. The decline in hypothalamic LH releasing activity by days 14 and 16 noted by these workers would appear to conflict with the present results where the peak hypothalamic potency was recorded on day 16. In their studies, hypothalamic LH releasing activity was lowest at 'pro-oestrus'. This term was not defined but may refer to the short period before the onset of oestrus when the ram is attracted to the ewe but the ewe refuses to accept the ram. If this interpretation is correct, 'pro-oestrus' would almost coincide with the 0-h group in the present experiments in which low values for hypothalamic LH releasing activity were also recorded.

Using a radioimmunoassay which employed as antigen the decapeptide claimed by Matsuo, Baba, Nair, Arimura & Schally (1971) to be a common FSH/LH releasing factor, Kerdelhué & Jutisz (1972) detected in one ewe increases in plasma content 2 days before the LH peak and again from 1 h before the start of the LH peak to 8 h after its end. The results of this and the present work are compatible although the fact that only one animal was used to obtain the radioimmunoassay data means that this information must be treated with caution. In the present study, hypothalamic LH releasing activity reached a minimal level not later than 40 min after the onset of oestrus. Plasma LH started to rise during the first 8 h of oestrus and the peak was reached either during this period or in the period between 8 and 16 h after onset. The experiment was not designed to follow accurately the time-course of these changes. Thus it is possible that the decline in hypothalamic LH releasing activity could have preceded the LH rise by as little as 1 h as found by the French workers, or by substantially longer.

While the present study concerned the LH releasing activity of the hypothalamus, the question of the control of FSH release must not be overlooked. The lack of sensitivity of the only FSH assay available, that of Steelman & Pohley (1953), precluded any attempt to assess changes in hypothalamic FSH releasing activity during the cycle. It was observed previously (Hartley *et al.* 1973) that FSH and LH releasing activity could not be separated in purified fractions obtained by gel filtration on Sephadex of an ovine hypothalamus extract. Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk & White (1971) and Amoss, Burgus, Blackwell, Vale, Fellows & Guillemin (1971) have reported that pure LH releasing factor contains FSH releasing activity, and Schally's group have proposed that there is a common FSH/LH releasing hormone. This suggests that changes in the LH releasing activity of the hypothalamus during the cycle would be accompanied by parallel changes in FSH releasing activity. The significance of the marked fall in hypothalamic LH releasing activity seen in the present work between day 16 of the cycle and the onset of oestrus (0 h) may therefore be related to FSH release rather than LH release or to FSH release in addition to LH release. A decline in the pituitary gland content of

FSH has been reported to occur in the 8 h preceding the onset of oestrus in the ewe (Robertson & Rakha, 1966) which adds further weight to this hypothesis but recent investigations with radioimmunoassay show a peak of serum FSH coinciding with that of LH (L'Hermite, Niswender, Reichert & Midgley, 1972) and no increase prior to the onset of oestrus.

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SIMULTANEOUS DETERMINATION OF LUTEINIZING HORMONE AND LUTEINIZING HORMONE RELEASING HORMONE IN THE JUGULAR VENOUS BLOOD OF THE SHEEP AT OESTRUS

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The occurrence of a peak of luteinizing hormone (LH) in the peripheral blood of the sheep within the first 16 h of oestrus is well established (Geschwind & Dewey, 1968; Niswender, Roche, Foster & Midgley, 1968; Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969). Changes in the LH releasing hormone (LH-RH) content of the hypothalamus have been correlated with the occurrence of the plasma LH peak and the accompanying decline in pituitary LH content (Crighton, Hartley & Lamming, 1973). Using a radioimmunoassay for LH-RH, Kerdelhué & Jutisz (1972) detected increases in plasma LH-RH content in one ewe 2 days before the LH peak and again from 1 h before the start of the LH peak to 8 h after its end. The present report describes the simultaneous determination of LH and LH-RH in samples taken at frequent intervals from onset of oestrus in the sheep.

Serial blood samples (2·5 ml) were taken from an indwelling jugular vein cannula at 15-min intervals for 18 h after the onset of oestrus in two Clun Forest ewes. The precise onset of oestrus was established by constant observation for mounting by a vasectomized ram which was removed once oestrus was observed. Plasma samples were stored at −20 °C until assayed. Both ewes were subjected to laparotomy 3 days later, and in both the ovaries showed recent ovulation. Aliquots of the plasma samples were assayed for LH using a specific double antibody radioimmunoassay (Crighton & Foster, 1972). Further aliquots were assayed for LH-RH using a specific radioimmunoassay described elsewhere (Jeffcoate, Fraser, Gunn & Holland, 1973; Jeffcoate, Fraser, Holland & Gunn, 1973). Methanol extracts of plasma were dissolved in assay buffer and assayed against standard synthetic LH-RH (Hoechst).

The results are shown in Fig. 1; LH peaks typical of oestrus were observed. The concentrations rose from baseline levels of 3–18 ng/ml to peaks of 250 and 236 ng/ml, the duration of the increased hormone level being about 10 h in each case. The LH-RH levels increased intermittently from less than 10 pg/ml to peaks ranging from several hundred pg/ml to > 10 ng/ml. These peaks occurred at 1·5- to 6-h intervals

and rapidly fell to very low or undetectable values in the next sample taken 15 min later. This fall is due in part to the dilution of jugular venous blood in the general circulation and in part to the rapid clearance of LH-RH ($T_{\frac{1}{2}}$ about 5 min in the sheep, unpublished observation).

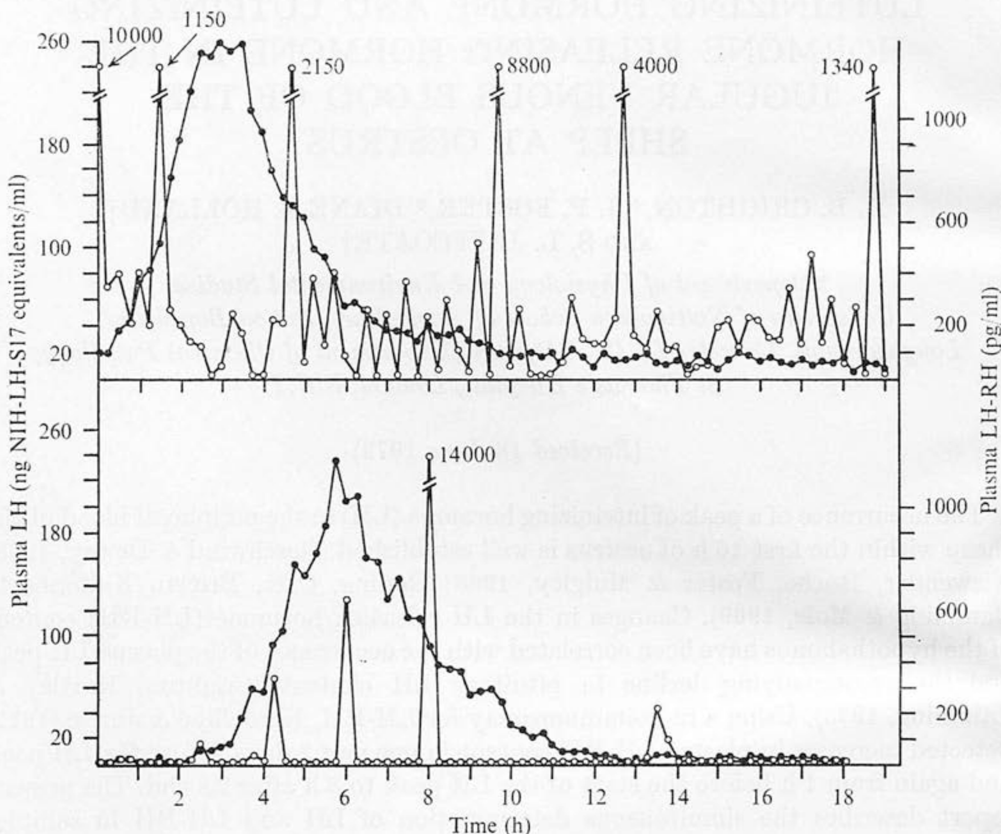

Fig. 1. Luteinizing hormone (LH) (●——●) and luteinizing hormone releasing hormone (LH-RH) (○——○) concentrations in jugular venous blood of two ewes at oestrus.

The finding of increases in plasma LH-RH has also been made in the rat on the afternoon of pro-oestrus (Fraser, Jeffcoate, Holland & Gunn, 1973) but our results differ from the pattern described by Kerdelhué & Jutisz (1972) in the ewe.

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LUTEINIZING HORMONE (LH) RELEASE AFTER SINGLE INJECTIONS OF A SYNTHETIC LH-RELEASING HORMONE (LH-RH) IN THE EWE AT THREE DIFFERENT REPRODUCTIVE STAGES AND COMPARISON WITH NATURAL LH RELEASE AT OESTRUS

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ABSTRACT

The possibility was investigated of using single i.v. injections of a synthetic luteinizing hormone-releasing hormone (LH-RH) to manipulate the reproductive pattern of the ewe.

Single i.v. injections of 150 µg synthetic LH-RH were given on Day 12 of the oestrous cycle, during seasonal anoestrus and on Day 16 post-partum in ewes which lambed during the breeding season. Blood samples were obtained at 5-, 10- or 15-minute intervals for 1 hour before and for 3 hours after treatment. Plasma LH concentrations were measured using a specific double antibody radioimmunoassay, the development of which is described. Laparotomy was performed on each animal 2-3 days after treatment.

The treatment induced LH peaks in all animals and ovulation in the majority. There was no significant difference between the groups in the LH response. The LH release was, however, much less than that found in untreated ewes sampled every 15 minutes for 18 hours during oestrus.

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INTRODUCTION

In 1971 a substance with LH-releasing activity was isolated from the porcine hypothalamus and found to have a decapeptide structure (1). This decapeptide was subsequently synthesized (2). In the experiments reported here the ability of a single intravenous injection of the synthetic decapeptide to release LH and cause ovulation in the Clun Forest ewe in different reproductive situations was tested. The response to treatment was compared among cyclic, seasonally anoestrous and post-partum anoestrous ewes and in each case with the natural release of LH at oestrus.

MATERIALS AND METHODS

A double antibody radioimmunoassay was used to estimate the LH concentrations of plasma samples. This assay was developed through all stages from the slaughterhouse collection of pituitary tissue from 2000 sheep. The LH preparations for raising antisera and for radioiodination were purified from the pituitary tissue by Dr. Anne Stockell Hartree, Department of Biochemistry, University of Cambridge and the LH activity was determined at each stage of purification at Sutton Bonington by the ovarian ascorbic acid depletion (OAAD) assay (3).

Raising of antisera: A partially purified extract with an LH activity in the OAAD assay approximately 0.5X NIH-LH-S1 was used for raising antisera. Rabbits were injected intradermally with an emulsion of 0.5-1.0 mg of the preparation in saline and Freund's complete adjuvant at 3-week intervals. Each rabbit was bled 10 days after the second and all subsequent injections.

Radioiodination: A highly purified preparation of LH derived from the original pituitary material was used. The LH activity of this material in the OAAD assay was 1.8 X NIH-LH-S1. A modified version of the method described by Greenwood, Hunter and Glover (4) was used.

Testing of the antiserum titres: The titre of each antiserum was tested by determining the percentage binding at different dilutions in the assay system with no unlabelled hormone present.

Assay method: A solution was prepared consisting of normal rabbit serum (NRS) at 1:400 dilution in assay diluent and antiserum at a dilution known to give approximately 50% binding of the labelled hormone. 0.2 ml of this solution was added to reaction tubes containing 0.5 ml of solutions of known concentration of standard LH (NIH-LH-S17, National Institutes of Health, Bethesda, U.S.A.) or known dilution (1:10 or 1:20) of test material. The tubes were incubated at 4°C for 24 hours and 0.2 ml of a solution of labelled LH diluted to give 10,000 counts per minute per 0.2 ml was added. The tubes were counted to determine total counts and incubated for 18 hours at 4°C. 0.1 ml of a 1:30 dilution of anti-rabbit gamma globulin (MR66, Burroughs Wellcome & Co., Beckenham, Kent, U.K.) was added.

The tubes were incubated for a further 6 hours at $4^{\circ}C$, centrifuged, the supernatants removed and the precipitates counted.

The LH concentrations of plasma samples were calculated by interpolating the percentage binding obtained on the standard curve.

Sensitivity and specificity: Antisera which gave sufficiently high binding of labelled hormone were tested for sensitivity and specificity. Standard curves were prepared for each antiserum with ovine NIH-LH and preparations of ovine follicle-stimulating hormone (FSH), growth hormone (GH) and prolactin.

Due to the lack of a suitably pure preparation of ovine thyrotrophin (TSH) the antisera were tested for cross reaction with TSH by assaying plasma samples believed to contain varying amounts of TSH but an unvarying amount of LH. The samples used were obtained from sheep before and after injection of 300 μg synthetic TRH. The most suitable antiserum was chosen for routine use.

Comparison of the radioimmunoassay with bioassay: Four ovine pituitary gonadotrophin preparations were assayed for LH using both the radioimmunoassay and the OAAD bioassay.

Test of the effect of sheep plasma on the standard curve: Standard curves were prepared using NIH-LH-S17 made up in: 1. Assay diluent alone, or 2. A 1:10 dilution of plasma from a hypophysectomized sheep (kindly supplied by Dr. J. Pelletier, INRA, Nouzilly, France).

Test for parallelism: A standard curve was prepared using NIH-LH-S17. At the same time various dilutions of a plasma sample were assayed. Two curves were plotted:

1. % binding against NIH-LH-S17 concentration (log scale) and
2. % binding against dilution of plasma sample (log scale).

The regression lines were calculated and tested for deviation from parallelism.

Experimental animals: Clun Forest ewes from the School of Agriculture flock were used.

Oestrous ewes: Two ewes were placed with a colour-marked vasectomized ram to establish cycle length and expected times of oestrus. The ewes were observed at frequent intervals when onset of oestrus was expected in the subsequent cycle. When onset of oestrus was observed blood samples (2.5 ml) were collected via a jugular vein cannula every 15 minutes for 18 hours. Plasma from these samples was stored at $-20^{\circ}C$ and later assayed for LH.

Treated ewes: Three groups of animals were treated: (i) Cyclic ewes. These were treated on Day 12 of the oestrous cycle during the period November, 1971 to March, 1972 and in October, 1972. (ii) Seasonally anoestrous ewes. These were treated during mid-seasonal anoestrus in June, 1972. (iii) Post-partum anoestrous ewes. The ewes used in this group were treated with a regime of shortening daylength from

Table I. Luteinizing hormone (LH) content of various ovine gonadotrophin preparations as measured by biological and radioimmunological assays

Preparation	LH content (µg NIH-LH-S17/mg)		Index of Discrimination
	bioassay λ	radioimmunoassay	
CM_1 (FSH-rich fraction from pit. extract)	209(132-315) 0.20	106	1.97
CM2 (LH-rich fraction from pit. extract)	477(336-683) 0.18	460	1.03
Crude extract (ewe pit.)	186(136-255) 0.16	160	1.16
Pit. extract (LH-rich)	360(220-570) 0.24	360	1.00
		Mean	1.29

Figures in parentheses are fiducial limits of error at P = 0.95

December, 1971, at a time when they were pregnant. These ewes lambed in April, 1972 and some showed oestrus 7-8 weeks later and were mated by a fertile ram. Following mating they were returned to natural lighting conditions with the rest of the School of Agriculture flock. They lambed in October or November, 1972, that is during the normal breeding season for this breed. They were treated with synthetic LH-RH on Day 16 post-partum.

Treatment of animals and collection of blood samples: All the ewes in each treated group were given a single injection of 150 µg synthetic LH-RH (Farbwerke Hoechst AG, Frankfurt (Main), W Germany) via a jugular vein cannula.

In the case of the cyclic and seasonally anoestrous ewes blood samples (2.5 ml) were collected from this cannula every 10 minutes for 1 hour before and every 5 minutes for 3 hours after injection. Blood samples were collected from the post-partum ewes every 15 minutes for the same time periods. Plasma from these samples was stored at -20°C for later assay of the LH concentration.

Surgical investigation: All ewes were laparotomized 2-3 days after treatment and the ovaries examined.

Detection of oestrus: All ewes were tested for oestrus for at least 20 days before and after treatment with a colour-marked vasectomized ram.

RESULTS

Testing of the antiserum: The most suitable antiserum obtained gave approximately 60% binding of labelled LH at a dilution of 1:20,000. 0.5 ng NIH-LH-S17 generally gave 10% inhibition of binding.

Negligible cross reactions were observed with all hormone preparations tested. There was no difference between the levels of LH detected by the assay in plasma samples obtained before and after treatment of ewes with 300 µg synthetic TRH.

When various preparations containing ovine LH were assayed by both biological and radioimmunological methods, the Index of Discrimination $\left(\frac{\text{biological assay}}{\text{radioimmunological assay}}\right)$ ranged from 1.00 to 1.97 (Mean 1.29) (Table I).

The effect of sheep plasma in the standard curve: Inclusion of 1:10 sheep plasma caused a slight (<2%) decrease in percentage binding at each point on the standard curve.

Test for parallelism: When serial dilutions of a solution of NIH-LH-S17 were compared for effects on percentage binding with serial dilutions of a plasma sample, curves which did not deviate significantly from parallelism were obtained.

Table II Mean heights of and areas under natural oestrous LH peaks and LH peaks induced by treatment with synthetic LH-RH

	Height of LH peak (ng NIH-LH-S17 equivalents/ml) (mean ± S.E.M.)	Area under LH peak (sq. mm) (mean ± S.E.M.)
Natural oestrous LH peaks	247 ± 11.0	7519 ± 610.9
Induced LH peaks		
Post-partum anoestrus	114 ± 15.5	2050 ± 329.7
Oestrous cycle	79 ± 11.8	1058 ± 167.1
Seasonal anoestrus	110 ± 19.6	1839 ± 397.5

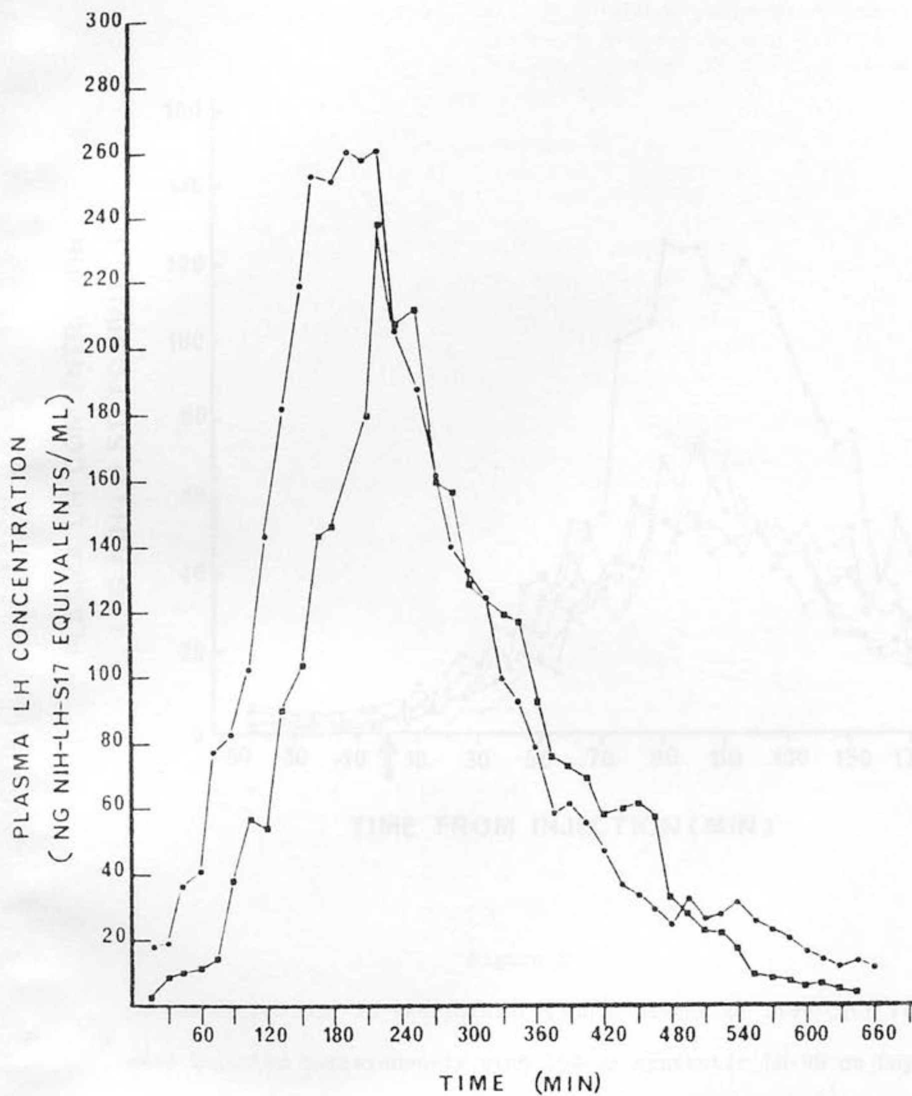

Figure 1

LH concentrations in the jugular venous plasma of two Clun Forest ewes at oestrus.

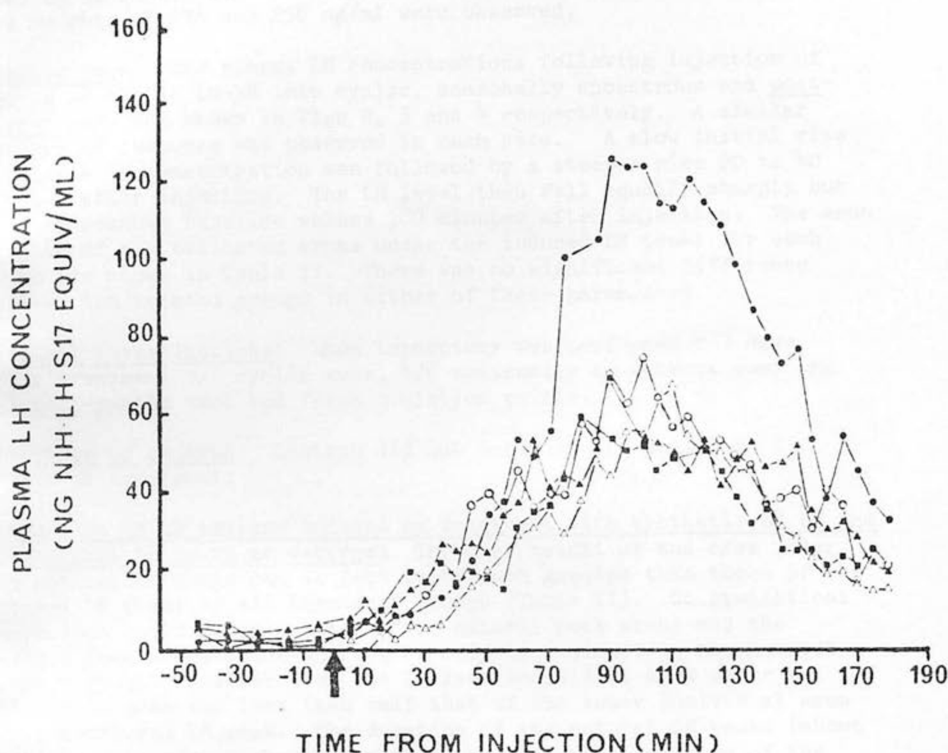

Figure 2

LH concentrations in the jugular venous plasma of five Clun Forest ewes injected intravenously with 150 μg synthetic LH-RH on Day 12 of the oestrous cycle. The arrow indicates the time of injection.

Oestrous ewes: The plasma LH concentrations during oestrus are shown in Fig. 1. Baseline LH levels ranged from 1-7 ng/ml in one ewe and 8-16 ng/ml in the other. LH peaks of approximately 10 hours duration and heights of 236 and 258 ng/ml were observed.

Treated ewes: The plasma LH concentrations following injection of 150 μg synthetic LH-RH into cyclic, seasonally anoestrous and *post-partum* ewes are shown in Figs 2, 3 and 4 respectively. A similar pattern of response was observed in each case. A slow initial rise in plasma LH concentration was followed by a steeper rise 20 to 40 minutes after injection. The LH level then fell equally sharply but had not reached baseline values 180 minutes after injection. The mean heights of and estimated areas under the induced LH peaks for each group are shown in Table II. There was no significant difference between the treated groups in either of these parameters.

Surgical investigations: When laparotomy was performed 2-3 days after treatment 5/5 cyclic ewes, 4/6 seasonally anoestrous ewes and 4/5 *post-partum* ewes had fresh ovulation points.

Detection of oestrus: Oestrus did not occur in any animal as a result of treatment.

Comparison of LH release induced by treatment with synthetic LH-RH and the natural LH peaks at oestrus: The mean height of and area under the natural LH peaks can be seen to be much greater than those of the induced LH peaks of all treatment groups (Table II). No statistical comparison could be made between the natural peak areas and the induced peak areas since there were only two animals in the natural oestrus group. However even the largest individual area under an induced LH peak was less than half that of the lower individual area under a natural LH peak. The duration of the natural LH peaks (about 10 hours) was also much greater than the estimated duration of the induced LH peaks (4-6 hours).

DISCUSSION

The radioimmunoassay for ovine LH was found to be sufficiently sensitive and specific to be used for routine determinations of the concentrations in plasma. A single intravenous injection of 150 μg synthetic LH-RH was found to cause LH release in Clun Forest ewes on Day 12 of the oestrous cycle, in mid-seasonal anoestrus and on Day 16 *post-partum*. A dose of 150 μg LH-RH was chosen because preliminary dose-response experiments in seasonally anoestrous ewes showed that this dose consistently provoked release of LH and ovulation. Lower doses released less LH and failed to cause ovulation consistently and higher doses did not cause a greater release of LH or improve on the percentage of animals ovulating (Foster and Crighton, unpublished data). The pattern of release was the same for all three groups. A slow initial rise in plasma LH after injection was followed by a steeper rise 20 to 40 minutes after injection which continued until a peak was reached 90 to 120 minutes after injection. The concentration then fell but was still above basal levels when sampling was terminated

180 minutes after injection. There was no significant difference between the groups in the mean heights of or mean areas under the LH peaks induced by this dose (Table II). There was, however, considerable individual variation in the height within each group. Thus it was not possible to demonstrate any difference in pituitary sensitivity to synthetic LH-RH between ewes on Day 12 of the oestrous cycle, in mid-seasonal anoestrus and on Day 16 post-partum. The doses used may have created non-physiological levels of LH-RH at the pituitary gland and hence the results do not rule out the possibility that there are differences in pituitary sensitivity between the groups. There was a tendency for the response to be lower on Day 12 of the cycle than in the other two groups (Table II) and it is possible that this may have reached significance if more animals had been used. This possible lower response could be attributed to the high plasma progesterone level present at this time.

In all animals given a single injection of 150 µg synthetic LH-RH the height and duration of the induced LH peak were much less than those of the natural LH peaks detected at oestrus. The total LH release as measured in terms of area under the peak was also much less (Table II). The LH release induced was however sufficient to induce ovulation in the majority of animals in all groups tested. Ovulation was held to have occurred when apparently normal ovulation points were present on the ovaries at laparotomy. No attempt was made to recover ova from these animals. However ova have been recovered from other ewes of the same breed treated with single injections of the same dose of synthetic LH-RH (W. Haresign, personal communication). There was no apparent correlation between the height of or area under the induced LH peak and the occurrence of ovulation.

The ovaries of Clun Forest ewes are therefore capable of ovulating during mid-seasonal anoestrus and during the third week post-partum. Thus the anoestrous state of these animals is apparently not due to low sensitivity of the ovaries to gonadotrophin stimulation, particularly since the induced LH release was less than that which occurs at normal oestrus.

Oestrus was not induced in any of the treated ewes. This is presumably due to the lack of a suitable steroid environment. In the anoestrous ewes there was no progesterone priming prior to treatment which is known to be necessary for oestrus to occur (5). In the cyclic ewes the progesterone level was maximal for the cycle at the time of treatment. Whether oestradiol release occurs as a result of follicular development induced by LH-RH treatment is not clear. There was no grossly-apparent steroid-induced stimulation of uterine development observed in the treated animals at laparotomy.

Whilst this work was in progress, and after its completion, reports were published confirming that the synthetic decapeptide causes LH release in the ewe *in vivo* (6, 7, 8, 9). It was also shown in our laboratory that the decapeptide causes LH and FSH release from the

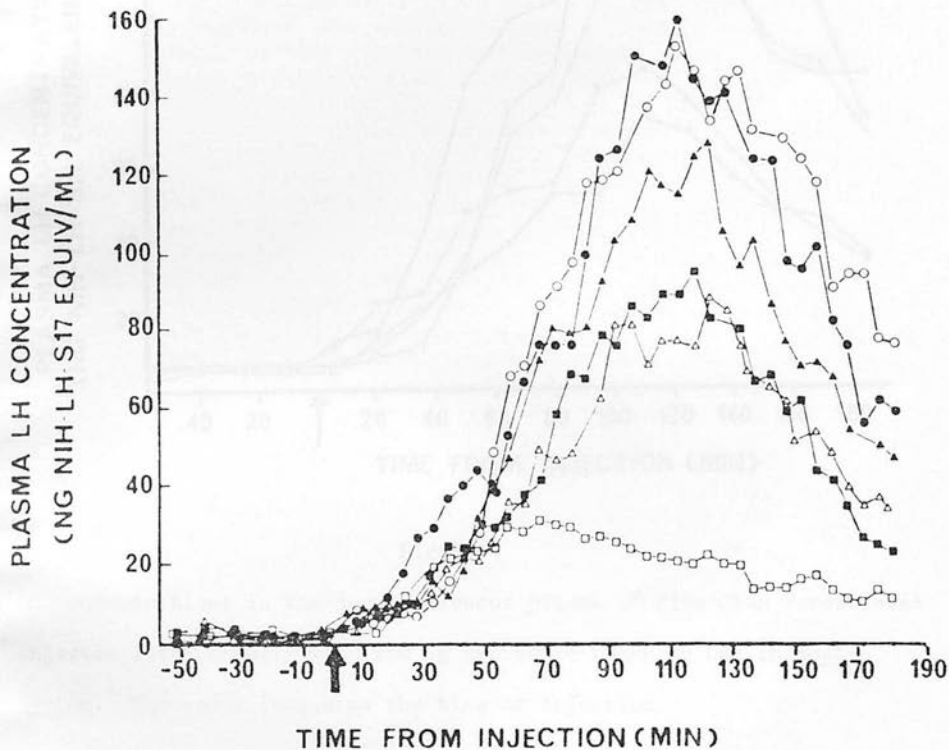

Figure 3

LH concentrations in the jugular venous plasma of six Clun Forest ewes injected intravenously with 150 µg synthetic LH-RH during seasonal anoestrus. The arrow indicates the time of injection.

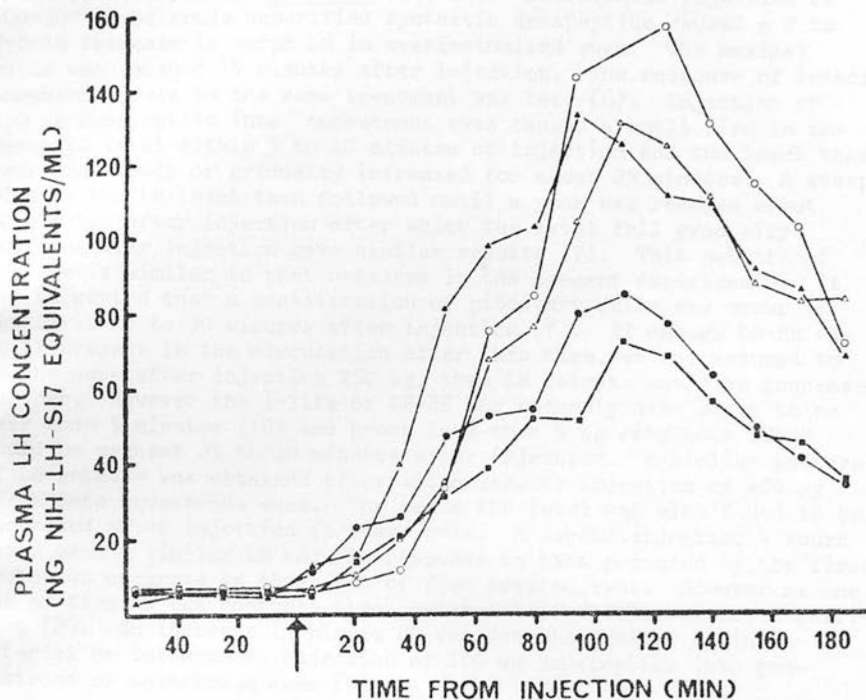

Figure 4

LH concentrations in the jugular venous plasma of five Clun Forest ewes injected intravenously with 150 μg synthetic LH-RH on Day 16 *post-partum*. The arrow indicates the time of injection.

ovine pituitary gland in vitro (10, 11). Intravenous injection of 100-300 μg of crude unpurified synthetic decapeptide caused a 2 to 7-fold increase in serum LH in ovariectomized ewes. The maximal value was reached 15 minutes after injection. The response of intact anoestrous ewes to the same treatment was less (6). Injection of 250 μg decapeptide into anoestrous ewes caused a small rise in the serum LH level within 5 to 10 minutes of injection and the level then remained steady or gradually increased for about 25 minutes. A steep rise in the LH level then followed until a peak was reached about 90 minutes after injection after which the level fell gradually. Intramuscular injection gave similar results (7). This pattern of release is similar to that obtained in the present experiments. It was suggested that a sensitization of pituitary cells may occur in the first 25 to 30 minutes after injection (7). If enough LH-RH was still present in the circulation after this time, as was assumed to be the case after injection 250 μg, then LH release would be increased further. However the ½-life of LH-RH has recently been shown to be less than 5 minutes (12) and hence less than 5 μg exogenous LH-RH would be present 25 to 30 minutes after injection. A similar pattern of LH release was obtained after intramuscular injection of 400 μg LH-RH into anoestrous ewes. The serum FSH level was also found to be increased after injection in these ewes. A second injection 4 hours later gave a similar LH and FSH response to that produced by the first. Ovulation occurred in three out of five treated ewes. However as one out of five of the controls also ovulated this result was not significant (8). An increase in plasma LH was detected following intra-arterial or intravenous injection of 100 μg decapeptide into pro-oestrous or anoestrous ewes (9).

This work demonstrates that single injection of the synthetic LH-RH will induce LH release and ovulation in different reproductive situations. It was not possible to demonstrate any clear differences in the response to treatment in the three situations tested although there was an indication of a lower release of LH in the ewes treated at a stage of the cycle when the progesterone level was maximal.

The LH release induced by single injection in all three groups was much less than natural LH release at oestrus. Work is currently in progress to determine whether alternative means of administration can be used to simulate more accurately the natural oestrous LH peak. The success of any treatment in practice is likely to depend on how closely the induced hormonal changes approach those which occur in the natural situation.

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PLASMA LH AND PROGESTERONE LEVELS AFTER SINGLE OR MULTIPLE INJECTIONS OF SYNTHETIC LH-RH IN ANOESTROUS EWES AND COMPARISON WITH LEVELS DURING THE OESTROUS CYCLE

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It has been shown that administration of a single intravenous injection of 150 or 300 μg synthetic LH-releasing hormone (LH-RH) to seasonally anoestrous Clun Forest ewes induced LH release in all animals and ovulation in the majority (Foster & Crighton, 1973, 1974). The induced LH peak was, however, much smaller than that observed at oestrus (Foster & Crighton, 1974). Luteal function, as assessed by peripheral plasma progesterone levels, was absent in the majority of treated animals (Crighton, Foster, Haresign, Haynes & Lamming, 1973; Haresign, Foster, Crighton, Haynes & Lamming, 1975), possibly because of an inadequate release of LH before ovulation. As an alternative means of administration to simulate more accurately the natural preovulatory LH peak, a single dose of 150 μg LH-RH was administered subcutaneously or intramuscularly instead of intravenously, but a greater release of LH was not obtained (S. C. Barnett, J. P. Foster and D. B. Crighton, unpublished data). Similarly, increasing the dose of the single intravenous injection from 150 to 300 μg failed to increase the height of (110 ± 20 and 118 ± 16 ng/ml respectively) or area under (1839 ± 398 and 2097 ± 247 mm^2 respectively) the induced LH peak (Foster & Crighton, 1973).

The development of a radioimmunoassay for LH-RH-like activity (Jeffcoate, Fraser, Gunn & Holland, 1974) allowed its simultaneous determination with that of LH in the jugular venous plasma of the sheep at oestrus (Crighton, Foster, Holland & Jeffcoate, 1973). This work suggested that, in order to simulate the natural LH peak in anoestrous ewes, administration of synthetic LH-RH intravenously as a series of injections might be appropriate.

In the present work, two cyclic and nine anoestrous Clun Forest ewes were used during the summer and autumn of 1973, respectively. All ewes were placed with a colour-marked vasectomized ram in order to detect the occurrence of oestrus. The cyclic ewes were bled (2 ml) from an indwelling jugular vein cannula at 4-min intervals from the onset of oestrus for 18 hr for the purposes of two separate studies. Every fourth sample only was used for the present work.

The anoestrous ewes were allocated to two groups: (1) five animals received a

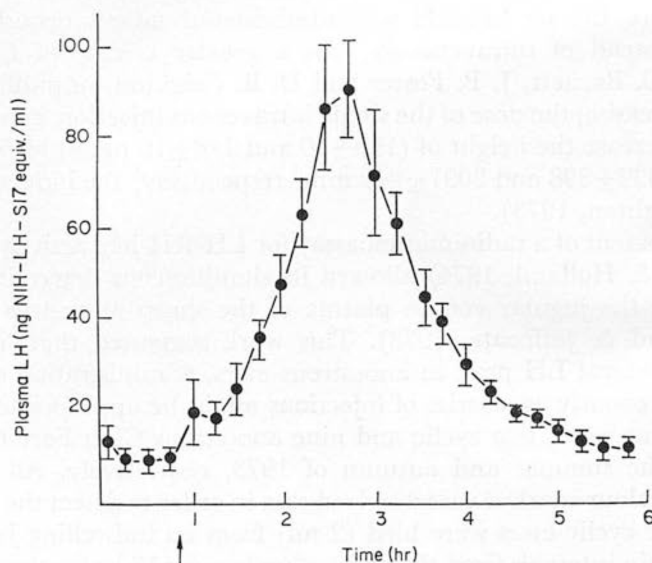
single injection of 150 μg synthetic LH-RH through a jugular vein cannula; (2) four animals received four injections each of 30 μg synthetic LH-RH at 90-min intervals followed by a fifth injection of 30 μg 5 hr after the fourth. A total dose of 150 μg was thus administered over a 9½-hr period. This sequence of injections was designed to mimic the pattern of natural discharge of LH-RH-like immunoreactivity previously observed at oestrus (Crighton, Foster, Holland & Jeffcoate, 1973). All treated animals were sampled (2 ml) from the jugular vein cannula at 15-min intervals for 1 hr before and up to 12 hr after the first or only injection of synthetic LH-RH. Plasma from all samples was stored at −20°C for later assay of the LH concentration by a double-antibody radioimmunoassay (Foster & Crighton, 1974). The standard used was NIH-LH-S17. In addition, blood samples (10 ml) were taken from the treated ewes daily for 3 days before and 24 days after treatment and progesterone levels were measured by radioimmunoassay (Haresign *et al.*, 1975).

Laparotomy was carried out on all treated ewes 4 days after treatment and the ovaries were examined.

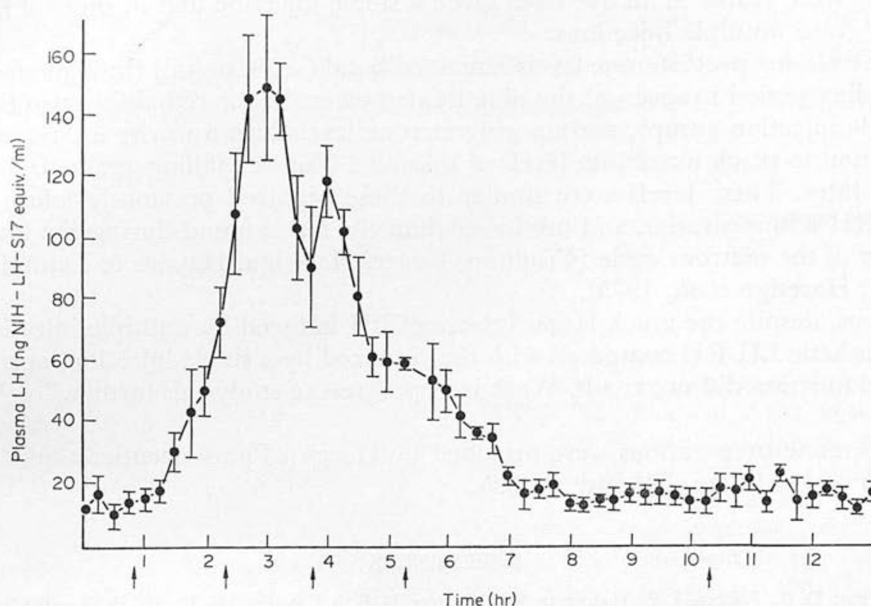
The two ewes sampled at oestrus showed preovulatory LH peaks similar to those observed previously (Foster & Crighton, 1974).

In each of the five anoestrous ewes treated with a single injection of synthetic LH-RH, the pattern of release following treatment (Text-fig. 1) was similar to that observed in ewes given identical treatment in previous experiments (Foster & Crighton, 1974).

All four anoestrous ewes treated with the multiple-injection sequence responded to the initial dose of synthetic LH-RH with a rise in the LH level. There was an accelerated rate of rise following the second injection, followed



TEXT-FIG. 1. Mean LH concentrations in the jugular venous blood of five ewes given a single injection of synthetic LH-RH. The arrow indicates the time of injection of 150 μg LH-RH. Vertical bars represent the S.E.M.



TEXT-FIG. 2. Mean LH concentrations in the jugular venous blood of four ewes given multiple injections of synthetic LH-RH. The arrows indicate the times of injection of 30 μg LH-RH (total dose 150 μg LH-RH). Vertical bars represent the S.E.M.

by a decline in the LH level. Another elevation in plasma LH occurred following the third injection. Thereafter, the level fell to baseline (Text-fig. 2). The height of the LH peak induced by multiple injections was significantly greater than that induced by single injection (172 ± 16 versus 98 ± 12 ng/ml, $P < 0{\cdot}01$), as was the duration of ($6{\cdot}87 \pm 0{\cdot}87$ versus $4{\cdot}30 \pm 0{\cdot}22$ hr, $P < 0{\cdot}05$) and the area under (7442 ± 815 versus 2477 ± 279 mm^2, $P < 0{\cdot}001$) the peak. Since the peak levels were not reached at exactly the same time after injection in the individual animals, the mean heights of the LH peaks are different from the maxima shown in Text-figs 1 and 2. The LH release induced by multiple injections of LH-RH resembled the natural preovulatory LH release (height 159 ± 24 ng/ml, duration $8{\cdot}8 \pm 0{\cdot}62$ hr, area $12{,}119 \pm 2719$ mm^2) more closely than that induced by a single injection. The results provide evidence, therefore, that the natural pattern of LH-RH-like immunoreactivity observed previously in jugular venous blood at oestrus (Crighton, Foster, Holland & Jeffcoate, 1973) may indicate the natural discharge pattern of LH-RH from the hypothalamus, although it is also possible that the pattern observed could have been influenced by the pituitary. It is noteworthy that the lack of response to the fourth and fifth injections is in agreement with the finding that endogenous peaks of LH-RH-like immunoreactivity which occur after the natural LH peak at oestrus are not associated with further increases in the LH level (Crighton, Foster, Holland & Jeffcoate, 1973). The doses of synthetic LH-RH used, however, may have resulted in non-physiological levels at the pituitary gland, thus making comparison of the natural and artificial situations hazardous.

When laparotomy was carried out 4 days after treatment, fresh ovulation

points were visible in all five ewes given a single injection and in three of four ewes given multiple injections.

The plasma progesterone levels remained basal (<0·4 ng/ml) throughout the sampling period in seven of the nine treated ewes. In the remaining two ewes (single injection group), plasma progesterone levels began to rise 3 days after injection to reach maximum levels of 1·4 and 1·0 ng/ml, falling again 10 to 12 days later. These levels were similar to those reported previously following LH-RH administration and are lower than the levels found during the luteal phase of the oestrous cycle (Crighton, Foster, Haresign, Haynes & Lamming, 1973; Haresign *et al.*, 1975).

Thus, despite the much larger release of LH induced by multiple injections of synthetic LH-RH compared with that induced by a single injection, normal luteal function did not result. Work is in progress to study this further.

Hormone preparations were provided by Hoechst Pharmaceuticals and the National Institutes of Health, U.S.A.

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PITUITARY RESPONSIVENESS TO A SINGLE INJECTION OF SYNTHETIC LUTEINIZING HORMONE RELEASING HORMONE BEFORE AND AFTER THE NATURAL PREOVULATORY PLASMA LUTEINIZING HORMONE PEAK IN THE SHEEP

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Peaks of immunoreactivity detected by the luteinizing hormone releasing hormone (LH-RH) radioimmunoassay of Jeffcoate, Fraser, Gunn & Holland (1973) occur in the jugular venous blood of the sheep before, during and after the luteinizing hormone (LH) peak at oestrus. Those occurring after the LH peak are not associated with further increases in the LH concentration (Crighton, Foster, Holland & Jeffcoate, 1973). If these immunoreactivity peaks give an indication of LH–RH release from the hypothalamus then changes in pituitary responsiveness must be important in the control of LH release. In the present experiment the responsiveness of the sheep pituitary gland to a single injection of 50 μg synthetic LH–RH was tested both before and after the natural preovulatory LH release.

Each ewe (Clun Forest) was given a single i.v. injection of 50 μg synthetic LH–RH (Hoechst) at either onset of oestrus (five ewes) or 20 h after onset (five ewes). The ewes were checked for oestrus using a vasectomized ram approximately every 30 min. Blood samples were obtained from each ewe via a jugular venous cannula, every 15 min for 25 h from onset of oestrus. Plasma was assayed for LH by radioimmunoassay (Foster & Crighton, 1974).

The mean LH concentrations (±S.E.M.) in the ewes injected at onset of oestrus are shown in Fig. 1 (*a*). The LH concentration reached a peak (mean 128, range 51–190 ng/ml) within 2 h of injection and had returned to baseline 7·7 h (mean) (range 5–11 h) after injection. There were no further LH peaks during the remainder of the sampling period with the exception of one ewe in which an increase (maximum 23 ng/ml) occurred between 9 and 12 h after onset of oestrus.

The earliest natural LH peak detected in this study began 3·5 h after onset of oestrus and previous work has indicated that the natural LH peak normally occurs between 4 and 16 h after onset (e.g. Geschwind & Dewey, 1968; Crighton *et al.* 1973). In the present experiment an increase in the LH concentration occurred within 30 min of LH–RH injection when this was administered within 30 min after onset of oestrus. This, together with the fact that the pattern of LH release was similar to that seen previously after a single injection of synthetic LH–RH (Foster & Crighton, 1974), suggests that the increased LH release was induced by synthetic LH–RH and was not the natural LH peak. Furthermore induction of an LH peak at the onset of oestrus seems to either abolish or reduce drastically the natural LH peak.

In the group injected 20 h after onset of oestrus, the preovulatory LH peak occurred before the injection, which resulted in a small LH response in each case. In two ewes, the LH concentrations were still in the declining phase at the time of injection (example in Fig. 1*b*). In the other ewes the injection was administered after the LH concentration had reached baseline (example in Fig. 1*b*). In both cases the responsiveness of the pituitary gland was greatly reduced compared with the onset of oestrus group. Thus the responsiveness of the pituitary gland to LH–RH is decreased after a natural LH peak.

This work extends the results of Reeves, Arimura & Schally (1971) which showed that the responsiveness of the sheep pituitary gland to purified LH–RH was high on the day of onset of oestrus, since it demonstrates that the nature of the response depends upon the relationship of the injection to the natural preovulatory LH release.

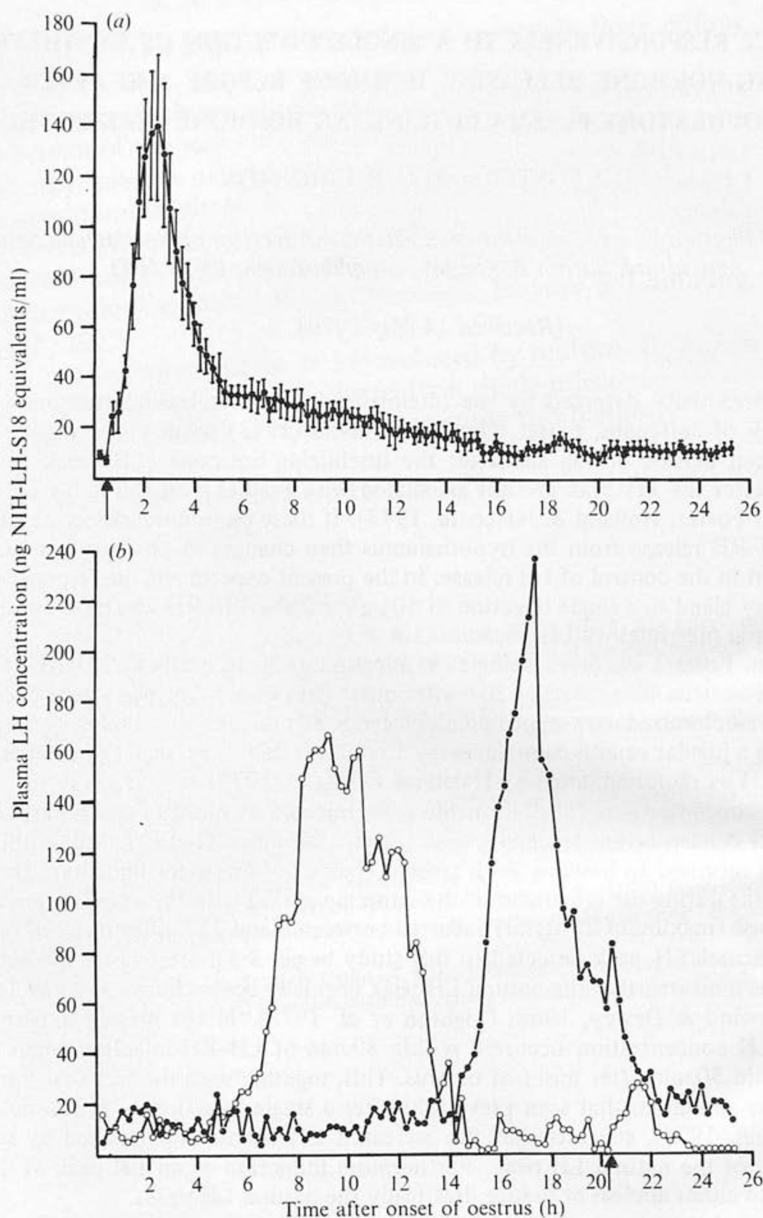

Fig. 1. LH levels in the jugular venous blood of ewes given a single i.v. injection (arrow) of 50 µg synthetic LH-RH. (*a*) Means of five animals injected at onset of oestrus, vertical lines represent ±S.E.M. (*b*) Individual results from two animals injected 20 h after onset of oestrus.

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LUTEINIZING HORMONE AND LUTEINIZING HORMONE RELEASING HORMONE-LIKE IMMUNOREACTIVITY IN THE JUGULAR VENOUS BLOOD OF SHEEP AT VARIOUS STAGES OF THE OESTROUS CYCLE

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SUMMARY

Luteinizing hormone and LH-RH-like immunoreactivity were measured in the jugular venous plasma of Clun Forest ewes at various stages of the oestrous cycle.

Blood samples were collected through jugular venous cannulae every 2 h for at least 20 days from three ewes during the breeding season. The ewes were checked twice daily for oestrus using a vasectomized ram. Plasma LH peaks of apparent height 112–192 ng NIH-LH-S17 equivalents/ml were detected at oestrus with basal levels of 2–15 ng/ml during most of the remainder of the 17-day oestrous cycle. Peaks of LH-RH-like immunoreactivity occurred at various times of the cycle. The apparent maximal level of these peaks was 220 pg/ml compared with basal levels of < 10 pg/ml. Further ewes (two for each group) were sampled at 4 min intervals for 12 h, (1) from onset of oestrus, (2) 36–48 h after onset of oestrus or (3) on day 10 of the oestrous cycle. In the ewes sampled at oestrus, peaks of LH-RH-like immunoreactivity were detected before, during and after the preovulatory LH peak. Those detected after the LH peak were unassociated with any further increases in the plasma LH level. In the ewes sampled 36–48 h after onset of oestrus and on day 10 of the cycle, several peaks of LH-RH-like immunoreactivity unassociated with any increases in the LH level were detected. These peaks, and those detected at oestrus, had durations of only one or two samples, and in some cases reached levels of several ng/ml compared with basal levels of < 10 pg/ml. The significance of these results is discussed.

INTRODUCTION

Radioimmunoassays for luteinizing hormone releasing hormone (LH-RH)-like immunoreactivity have recently been developed using synthetic LH-RH for radioisotopic labelling and as an antigen for raising antisera (Jeffcoate, Fraser, Gunn & Holland, 1973; Kerdelhué, Jutisz, Gillessen & Studer, 1973; Nett, Akbar, Niswender, Hedlund & White, 1973). Using such methods, detectable levels have been found in the blood of the rat (Fraser, Jeffcoate, Holland & Gunn, 1973) and the sheep (Crighton, Foster, Holland & Jeffcoate, 1973*a*; Kerdelhué *et al.* 1973; Nett, Akbar & Niswender, 1974). Crighton *et al.* (1973*a*) detected short-lived peaks of LH-RH-like immunoreactivity in the jugular venous plasma of ewes at 1·5–6 h intervals before, during and after the LH peak at oestrus when sampling

was carried out at 15 min intervals. Each peak had a duration of only one sample. For this reason and also because the half-life of injected LH-RH was found to be about 5 min, it was considered necessary for sampling to be carried out at even greater frequency in further work if the detailed pattern was to be determined. In this paper, work is described in which the LH and LH-RH-like immunoreactivity were measured in samples of jugular venous plasma obtained from cyclic ewes: (1) every 2 h for at least 20 days in order to obtain some idea of the times of the oestrous cycle at which peaks of LH-RH-like immunoreactivity occur, and (2) every 4 min for 12 h at particular stages of the oestrous cycle to determine the detailed pattern.

MATERIALS AND METHODS

Animals

Clun Forest ewes from the School of Agriculture flock were used during the breeding season.

Collection of blood samples

Blood samples were collected through indwelling jugular venous cannulae. Three ewes were sampled every 2 h for at least 20 days. Further ewes were sampled every 4 min for 12 h, (1) from the onset of oestrus, (2) 36–48 h after onset of oestrus, or (3) on day 10 of the oestrous cycle. Two animals were sampled in each group.

Each blood sample was centrifuged at 3000 ***g*** for 10 min within 1 h of collection and the plasma removed and stored at −20 °C as two aliquots. One aliquot was later assayed for LH-RH-like immunoreactivity. In the case of samples obtained at 4 min intervals, every third sample only was assayed for LH.

Assay methods

Plasma LH concentrations were measured using a specific double antibody radioimmunoassay (Foster & Crighton, 1974).

LH-RH-like immunoreactivity was measured by the radioimmunoassay method of Jeffcoate *et al.* (1973). The specificity of this assay is such that there is no cross-reaction with known peptide hormones such as thyrotrophin releasing hormone, vasopressin or oxytocin. Cross-reaction does occur however with peptides closely related to the decapeptide molecule of LH-RH, such as the CO_2H-terminal octapeptide and nonapeptide (see Discussion).

Detection of oestrus

The ewes sampled every 2 h were checked for oestrus twice daily using a vasectomized ram. The ewes sampled in the other groups were housed with a colour-marked vasectomized ram for at least a complete oestrous cycle before sampling to establish cycle length and expected times of oestrus. The ewes sampled from onset of oestrus or from 36 to 48 h after onset were observed approximately every 30 min throughout the day and night when oestrus was expected in the subsequent cycle so that the time of onset of oestrus could be determined accurately and sampling could commence at the appropriate time. In the onset of oestrus group this was within 30 min of first detection of oestrus.

RESULTS

The LH levels and LH-RH-like immunoreactivity of plasma samples obtained from one of the three ewes sampled every 2 h for 20 days are shown in Fig. 1. Baseline LH levels ranging from 2 to 15 ng NIH-LH-S17 equivalents/ml were present throughout most of the sampling periods in the three ewes sampled on this schedule. In all ewes an LH peak of apparent

height 112–192 ng/ml was observed at each oestrous period (Fig. 1). In addition in one ewe an LH peak of apparent height 59 ng/ml was observed on day 11 of the first cycle but not in the subsequent cycle (Fig. 1). In another ewe a peak of 115 ng/ml was observed on day 16 of the second cycle but not in the preceding cycle.

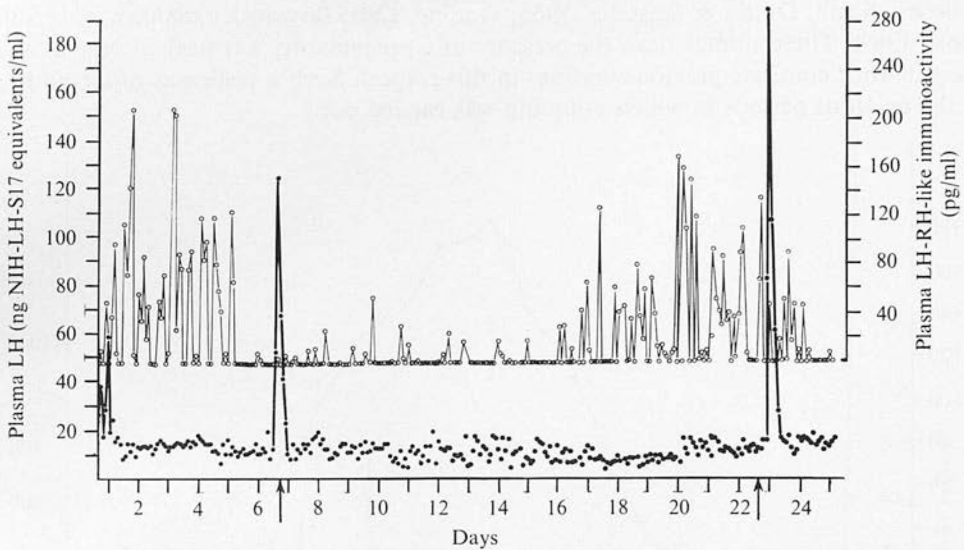

Fig. 1. LH (●) and LH-releasing hormone (LH-RH)-like immunoactivity (○) levels in the jugular venous plasma of a ewe sampled every 2 h throughout the oestrous cycle. The arrows indicate the times of first detection of oestrus.

Peaks of LH-RH-like immunoreactivity were detected at various times of the cycle. The apparent maximal level of these peaks was 220 pg/ml compared with basal levels of < 10 pg/ml. Each peak had a duration of only one sample. The peaks which occurred at times other than oestrus showed no obvious correlation with subsidiary LH peaks.

The LH levels and LH-RH-like immunoreactivity of plasma samples obtained from the ewes sampled for 12 h from onset of oestrus are shown in Fig. 2. LH peaks of height 139 and 183 ng/ml respectively were detected in the two ewes sampled at this time. Peaks of LH-RH-like immunoreactivity were detected before, during and after the preovulatory LH peak in both ewes, in a pattern similar to that observed previously when a 15-min sampling interval was used (Crighton *et al.* 1973*a*). However, in one of the animals the peak levels were substantially lower than most of those detected when frequent sampling was employed. Those peaks which occurred after the LH peak were not associated with any further increases in the LH level.

The LH levels and LH-RH-like immunoreactivity of plasma samples obtained from the ewes sampled from 36 to 48 h after onset of oestrus and for 12 h on day 10 of the oestrous cycle are shown in Figs 3 and 4 respectively. In both ewes in each of these two groups basal LH levels of 2–14 ng/ml were present throughout the sampling period. Several peaks of LH-RH-like immunoreactivity were detected during these periods, however, and these were unassociated with any increases in the LH level.

All the peaks of LH-RH-like immunoreactivity detected in the ewes sampled at 4-min intervals had a duration of only one or two samples and reached concentrations of up to 10 ng/ml compared with baseline levels of < 10 pg/ml.

DISCUSSION

Plasma LH concentrations during the oestrous cycle of the sheep have been measured previously (e.g. Geschwind & Dewey, 1968; Niswender, Roche, Foster & Midgley, 1968; Pelletier, Kann, Dolais & Rosselin, 1968; Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969). These studies show the presence of a preovulatory LH peak at oestrus and the present work confirms previous findings in this respect. Such a peak was observed in each of the oestrous periods in which sampling was carried out.

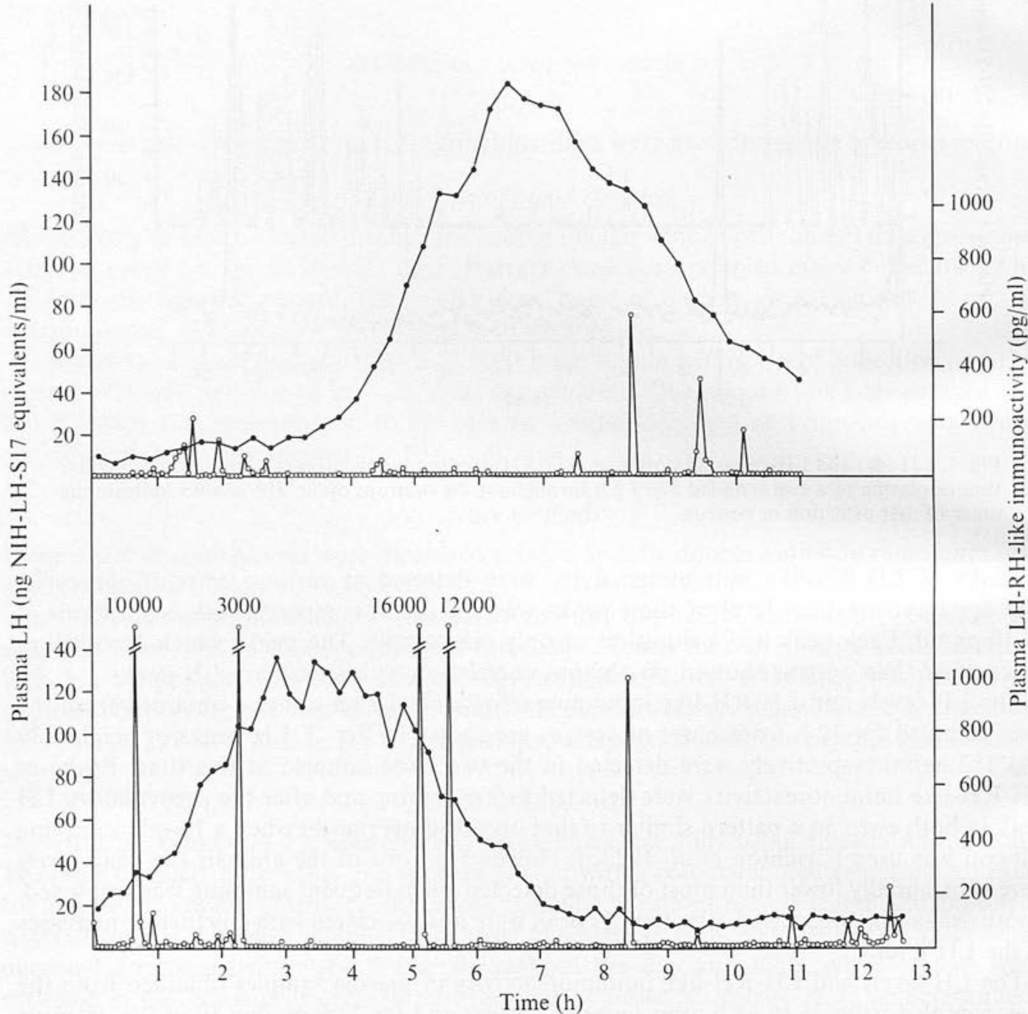

Fig. 2. LH (●) and LH-releasing hormone (LH-RH)-like immunoactivity (○) levels in the jugular venous plasma of two ewes sampled every 4 min from the onset of oestrus. Every third sample only was assayed for LH.

In the present work, sampling at 2 h intervals revealed that peaks other than the preovulatory one may occur in some cycles of some individuals. In one ewe a subsidiary peak occurred on day 11 of the cycle (Fig. 1). In a second ewe in which a subsidiary peak was observed on day 16 of the cycle, the expected preovulatory peak occurred on the following day. The purpose of these subsidiary peaks is not clear.

The LH-RH-like immunoreactivity measurements in the plasma samples obtained every 2 h for 20 days from cyclic ewes indicate that peaks occur at various times of the cycle and not only at oestrus as might be expected from previous considerations. Jackson, Roche, Foster & Dziuk (1971) assayed sheep hypothalamic extracts obtained at various stages of the oestrous cycle for LH-RH-activity using a rat anterior pituitary incubation system.

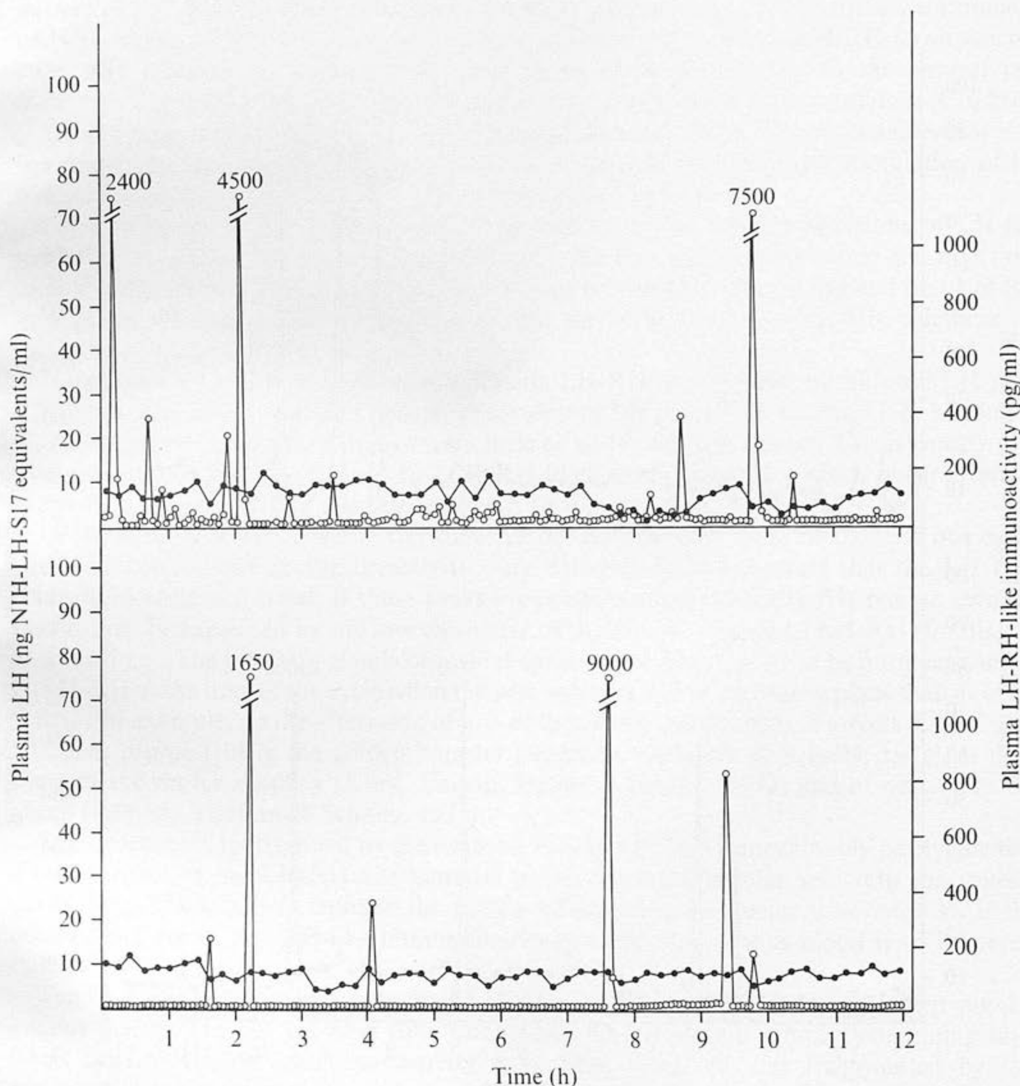

Fig. 3. LH (●) and LH-releasing hormone (LH-RH)-like immunoactivity (○) levels in the jugular venous plasma of two ewes sampled every 4 min from 36 to 48 h after onset of oestrus. Every third sample only was assayed for LH.

LH releasing activity was found to rise gradually during the cycle and then fall abruptly at pro-oestrus. Crighton, Hartley & Lamming (1973*b*) assayed the hypothalamic content of LH-RH at various stages of the sheep oestrous cycle using an ovine anterior pituitary tissue incubation system. Pituitary LH content and plasma LH concentration were also measured in the same animals by bioassay and radioimmunoassay respectively. The hypothalamic

LH-RH content and pituitary LH content were found to increase during the cycle from low levels at the end of oestrus whilst plasma LH remained at basal levels. On day 16 of the cycle the hypothalamic LH-RH content reached a maximal level and then fell before the onset of oestrus. Pituitary LH content fell and plasma LH rose during the first 12 h of oestrus. These results were interpreted as suggesting a release of LH-RH shortly before the onset of oestrus followed by a release of LH from the pituitary during the first 12 h of oestrus. The

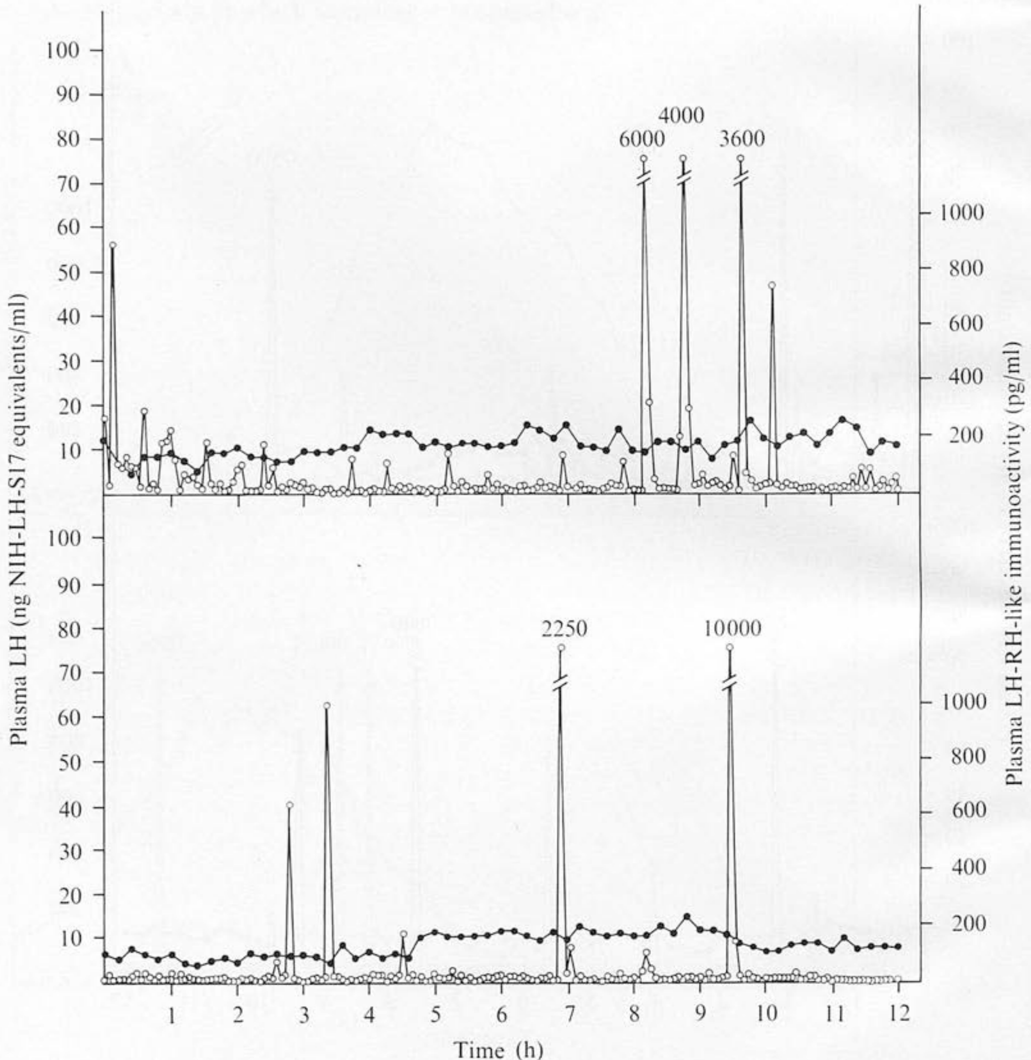

Fig. 4. LH (●) and LH-releasing hormone (LH-RH)-like immunoactivity (○) levels in the jugular venous plasma of two ewes sampled every 4 min on day 10 of the oestrous cycle. Every third sample only was assayed for LH.

levels of releasing activity in the hypothalamus probably represent a balance between synthesis and release and hence interpretation of changes in the level can only be speculative. Also since only one sample can be obtained from each animal, it is not possible to determine the detailed pattern of changes in content using these methods. In the present work the times

of sampling were chosen because they corresponded to those at which Crighton *et al.* (1973*b*) measured hypothalamic LH-RH activity.

Peaks of LH-RH-like immunoreactivity were detected before, during and after the preovulatory LH peak at oestrus. The peaks which occurred after the LH peak were unassociated with any further increases in the LH level. The results were similar to those obtained when a 15-min sampling interval was used (Crighton *et al.* 1973*a*). When the pattern of LH-RH-like immunoreactivity found in this physiological situation was simulated pharmacologically, by administering multiple injections of synthetic LH-RH to anoestrous ewes, LH release was induced which was much closer in quantity to the natural preovulatory LH release than that induced by single injection of the same total dose (Crighton, Scott & Foster, 1974; Crighton, Foster, Haresign & Scott, 1975). This is in agreement with the possibility that the preovulatory LH peak is induced by prolonged stimulation of the pituitary gland and not by a single short-term pulse of LH-RH.

Aiyer, Chiappa & Fink (1974) found that the second of two i.v. injections of LH-RH given to rats gave a greater LH response than the first, particularly when the injections were given at 60 min intervals on the afternoon of pro-oestrus. They suggested that LH-RH may prime the anterior pituitary gland so that further exposure to LH-RH enhances the response of the gonadotrophs.

It has been shown that injection of synthetic LH-RH shortly after an induced LH peak (Crighton *et al.* 1975) or after a natural preovulatory LH peak (J. P. Foster, G. F. M. Ball & D. B. Crighton, unpublished data) causes little or no further LH release. This is comparable with the finding that natural peaks of LH-RH-like immunoreactivity which occur after the preovulatory LH peak are unassociated with further increases in the LH level.

In the ewes sampled 36–48 h after onset of oestrus and on day 10 of the oestrous cycle, peaks of LH-RH-like immunoreactivity were detected despite the fact that the LH concentrations remained basal. If these peaks are related to increased LH-RH release then the results may be explained by the low sensitivity of the pituitary gland to LH-RH stimulation at these times. The pituitary glands of several species have been shown to be more responsive to LH-RH at the time of the cycle when the preovulatory LH release takes place than at other times. For example, on the afternoon of pro-oestrus in the rat (Cooper, Fawcett & McCann, 1973), at pro-oestrus in the golden hamster (Arimura, Debeljuk & Schally, 1972), at midcycle in the rhesus monkey (Krey, Yamaji, Butler & Knobil, 1972) and at oestrus in the sheep (Reeves, Arimura & Schally, 1971).

The clearance rate suggested by the results is very rapid. This is presumably partly due to a dilution effect as the LH-RH-like material passes from the jugular vein into the general circulation. These results indicate the futility of sampling at greater time intervals if the detailed pattern of LH-RH-like immunoreactivity in jugular venous blood is to be determined.

The identity of the immunoreactive LH-RH-like components found in sheep jugular venous plasma has not yet been fully established. Sheep plasma samples containing high levels of LH-RH-like immunoreactivity have been extracted and fractionated by ion exchange chromatography on carboxymethyl-cellulose followed by radioimmunoassay of the fractions (Jeffcoate & Holland, 1974). Three non-LH-RH peaks were identified on these columns. It has been shown that these components are not artifacts since if LH-RH is added to serum *in vitro* or *in vivo* the properties of the decapeptide are unchanged after extraction, chromatography and assay. The specificity of the antiserum used in the radioimmunoassay is directed towards the CO_2H-terminus of the decapeptide since changes in this end of the molecule result in a complete loss of immunoreactivity. It therefore appears more likely that if the components are formed by metabolism of LH-RH then they are formed as a result of changes in the NH_2-terminus. Nett *et al.* (1974) did not detect any

peaks of LH-RH-like immunoreactivity in the jugular venous plasma of sheep sampled every 4 min for 4 h at oestrus. This may be explained by the fact that the antiserum used in the radioimmunoassay of Nett *et al.* (1974) is specific for the decapeptide and has little cross-reaction with fragments of LH-RH.

If the LH-RH-like immunoreactive molecules detected in sheep jugular venous blood are metabolites of LH-RH then the levels of these molecules are presumably related to LH-RH release from the hypothalamus. Work is currently in progress to determine the identity of these molecules and the nature of their relationship with LH-RH, if indeed any exists.

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LUTEINIZING HORMONE RELEASE AFTER TWO INJECTIONS OF SYNTHETIC LUTEINIZING HORMONE RELEASING HORMONE IN THE EWE

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SUMMARY

Anoestrous ewes were given two injections of 30 μg synthetic luteinizing hormone releasing hormone (LH-RH) separated by one of the following intervals: 1·5, 3, 6, 12 or 24 h. The first injection caused an increase in the plasma LH concentration in each animal. The response to the second injection was dependent on the interval between the injections. When the second injection was administered 1·5 h after the first it caused a further increase in the LH concentration to maximal levels which were significantly greater than those induced in the other anoestrous groups. When the second injection was administered 3 h after the first, there was no significant difference between the responses to the two injections although the time to reach the maximal LH concentration was shorter and the height of the LH peak was greater in each animal following the second injection. When the second injection was administered 6, 12 or 24 h after the first, the LH response was significantly less, in terms of height and area of the induced peak, than following the first injection. The LH response to the second injection was particularly low in the 12 and 24 h groups. Two injections of 30 μg synthetic LH-RH were also administered at 1·5 h intervals to ewes on either day 10 of the oestrous cycle or at onset of oestrus. The pattern of LH responses in all these animals was similar to that observed in anoestrous ewes injected at 1·5 h intervals. The total LH release, as assessed in terms of area of the induced peaks, was significantly greater in the onset of oestrus group than in the day 10 group or any of the anoestrous groups.

Presumably the sensitization–desensitization sequence of the pituitary gland to LH-RH which has been demonstrated, together with the effects of sex steroid hormones, must play an important part in the development and decay of the natural preovulatory LH peak.

INTRODUCTION

It has been shown that multiple injections of synthetic luteinizing hormone releasing hormone (LH-RH) induce a much greater release of luteinizing hormone (LH) in anoestrous ewes than a single injection of the same total dose (Crighton, Foster, Haresign & Scott, 1975). The multiple injection schedule used was four injections of 30 μg synthetic LH-RH at 1·5 h intervals followed by a fifth injection, also of 30 μg, 5 h after the fourth. This sequence was based on the pattern of peaks of immunoreactivity detected by the LH-RH assay of Jeffcoate, Fraser, Gunn & Holland (1973) in the jugular venous blood of the ewe at oestrus (Crighton, Foster, Holland & Jeffcoate, 1973*a*). Most of the LH release was apparently induced by the first two injections, the second injection appearing to accelerate the increase in the plasma LH concentration caused by the first. Aiyer, Chiappa & Fink (1974) and

Edwardson & Gilbert (1976) have shown *in vivo* and *in vitro* respectively that LH-RH not only stimulates the release of LH but also has the ability to sensitize the pituitary gland of the rat so that an appropriately spaced second exposure to LH-RH causes a greater LH release than that produced by the first.

In the present work the apparent sensitizing effect of LH-RH observed by Crighton *et al.* (1975) in the anoestrous ewe was investigated further using different time-intervals between two equal injections. Responses to a fixed time-interval between two equal injections were also compared in reproductive states in which pituitary responsiveness to LH-RH was known to be either low or high (Foster & Crighton, 1976).

MATERIALS AND METHODS

Clun Forest ewes from the School of Agriculture flock were used.

Anoestrous ewes

These ewes were treated in mid-anoestrus. They were given two injections of 30 μg synthetic LH-RH separated by one of the following time-intervals: 1·5, 3, 6, 12 and 24 h. There were four ewes in each group. Blood samples (2·0 ml) were obtained from each ewe via an indwelling jugular vein cannula every 15 min for 1 h before and at least 6 h after each injection.

Cyclic ewes

Two injections of 30 μg synthetic LH-RH were administered at 1·5 h intervals to ewes at two stages of the oestrous cycle. The injections were made at either day 10 of the cycle or at onset of oestrus. Blood samples (2·0 ml) were obtained from these ewes every 15 min, two samples being taken before the first injection and sampling being continued until 7 h after the second injection. Oestrus was detected using a vasectomized ram. In the onset of oestrus group, the ewes were checked for oestrus at least every 30 min in the period when oestrus was expected and sampling was begun immediately after onset of oestrus was detected.

Each blood sample was centrifuged at 3000 ***g*** for 15 min and the plasma removed and stored at -20 °C for later assay by a specific radioimmunoassay for LH (Foster & Crighton, 1974). In addition, a further blood sample (5 ml) was obtained from each cyclic ewe just before injection and the plasma from this was assayed for progesterone by the method of Haresign, Foster, Haynes, Crighton & Lamming (1975). The LH results were analysed statistically using analysis of variance and Student's *t*-test to determine between- and within-group differences in terms of the heights and areas of the induced peaks.

RESULTS

Anoestrous ewes

The mean plasma LH concentrations ($\pm$ S.E.M.) in the anoestrous ewes are shown in Figs 1–3. Injection of the first dose of synthetic LH-RH resulted in a short-lived (approximately 3 h) increase in plasma LH concentration. The height and area of the peak varied considerably between individuals but the mean responses in the four groups in which it was possible to make accurate measurements (3, 6, 12 and 24 h groups) were not significantly different in terms of these parameters.

When the injections were separated by 1·5 h (Fig. 1) the LH concentration was still increasing in two animals and had just reached a peak in the other two when the second injection was given. The second injection caused a further increase in the LH concentration

to maximal levels which were significantly greater ($P < 0{\cdot}05$) than those in the other anoestrous groups. The mean total area of the LH peak was not significantly greater than that in any of the other groups, which were also not significantly different from each other.

When the injections were separated by 3 h (Fig. 2, upper graph) the plasma LH concentration had almost returned to baseline when the second injection was given. There was no significant difference between the heights or areas of the LH peaks induced by the two injections although the time required to reach the maximal LH concentration was shorter and the height of the LH peak was greater in each animal following the second injection.

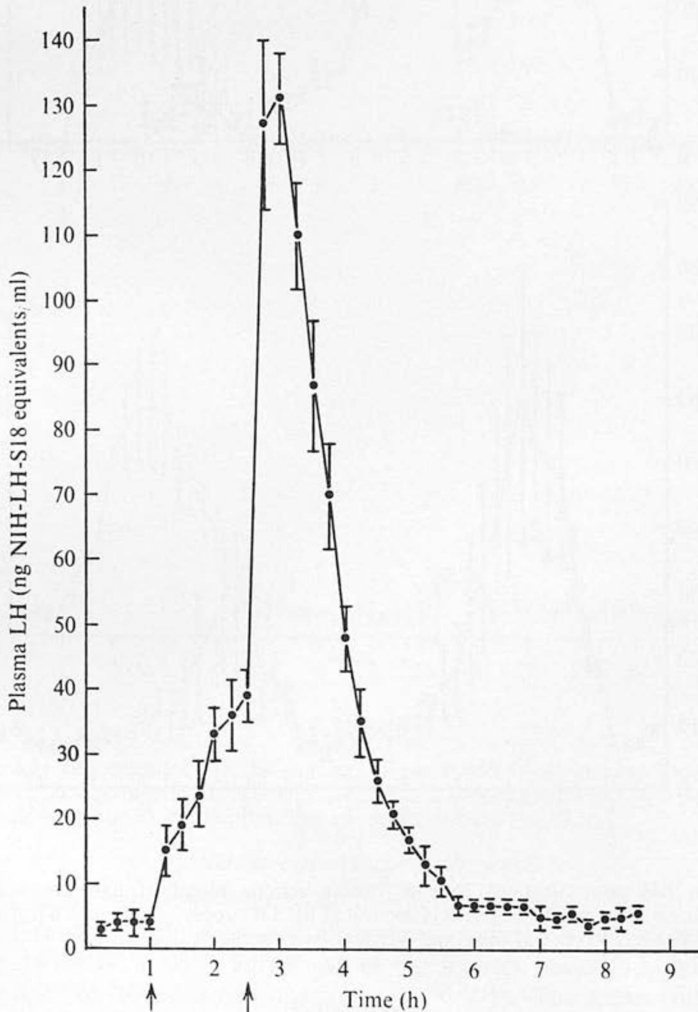

Fig. 1. Mean LH concentrations in the jugular venous blood of anoestrous ewes given two injections each of 30 μg synthetic LH-RH separated by 1·5 h. The arrows indicate the times of injection. Vertical bars represent the S.E.M.

When the second dose was administered 6, 12 or 24 h after the first, the LH peak height reached and the area of the peak achieved in response to the second injection were lower in each animal than the comparable responses to the first injection. These reductions were

significant in each case in terms of both mean peak height (6 h, $P < 0{\cdot}01$; 12 h, $P < 0{\cdot}01$; 24 h, $P < 0{\cdot}001$) and mean area of the peak (6 h, $P < 0{\cdot}01$; 12 h, $P < 0{\cdot}01$; 24 h, $P < 0{\cdot}001$).

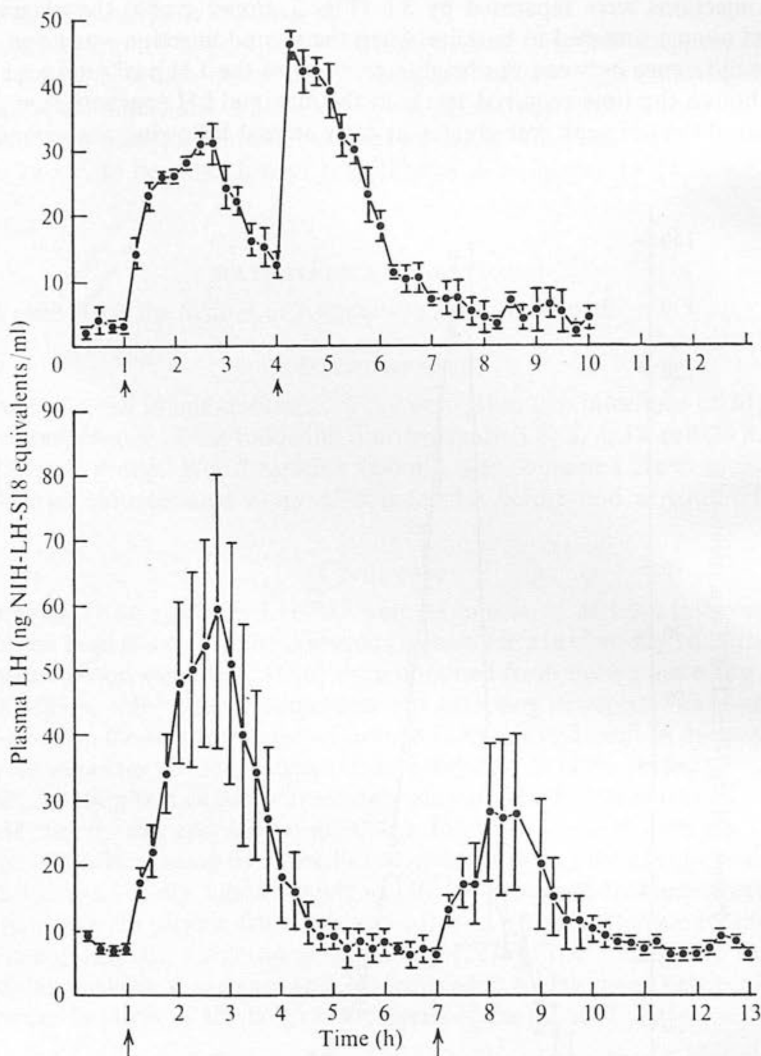

Fig. 2. Mean LH concentrations in the jugular venous blood of anoestrous ewes given two injections each of 30 μg synthetic LH-RH separated by 3 h (upper graph) and 6 h (lower graph). The arrows indicate the times of injection. Vertical bars represent the S.E.M.

Cyclic ewes

The mean plasma LH concentrations (±S.E.M.) in the cyclic ewes are shown in Fig. 4. In each animal the pattern of the response was similar to that seen in anoestrous ewes when the second dose of LH-RH was administered 1·5 h after the first. Although the mean heights of the LH peaks were not significantly different, the mean total area of the peak in ewes injected at onset of oestrus was significantly greater ($P < 0{\cdot}01$) than that in ewes injected on day 10. The plasma LH concentration attained just before the second injection was significantly greater ($P < 0{\cdot}05$) in the onset of oestrus group than in the day 10 group. The

plasma progesterone concentration just before treatment was non-detectable (< 0·1 ng/ml) in the onset of oestrus group and at typical mid-luteal levels in the day 10 group (3·12 ± 0·81 ng/ml, mean ± S.E.M.).

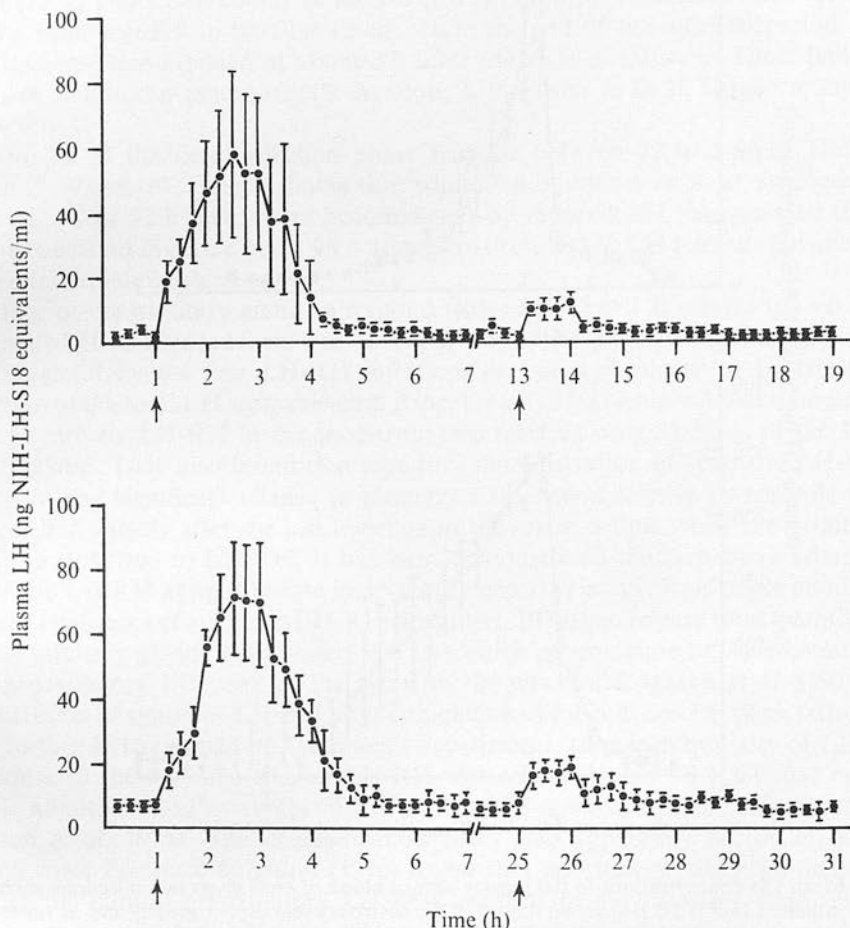

Fig. 3. Mean LH concentrations in the jugular venous blood of anoestrous ewes given two injections each of 30 μg synthetic LH-RH separated by 12 h (upper graph) and 24 h (lower graph). The arrows indicate the times of injection. Vertical bars represent the S.E.M.

Anoestrous ewes versus cyclic ewes

The mean height of the LH peaks induced by two injections separated by 1·5 h at onset of oestrus was significantly greater than that of the peaks induced by injections separated by 1·5 h during anoestrus ($P < 0{\cdot}01$) and those of the highest peaks induced by injections separated by 3, 6, 12 or 24 h during anoestrus ($P < 0{\cdot}001$). The mean total area of the induced LH peaks in the onset of oestrus group was significantly greater ($P < 0{\cdot}01$) than the mean total areas under the induced LH peaks in all the anoestrous groups.

The mean height and area of the peaks induced by two injections separated by 1·5 h on day 10 of the oestrous cycle were not significantly different from those of the peaks induced by the same treatment during anoestrus. The mean height of the LH peaks in the day 10 group was significantly greater ($P < 0{\cdot}01$) than the mean height of the largest peaks induced by injections separated by 3, 6, 12 and 24 h during anoestrus. In the case of the mean total areas of the peaks, no significant differences were apparent.

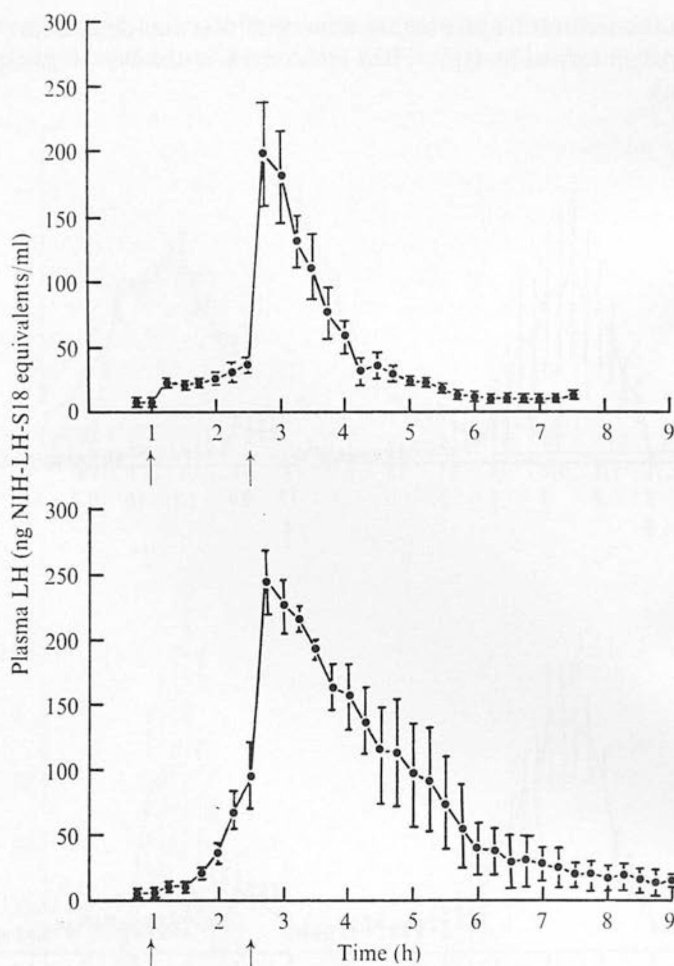

Fig. 4. Mean LH concentrations in the jugular venous blood of ewes given two injections each of 30 μg synthetic LH-RH 1·5 h apart on day 10 of the oestrous cycle (upper graph) and at onset of oestrus (lower graph). The arrows indicate the times of injection. Vertical bars represent the S.E.M.

DISCUSSION

These results indicate that the sensitivity of the ovine pituitary gland to synthetic LH-RH *in vivo* is increased for approximately 3 h after initial exposure to LH-RH but that this state is replaced by a desensitized one which lasts for at least 24 h. There is some suggestion from comparison of the 3, 6, 12 and 24 h groups that the achievement of the desensitized state is progressive, reaching a maximum at about 12 h after first exposure to LH-RH.

The sensitization effect appears to depend mainly on time elapsing after initial exposure to LH-RH. Reeves, Arimura, Schally, Kragt, Beck & Casey (1972) found that in the anoestrous ewe, there was no difference in the pituitary responsiveness to two injections each of 200 μg synthetic LH-RH given 4 h apart, while in a study using only two animals Symons, Cunningham & Saba (1974) observed an increased response to the second of two injections of 100 μg synthetic LH-RH when the injections were separated by 3 h. The administration of four injections of 30 μg synthetic LH-RH at 1·5 h intervals did not result in augmented responses beyond the second injection (Crighton *et al.* 1975) and the data of

Adams, Kinder, Chakraborty, Estergreen & Reeves (1975) seem to show a similar effect when injections of 10 μg LH-RH were given at 2 h intervals. In LH-RH infusion studies in the anoestrous ewe, both Cumming, Buckmaster, Cerini, Cerini, Chamley, Findlay & Goding (1972) and Chakraborty & Reeves (1973) found that the serum LH concentration rose to a peak and fell to baseline levels before the end of the infusion period. Maximal concentrations were attained at about 3 h after the start of infusion. These findings have been confirmed in our laboratory (S. A. Scott, J. P. Foster & D. B. Crighton, unpublished observations).

The length of the desensitization phase may be between 72 and 96 h. Thus Rippel, Johnson & White (1974) have shown that while two injections of 50 μg synthetic LH-RH spaced 24, 48 or 72 h apart were accompanied by reduced LH responses to the second injection, a second injection given 96 h after a first resulted in LH release equivalent to that after the initial administration.

Inability of the pituitary gland to respond with equivalent LH release to two injections of synthetic LH-RH spaced from 6 to 72 h apart cannot be due to complete depletion of LH from the gland by the first LH-RH injection, nor does it appear to be related to the proportion of the total LH store released. Rippel *et al* (1974) estimated that a single injection of 50 μg synthetic LH-RH in the anoestrous ewe released only 20–30 % of the LH in the pituitary gland. They also found that repetitive administration of synthetic LH-RH failed to result in any significant change in pituitary LH content relative to controls when this was measured shortly after the last injection in the series, a time when the pituitary gland was still desensitized to LH-RH. It has been demonstrated that repetitive administration of synthetic LH-RH at appropriate intervals (Crighton *et al.* 1975) or single administration of certain analogues of synthetic LH-RH (Crighton, 1976) can release total quantities of LH from the pituitary gland of the anoestrous ewe which approximate to the amounts released at the preovulatory LH peak of the cycle. In the work of Crighton *et al.* (1975) further administration of synthetic LH-RH after completion of the induced LH peak failed to result in any further LH response. In the present experiments, the mean quantity of LH released in response to the first injection of LH-RH was approximately 30 % of that released by multiple administration of synthetic LH-RH during anoestrus in the same breed of ewe (Crighton *et al.* 1975). The desensitization effect also apparently occurs in the natural situation since Foster & Crighton (1976) found that injection of 50 μg synthetic LH-RH just after the natural preovulatory LH peak resulted in only a low release of LH. The depletion of the pituitary LH store which is associated with the natural preovulatory LH peak in the ewe has been estimated as 62 % (Robertson & Rakha, 1966), 67 % (Dierschke & Clegg, 1968) and 88 % (Roche, Foster, Karsch, Cook & Dziuk, 1970). There are undoubtedly quite large quantities of LH remaining in the pituitary gland after the preovulatory LH peak (Crighton, Hartley & Lamming, 1973*b*).

The possibility that LH in the pituitary gland may be convertible between a 'releasable' and 'non-releasable' form (e.g. Bremner & Paulsen, 1974) would explain the present observations in the anoestrous ewe if it were postulated that in the short-term after initial exposure to LH-RH, predominance of the 'releasable' form is favoured while in the long-term after exposure, the 'non-releasable' form predominates. Diebel, Yamamoto & Bogdanove (1973) have suggested that gonadal steroids may affect pituitary gonadotrophins qualitatively as well as quantitatively and it is conceivable that LH-RH could also influence the type of LH in the pituitary gland. However, other factors such as changes in the LH-RH receptors of the pituitary gland could also provide an explanation.

Although the sensitizing effect of LH-RH was observed in both cyclic groups, the rising portion of the LH peak was biphasic when the injections were made at day 10 (Fig. 4, upper graph) and rose almost smoothly when the injections were made at onset of oestrus

(Fig. 4, lower graph). This difference was presumably determined by differences in the steroid environment influencing the pituitary gland in the period immediately preceding the injections. When the injections were made at onset of oestrus the resulting LH peak was similar to the natural preovulatory LH peak except in terms of duration of the LH increase. This was $6{\cdot}7 \pm 1{\cdot}2$ h compared with $10{\cdot}3 \pm 0{\cdot}6$ h (means ± S.E.M.) for the natural LH peak in a sample of ten animals from the same breed (J. P. Foster & D. B. Crighton, unpublished).

The changes in pituitary sensitivity induced by LH-RH demonstrated in the present work presumably play an important part in the development and decay of the natural preovulatory LH peak. Whether the LH peak were induced by a pulsatile LH-RH release, a possibility indicated by the work of Crighton *et al.* (1973*a*) and Foster, Jeffcoate, Crighton & Holland (1976), or by a period of increased but relatively constant LH-RH stimulation, the sensitization-desensitization sequence would play a part in determining its height, duration and form.

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Changes in the luteinizing hormone-releasing factor (LH-RF) content of the hypothalamus of the sheep during the oestrous cycle

By D. B. Crighton, Barbara M. Hartley and G. E. Lamming. *Department of Physiology and Environmental Studies, University of Nottingham*

Few studies have been reported on variations in the levels of gonadotrophin-releasing factors in the hypothalamus during the oestrous cycle. The situation in the rat has received some attention (Chowers & McCann, 1965; Ramirez & Sawyer, 1965) as has that in the cow (Hackett & Hafs, 1969).

Following the development of a technique employing the *in vitro* incubation of ovine anterior pituitary gland tissue coupled with a specific biological assay for LH (Hartley, Crighton & Lamming, 1972) assessments were made of the LH-RF activity of hypothalamic extracts from sheep at various stages of the oestrous cycle.

Mature ewes were placed with a vasectomized ram and observed for a period long enough to establish normality of oestrous cycles. Thirty animals with regular oestrous cycles were divided into six groups for slaughter relative to the time of first acceptance of the ram. The slaughter times were: 0 hr (in practice within 40 min of onset of oestrus), 6, 12 and 36 hr after onset and days 10 and 16 of the cycle. The hypothalamus with attached pituitary stalk was extracted in 0·1 N-HCl and stored at -15° C. Equal aliquots of the supernatants obtained after centrifuging the five individual extracts in each group were pooled. The pooled extracts were tested for LH-RF activity by adding them to the medium in which anterior pituitary tissue from castrate male sheep was incubated. The LH content of the medium after a 6 hr incubation was measured by the ovarian ascorbic acid depletion method (Parlow, 1958) and compared with the LH content of medium to which an equivalent volume of acid only had been added.

The minimal effective dose (MED) of extract, i.e. the lowest dose which significantly increased the LH content of the medium, was expressed in hypothalamic equivalents (HE).

The activity of the extract from the group slaughtered on day 16 of the cycle was high (MED = 0·00625 HE). The potency declined with the onset of oestrus and remained low at 6 and 36 hr after onset (MED in each case 0·025 HE) with intermediate potencies at 12 hr and 10 days after onset (MED in each case 0·0125 HE).

These results are, in general, similar to those of Ramirez & Sawyer (1965) in the rat but differ from those of Hackett and Hafs (1969) in the

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cow in which hypothalamic LH-RF content was found to be high at oestrus and for the first 7 days of the cycle.

The present results suggest that LH-RF is released from the hypothalamus just before the onset of oestrus but additional studies are required to examine more closely the physiological significance of the observed changes.

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Preliminary observations on the administration of a synthetic preparation of gonadotrophin releasing factor to cyclic and anoestrous ewes. By J. P. FOSTER and D. B. CRIGHTON. *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire*

The determination of a decapeptide structure for luteinizing hormone releasing factor by Matsuo, Baba, Nair, Arimura & Schally (1971) has led to the synthesis of this material (Geiger, König, Wissman, Geisen & Enzmann, 1971) and its availability for testing in various situations.

The synthetic decapeptide was administered to Clun Forest ewes both on day 12 of the oestrous cycle and during seasonal anoestrus. Twenty-four hours prior to administration, a catheter was placed in the jugular vein. Blood samples were obtained from the catheter every 10 min for 1 h before, and every 5 min for 3 h after, administration of the decapeptide via the catheter. The luteinizing hormone (LH) content of the plasma obtained from these samples was measured by a specific double antibody radioimmunoassay developed by the authors. The standard used was NIH-LH-S17 (National Institutes of Health, U.S.A.).

In cyclic animals, single administrations of 50 and 150 μg of the decapeptide were tested initially in order to establish the dose required to provoke a substantial rise in plasma LH. Two ewes were treated at each dose level. The administration of 50 μg caused an increase in plasma LH from 1·0–2·0 to 10·0–30·0 ng/ml. The administration of 150 μg resulted in peak levels of 65–75 ng/ml. Peak levels were achieved 90–120 min after injection, and when sampling was stopped after 180 min levels had declined to near baseline. Ovulation was observed in both ewes treated with 150 μg decapeptide when laparotomy was performed 2 days after treatment, but the ewes treated with 50 μg had not ovulated at this time.

In anoestrous animals, single administrations of 150 or 300 μg of the decapeptide were used. Peaks of LH of similar form to those observed in cyclic ewes were achieved and the mean heights of the peaks at both doses were similar, averaging about 110 ng/ml. Ovulation was observed in the majority of animals when laparotomy was performed 2–5 days after treatment. Those animals which failed to ovulate as a result of treatment responded to the extent of showing elevated LH levels and some showed massive follicular development. In the small number of ewes treated, individual variation in the height of LH peaks was too great to allow correlation of occurrence of ovulation with height of LH peak.

Both cyclic and anoestrous ewes were also treated in the same manner with 300 μg of the synthetic tripeptide thyrotrophin releasing factor (Pyroglu-His-Pro-NH_2) and the synthetic tripeptide Pyroglu-Val-Ser-NH_2. These materials failed to elevate LH levels in the plasma of treated ewes and ovulation failed to occur.

The synthetic peptides were kindly provided by Mr J. Best, Hoechst Pharmaceuticals.

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Comparison of LH levels in *post-partum* anoestrous and cycling ewes and the effects of synthetic gonadotrophin-releasing factor. J. P. Foster and D. B. Crighton, *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicester LE*12 5*RD.*

Plasma luteinizing hormone (LH) levels measured by a specific radioimmunoassay were compared in Clun Forest ewes during the early *post-partum* period and the oestrous cycle. Concurrent sampling in the two groups was made possible by inducing some ewes to lamb during the breeding season in the autumn by manipulating the diurnal light: dark ratio causing mating in the spring.

Blood sampling was carried out from a cannula placed in the jugular vein. Three ewes were tested at 2-hr intervals for the first 23 days *post-partum* and three similarly for at least 20 days of the cycle including two oestrous periods. All ewes were checked twice daily for oestrus with a vasectomized ram.

The cycling ewes showed basal LH levels of 4 to 18 ng NIH-LH-S17 equivalents/ml during most of the cycle. A peak of plasma LH (apparent height 112 to 192 ng/ml) was observed in all ewes at each oestrus. An additional peak of plasma LH (apparent height 58 ng/ml) occurred in one ewe on Day 11 of one cycle but was absent from the following cycle. A second ewe showed a peak of plasma LH (apparent height 115 ng/ml) on Day 16 of one cycle but not in the preceding cycle.

Two of the three ewes tested *post-partum* were suckling lambs, the remaining one was not lactating since the lambs were dead at birth. The lactating ewes showed levels of LH similar to basal values of the cycle throughout the sampling period. The non-lactating ewe showed similar levels except on Day 17 *post-partum* when a peak of LH was observed (apparent height 69 ng/ml). None of the ewes tested *post-partum* showed oestrus during the sampling period and the first oestrus observed in these ewes was on Day 54 *post-partum.*

At laparotomy, 3 days after the second oestrus observed in the cycling ewes, all were seen to have ovulated recently. In the *post-partum* group, laparotomy was carried out on Day 34 and at this time there were no CL, corpora albicantia or even large follicles visible.

Five lactating ewes were given 150 μg synthetic gonadotrophin-releasing factor (kindly supplied by Mr Julian Best, Hoechst Pharmaceuticals) intravenously on Day 16 of lactation and the results were compared with those from five ewes treated identically on Day 12 of the oestrous cycle. Blood samples were obtained at 15-min intervals for 1 hr before injection and for 3 hr after-

wards. Both groups of ewes responded with increased plasma LH levels, the mean heights of the peaks being 114 and 79 ng/ml in lactating and cycling ewes, respectively. Laparotomy 3 days after treatment revealed that recent ovulation had occurred in four lactating ewes and all five cycling ewes.

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The effects of a synthetic preparation of gonadotrophin releasing factor on pituitary and ovarian function in anoestrous ewes

By D. B. Crighton, J. P. Foster, W. Haresign, N. B. Haynes and G. E. Lamming. *Department of Physiology and Environmental Studies, University of Nottingham*

It has been established that a synthetic decapeptide will cause release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in anoestrous ewes. Ovulation was observed in some animals. This led to speculation that the material may have a practical application in controlling ovulation in domestic animals (Reeves, Arimura, Schally, Kragt, Beck & Casey, 1972). No investigations were carried out, however, on post-ovulation ovarian activity resulting from treatment.

The present study was undertaken to assess luteal function in seasonally anoestrous Clun Forest ewes treated with the synthetic decapeptide. In July 1972 blood was taken from jugular vein cannulae in fourteen ewes at intervals of 10 min for 1 hr before, and 5 min for 3 hr after, administration via the cannulae of 150 μg decapeptide (five ewes), 300 μg decapeptide (five ewes), saline or 300 μg of the synthetic tripeptide thyrotrophin releasing factor (four control ewes). Thereafter, samples were taken daily for 25 days. Laparotomies were performed 2–3 days after administration and the ovaries were examined. Plasma LH content during the initial 4 hr period was assessed and daily plasma progesterone concentrations were determined throughout by radioimmuno-assays (Crighton & Foster, 1972; N. B. Haynes & W. Haresign, unpublished results).

In all sheep given the decapeptide, plasma LH rose after treatment, reached a peak at approximately 110 min and declined thereafter. No LH peaks were observed in control animals. At laparotomy, four animals at each dose level of decapeptide had one apparent ovulation point, macroscopically resembling those seen in normally cycling animals at the same interval after observation of an LH peak. No ovulation points were observed in two treated animals and the four controls. In five treated animals with ovulation points progesterone levels were low throughout and were equivalent to those in control sheep and the two treated animals which had no ovulation points. In three treated sheep with ovulation points progesterone levels rose 2–4 days after administration, indicative of some luteal function, and fell at 10–13 days. The maximum value attained (2·0 ng/ml) was, however, lower than values (3–6 ng/ml) found during the luteal phase of the normal cycle. Laparotomy seemed not to be responsible for reduced progesterone production since three ewes treated with the

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decapeptide on day 12 of a normal cycle and laparotomized on days 14 and 20, showed normal plasma progesterone levels during the subsequent cycle.

The results demonstrate that administration of the decapeptide caused LH release and ovarian changes characteristic of ovulation in anoestrous ewes. The treatment did not, however, result in normal luteal function.

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Simultaneous determination by radioimmunoassay of luteinizing hormone and luteinizing hormone releasing hormone at different stages of the oestrous cycle in the sheep. By J. P. FOSTER, DIANE T. HOLLAND*, S. L. JEFFCOATE* and D. B. CRIGHTON. *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire and *Department of Chemical Pathology, St Thomas's Hospital, London, SE 1 7EH*

Blood samples were collected from Clun Forest ewes through jugular vein cannulae. Plasma from these samples was assayed for luteinizing hormone (LH) and luteinizing hormone releasing hormone (LH-RH) by radioimmunoassays (J. P. Foster & D. B. Crighton, unpublished observations; Jeffcoate, Fraser, Holland & Gunn, 1974). Samples were collected every 2 h for at least 20 days from three ewes during the breeding season. The ewes were checked twice daily for oestrus with a vasectomized ram. Luteinizing hormone peaks of 120–200 ng NIH-LH-S17 equivalents/ml were detected at oestrus with basal levels of 5–15 ng/ml during most of the rest of the 17-day oestrous cycle. Peaks of LH-RH-like immunoactivity occurred at various times of the cycle. The apparent maximum level of these peaks was 220 pg/ml compared with basal levels of < 10 pg/ml. Few peaks were detected during the few days after oestrus, the frequency being greater during the latter part of the cycle. To characterize the LH and LH-RH peaks more fully, ewes were sampled more frequently for limited periods at particular times of the oestrous cycle. The times chosen were: (1) from onset of oestrus, (2) 36 to 48 h after onset of oestrus and (3) day 10 of the cycle, as these represented respectively the time of the preovulatory LH peak, a time of apparently low LH and LH-RH release and a time when LH-RH peaks were observed unassociated with any rises in the LH level. Sampling was carried out in the first instance every 15 min at oestrus (Crighton, Foster, Holland & Jeffcoate, 1973). However, since the half-life of exogenous LH-RH in the sheep was found to be only 5 min, sampling was carried out more frequently in further animals. A frequency of 4 min for a period of 12 h was used. The patterns of LH and LH-RH levels at oestrus at this frequency of sampling were similar to those found in the ewes sampled at 15-min intervals, i.e. LH-RH peaks were detected before, during and after the preovulatory LH peak. The LH-RH peaks detected after the preovulatory LH peak were unassociated with any further rises in the LH level. In the ewes sampled 36 to 48 h after onset of oestrus and on day 10 of the cycle several LH-RH peaks unassociated with any rises in the LH level were detected. All the LH-RH peaks detected in the ewes sampled at 4-min intervals had a duration of only one or two samples and in some cases reached levels of several ng/ml compared with basal levels of < 10 pg/ml. The rate of disappearance of LH-RH suggested by these results was greater than would be expected for a half-life of 5 min. This may be due to a dilution effect as the LH-RH-like material passes from the jugular vein into the general circulation.

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An attempt to simulate, by injection of luteinizing hormone releasing hormone in the anoestrous sheep, the pattern of release observed at oestrus and the effects of this on luteinizing hormone release. By D. B. Crighton, Susan A. Scott and J. P. Foster. *University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire*

It was reported previously (Foster & Crighton, 1973) that administration of a single intravenous injection of 150 μg luteinizing hormone releasing hormone (LH-RH) to the seasonally anoestrous ewe resulted in an LH peak and the appearance of ovulation points on the ovaries. Although the LH peak was of similar form to that seen during the oestrous cycle, the area under the induced peak was in fact only about 25% of that of the preovulatory peak of the oestrous cycle. This situation was not altered by administering the 150 μg dose subcutaneously or intramuscularly (S. C. Barnett, J. P. Foster & D. B. Crighton, unpublished observations) or by increasing the intravenous dose to 300 μg. Ewes treated with LH-RH failed to show normal luteal function (Crighton, Foster, Haresign, Haynes & Lamming, 1973).

During the oestrous cycle it was shown that LH-RH appeared to be released as a series of pulses (Crighton, Foster, Holland & Jeffcoate, 1973; Foster, Holland, Jeffcoate & Crighton, 1974). It was decided to mimic this situation by administering the previously used dose of 150 μg LH-RH as five injections of 30 μg spaced as follows: four injections at 90-min intervals followed by a fifth injection 5 h after the fourth. This multiple injection schedule (four animals, three of which showed ovulation points) was compared with the previously used single injection (five animals, all of which ovulated) in Clun Forest ewes. Blood samples were obtained from jugular vein cannulae every 15 min for 45 min before the first or only LH-RH injection and for up to 13 h thereafter. The plasma from these samples was assayed for LH by a specific double-antibody radioimmunoassay. Three months later during the breeding season of the same year samples were obtained during oestrus (two ewes) and also subjected to radioimmunoassay to allow characterization of the preovulatory LH peak.

For the single-injection, multiple-injection and cyclic ewes respectively, the mean LH peak heights were 98 ± 12, 172 ± 16 and 159 ± 24 (s.e.m.) ng NIH-LH-S17 equivalents/ml, the lengths of the LH peaks were $4{\cdot}30 \pm 0{\cdot}22$, $6{\cdot}87 \pm 0{\cdot}87$ and $8{\cdot}88 \pm 0{\cdot}62$ h and the areas under the curves were 2477 ± 279, 7442 ± 815 and $12\,119 \pm 2719$ mm^2 when plotted appropriately. Thus the multiple LH-RH injection schedule was effective in producing an LH peak approaching that of the oestrous cycle as judged by the criteria used, whereas the single injection produced a much smaller LH peak as observed previously.

The better response of anoestrous ewes to the multiple LH-RH injection schedule designed to simulate the natural discharge pattern disclosed by radioimmunoassay suggests that this pattern may play a role in generating the natural LH peak at oestrus. It must be borne in mind, however, that the LH-RH doses used may well have resulted in non-physiological levels at the pituitary gland. The results should be interpreted with caution at this stage in view of the finding that pulses of LH-RH-like activity are frequently unaccompanied by LH rises during the natural cycle (Foster *et al.* 1974).

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Luteinizing hormone release after injection of synthetic luteinizing hormone releasing hormone at various stages of the oestrous cycle in the sheep. By J. P. Foster and D. B. Crighton. *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leics.*

It has been shown previously that peaks of luteinizing hormone releasing hormone (LH-RH)-like immunoreactivity occur in the jugular venous blood of the sheep at various stages of the oestrous cycle and are not always associated with increases in LH release (Foster, Holland, Jeffcoate & Crighton, 1974). If these peaks of immunoreactivity give an indication of LH-RH release from the hypothalamus then changes in pituitary responsiveness to LH-RH during the cycle are presumably important in the control of LH release.

To investigate the responsiveness of the sheep pituitary gland to LH-RH at various stages of the oestrous cycle, ewes were injected with a submaximal dose (50 μg) of synthetic LH-RH at onset of oestrus, 20 h after onset of oestrus, 48 h after onset of oestrus or on days 10 or 16 of the cycle. These stages correspond to those at which hypothalamic LH-RH content, pituitary LH content and the levels of LH and LH-RH-like immunoreactivity in jugular venous blood had previously been measured (Crighton, Hartley & Lamming, 1973; Foster *et al.* 1974). When the injection was administered at onset of oestrus or on day 16 of the cycle the LH release was significantly greater than that when the ewes were injected on day 10 of the cycle or 20 or 48 h after onset of oestrus. The LH release resulting from injection 20 h after onset of oestrus (that is just after the natural LH peak had occurred) was significantly less than the LH release resulting from injection at all other stages of the cycle.

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Effects of duplicate injections of synthetic luteinizing hormone releasing hormone at various intervals on luteinizing hormone release in the anoestrous ewe. By D. B. Crighton and J. P. Foster. *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicestershire, LE12 5RD*

Previous studies have shown that multiple i.v. injections of synthetic luteinizing hormone releasing hormone (LH-RH) into anoestrous ewes induce a much greater release of LH than a single injection of the same total dose. Four injections of 30 μg were given at 1·5 h intervals followed by a fifth injection 5 h after the fourth. The first injection induced an increase in the LH level and this was accelerated by the second injection. The remaining injections were administered after the LH level had reached a peak and they apparently had progressively less effect (Crighton, Foster, Haresign & Scott, 1975). To investigate possible changes in the responsiveness of the pituitary gland to synthetic LH-RH at various times after an initial stimulation by synthetic LH-RH, anoestrous Clun Forest ewes were given two i.v. injections of 30 μg synthetic LH-RH at intervals of 1·5, 3, 6, 12 or 24 h. There were four ewes in each group. In the ewes given injections 1·5 h apart the first injection caused an increase in the LH level and the second injection accelerated this rise confirming previous findings. When the injections were given 3 h apart, two distinct peaks were induced, the second peak being greater in terms of height and area than the first in each animal. Two distinct peaks were also induced in the ewes given injections 6, 12 and 24 h apart. However, in each animal in these groups, the second peak was smaller than the first. In the 12 and 24 h groups, the second LH response was very small indeed. These results indicate a change in the responsiveness of the pituitary gland with time after an initial 'priming' injection of synthetic LH-RH, there being an increase in responsiveness observed as a dramatic effect 1·5 h after first exposure to LH-RH and lasting for between 3 and 6 h. This is replaced at some time during that period by a desensitizing effect as observed when injections were spaced 6, 12 or 24 h apart.

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EFFECTS OF LACTATION ON THE PITUITARY GONADOTROPHINS OF THE SOW

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MECHANISMS OF LACTATION

THE REGULAR oestrous cycles of the sow are interrupted by two situations during its normal reproductive life, namely pregnancy and lactation. It is with the mechanisms underlying the second of these two anoestrous periods that this paper is concerned.

It is apparent that in the sow, the corpora lutea of pregnancy start to regress just before parturition. At this time, Kimura and Cornwell (1938) detected, by means of biological assay in adult rabbits, a decline in the progesterone content of the corpora lutea. Lauderdale, Kirkpatrick, First, Hauser and Casida (1965) showed that the total weight of luteal tissue in the ovaries and the progesterone content of the corpora lutea, measured by means of a chemical assay, were significantly lower on the day of parturition than 2 days earlier. Short (1960), using a chemical assay, was able to demonstrate in addition that the systemic blood progesterone level showed a decline at this time. The process of regression continues rapidly after parturition (Corner, 1919; Warnick, Casida and Grummer, 1950; Burger, 1952; Palmer, Teague and Venzke, 1965a, b). By the fifty-third to sixty-third day of lactation, the corpora lutea of pregnancy were represented only by dark brown spots in the ovarian tissue (Palmer *et al.*, 1965a).

There is agreement that post-partum oestrus occurs in the sow but the percentage of sows exhibiting oestrus has varied widely in different studies (Warnick *et al.*, 1950; Burger, 1952; Heitman and Cole, 1956; Self and Grummer, 1958). The variation in the expression of post-partum oestrus may be due in part to the widely differing breeds and strains studied and the different climatic and environmental conditions prevailing.

Post-partum ovulation does not normally occur in the sow, regardless of whether or not there is a post-partum oestrus. This has been

established by examining the ovaries of sows slaughtered shortly after the post-partum oestrus (Warnick *et al.*, 1950; Burger, 1952) and about 10 days after parturition where post-partum oestrus had not occurred (Warnick *et al.*, 1950). Post-partum oestrus occurs at a time when large follicles are absent from the ovaries of the sow (Warnick *et al.*, 1950; Burger, 1952; Palmer *et al.*, 1965a). In addition, Palmer *et al.* (1965b), found that during the first week of lactation approximately 50 per cent of the ovarian follicles showed signs of atresia. The percentage of atretic follicles was much higher during this period than in later lactation.

Throughout the remainder of lactation subsequent to the immediate post-partum period, the sow does not normally exhibit oestrus or ovulation (Marshall and Hammond, 1937; Burger, 1952; Heitman and Cole, 1956; Allen, Lasley and Uren, 1957; Self and Grummer, 1958; Palmer *et al.*, 1965a). When lactation is terminated by removal of the young some 8 weeks after parturition, oestrus and ovulation occur within about 4–7 days (Marshall and Hammond, 1937; Burger, 1952; Self and Grummer, 1958; Smidt, Scheven and Steinbach, 1965). This is, in fact, a matter of common observation, being the basis of the traditional husbandry pattern whereby conception occurs at the post-weaning ovulation after an 8 week lactation. Thus lactation in the sow is accompanied by a state of follicular quiescence dating from parturition.

Weaning was found by Palmer *et al.* (1965a) to produce a marked increase in mean follicular diameter, which was most noticeable 3–4 days post-weaning. A similar pattern was seen when the number of ovarian follicles equal to or greater than 5·0 mm was recorded. This effect of weaning on follicular growth has also been observed in early lactation. Warnick *et al.* (1950) found that the total follicular volumes in sows killed about the sixth or tenth days of lactation were significantly less in animals which had been suckled up to slaughter than in those where the litters had been removed at birth. This finding is confirmed by the work of Lauderdale *et al.* (1965), who studied the effect of weaning 5 days before slaughter on the total follicular fluid weight and mean diameter of the four largest follicles in the ovaries of sows killed on the sixth, eleventh and sixteenth days post-partum. These workers found that the values for both criteria were significantly greater in the weaned than in the suckled groups of sows. There is evidence that the follicular quiescence during lactation demonstrated by these investigations is accompanied by atresia in about 30 per cent of the ovarian follicles (Palmer *et al.*, 1965b).

A number of these suggestions as to the underlying causes of the lactational anoestrus of the sow have been found in the literature. Warnick *et al.* (1950) suggested that suckling might operate via the pituitary gland to inhibit follicular development but did not contribute any evidence of altered levels of pituitary hormones during lactation to support this theory. Self and Grummer (1958) postulated a two-stage process responsible for the inhibition of ovulatory oestrus during lactation. The first stage envisaged so-called 'residual endogenous elements of the initiation process of lactation associated with parturition' combined with an unspecified effect of suckling. It was suggested that weaning during the period of dual responsibility for the suppression of ovulatory oestrus would result in the 'endogenous elements' operating for variable periods prior to their exhaustion, whereas weaning during the suckling-dependent anoestrous period would result in a more uniform return to oestrus. This hypothesis was used to explain results obtained from trials with 10-, 21- and 56-day weaning but was unsupported by experimental evidence. Smidt *et al.* (1965) stated that the lactational anoestrus of the sow is probably due to blockage of the FSH-releasing factor of the hypothalamus but again no evidence was provided for this hypothesis. Palmer *et al.* (1965a) suggested, on the basis of data collected on ovarian follicular and uterine changes during lactation and the early post-weaning period, that lactation, or the suckling stimulus, or both, may bring about a decrease in the synthesis or release of the pituitary gonadotrophic hormones which are responsible for follicular development and that this effect may be most marked during early lactation. These workers pointed out that two other pituitary hormones, prolactin and oxytocin, are known to be physiologically active during lactation and suggested that perhaps one or both of these may counteract the action of the gonadotrophic hormones or prevent their release from the pituitary gland. While these conclusions from ovarian and uterine data were reasonable, although unproven in their implication of a deficiency of the pituitary gonadotrophins, the hypotheses concerning prolactin and oxytocin in the sow are without supporting evidence.

There is, in fact, very little sound evidence to implicate the sort of gonadotrophic insufficiency postulated directly or indirectly by the above authors. One study has been made by Lauderdale *et al.* (1965) on changes in pituitary gonadotrophin levels coincident with early lactation. No significant differences in anterior pituitary FSH or LH levels were found during the stages of pregnancy or lactation studied. The anterior pituitary content of FSH during

lactation up to the sixteenth day was significantly greater in suckled than in weaned sows but the LH content was unaffected by weaning. The observed increase in anterior pituitary FSH, coupled with the inhibition of follicular development over the same period was held by the authors to demonstrate an effect of suckling blocking the release of FSH from the anterior pituitary gland.

LACTATIONAL ANOESTRUS

We have become interested in the situation of lactational anoestrus because it is a factor restricting the annual production per sow and we have been examining the possibility of inducing oestrus and ovulation in the lactating sow and thus obtaining pregnancy concurrent with lactation. The results of experiments in the rat described by Rothchild (1960); McCann, Graves and Taleisnik (1961); McCann and Ramirez (1964) and others, suggested that the use of similar approaches in the sow might provide information on the underlying causes of lactational anoestrus. Accordingly, the ovarian and pituitary gland changes associated with lactation in the sow were investigated, partly in the hope that the results might provide a sound basis for any treatment for the induction of oestrus and ovulation in the lactating sow.

Two such experiments are described here. The first experiment was designed to provide information on the period between late lactation and the post-weaning oestrus and ovulation by examining any changes in the ovarian follicles and anterior pituitary gland content of FSH and LH which might take place over that period.

Thirteen Large White sows from the School of Agriculture herd were used in this experiment. These sows entered the experiment after approximately 45 days of lactation at which time they were nursing litters ranging from four to eleven piglets. All had normal reproductive histories, having reared at least two previous litters. They had been subjected to normal management during the existing lactation. The sows were divided into four groups on the basis of time of slaughter relative to a 56-day lactation, the presence or absence of oestrous behaviour immediately prior to slaughter and the presence or absence of ovulations on the ovaries obtained at slaughter. Details of the four groups are given in Table 1.

The second experiment was carried out in order to determine whether ovariectomy had any effect on the anterior pituitary LH potency of lactating sows and to compare any response to ovariectomy with that of sows ovariectomized during the oestrous cycle. A definite stage (day 5) of the oestrous cycle was chosen in order to

allow a comparison of this with a definite stage of lactation. In addition, it was hoped that ovariectomy might raise serum levels of LH to a point where they might be detected by biological assay, thus providing an index of the relative abilities of lactating and cycling sows to release LH.

Fourteen Large White sows from the School of Agriculture Herd were used. Six entered the experiment after 10 days of lactation and the remaining eight during various stages of oestrous cycles initiated as a result of weaning.

The lactating sows were nursing litters of from 6–11 piglets at the start of the experiment and were divided at random into two groups

Table 1

Allocation of sows into groups in the first experiment

Group	*No. of sows*	*Reproductive status at slaughter*	*Time of slaughter*
1	4	Lactating, anoestrous	52–53 days of lactation
2	3	Post-weaning, anoestrous	3–4 days post-weaning after 56-day lactation
3	3	Post-weaning, oestrous, no ovulation	4 days post-weaning after 56-day lactation
4	3	Post-weaning, oestrous, recent ovulation	4–6 days post-weaning after 56-day lactation

on the basis of whether a laparotomy or ovariectomy was to be carried out on day 20 to 21 of lactation. Each laparotomized sow was tested daily with a boar from day 10 until slaughter, 25 days post-operation on day 45 or 46 of lactation. Each ovariectomized sow was tested daily with a boar from day 10 until ovariectomy on day 20 to 21 of lactation.

The cycling sows were at various stages of the oestrous cycle when they entered the experiment. Each sow was observed through two successive oestrous periods prior to surgery, which was carried out on day 5 of the cycle (the first day being designated day 1). The sows were divided on the basis of whether a laparotomy or ovariectomy was to be carried out on day 5.

In both experiments, quantitative, differential bio-assays were used to assess the anterior pituitary levels of FSH and LH. The

assay used for FSH was the Augmentation Assay of Steelman and Pohley (1953) as modified by Brown (1955) for use in mice. For LH the Ovarian Ascorbic Acid Depletion assay of Parlow (1958) was used with two modifications, a 4-h test as described by Schmidt-Elmendorff and Lorraine (1962) but using intraperitoneal injection of the test material, and a 1 h test as described by McCann and Ramirez (1964). The former was used for anterior pituitary material and the latter when serum was being injected. All anterior pituitary assays were carried out against standard preparations given by the National Institute of Health, U.S.A. Over 90 per cent of

Table 2

Allocation of sows into groups in the second experiment

Group	*No. of sows*	*Reproductive status at operation*	*Operation performed*	*Time of slaughter*
5	3	Lactating, 20–21 days, anoestrus	Laparotomy	25 days after operation
6	3	Lactating, 20–21 days, anoestrus	Ovariectomy	25 days after operation
7	4	Cycling, day 5 of cycle	Laparotomy	Day 5 of oestrous cycle subsequent to that of operation
8	4	Cycling, day 5 of cycle	Ovariectomy	25 days after operation

the anterior pituitary determinations were four-point assays, the remainder being of the three-point design.

The sows in both experiments were slaughtered in the following manner: Firstly they were stunned with a captive bolt pistol and then bled immediately, at which time a sample of blood was taken from the jugular venous effluent for serum collection. The pituitary glands were removed as soon as possible after slaughter and the anterior lobes were stored at $-20°C$. The reproductive tracts of these animals were also examined.

The mean diameter of the largest follicle present in either ovary showed an increase after weaning, reaching a maximum at oestrus, followed by a fall again when the follicles ovulated (*Figure 1*). These changes approach significance at $P=0{\cdot}05$ but do not reach it.

The number of large follicles (over 5·0 mm in diameter) increased significantly after weaning ($P<0·001$) and decreased significantly ($P<0·001$) after ovulation.

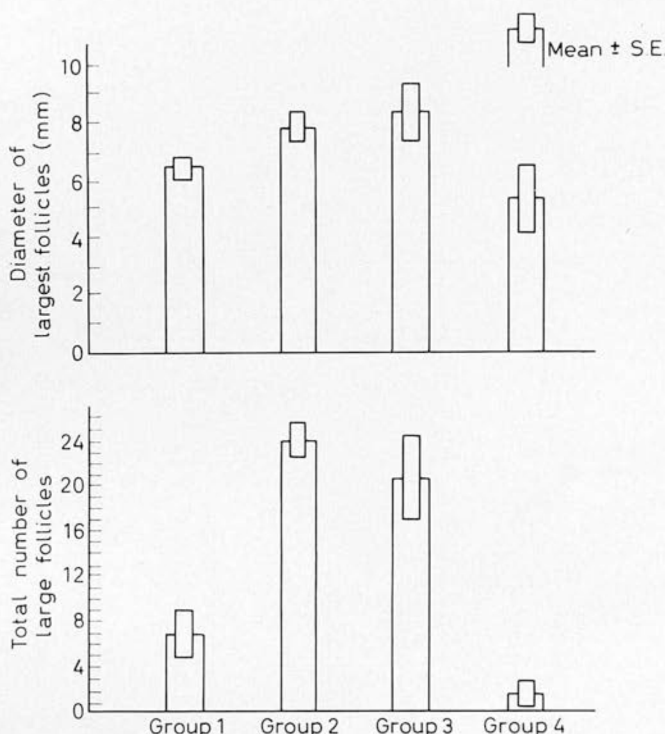

Figure 1. Changes in the ovarian follicles of sows between late lactation and the post-weaning ovulation

From the second experiment a comparison can be made between the four groups for the weight and length of the two uterine horns of each animal taken together (*Figure 2*). The mean weight for the normal lactating sows on days 45–46 of lactation (Group 5) is not significantly different from that of sows at the same stage of lactation but which had at that time been ovariectomized for 25 days (Group 6) or from cycling sows which had been ovariectomized for 25 days (Group 8). The value for cycling sows on day 5 of the cycle is significantly higher than any of the other three values ($P<0·001$ in each case). When uterine development is expressed in terms of

total length of the uterine horns, the picture is essentially similar to that seen for weight. Thus there is evidence that follicular growth is markedly suppressed during lactation, resulting in a substantial decrease in oestrogen secretion.

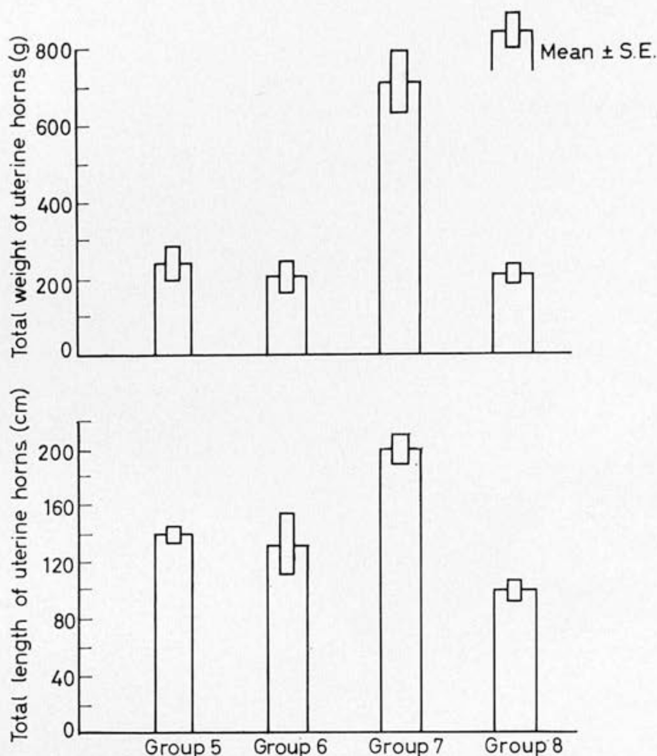

Figure 2. *Total weight and total lengths of uterine horns of intact and ovariectomized lactating and cycling sows*

With regard to the anterior pituitary gland assays from the first experiment, the results calculated for FSH are shown in *Figure 3.* The data presented in the form of the mean relative potencies per milligramme for each group show a slight and non-significant rise from late lactation to the post-weaning period before oestrus followed by a significant fall with the onset of oestrus. When these figures are converted to compare the total anterior pituitary contents of FSH the same picture is seen, with no significant change from

lactation to the period immediately after weaning (in fact a slight fall in content is indicated).

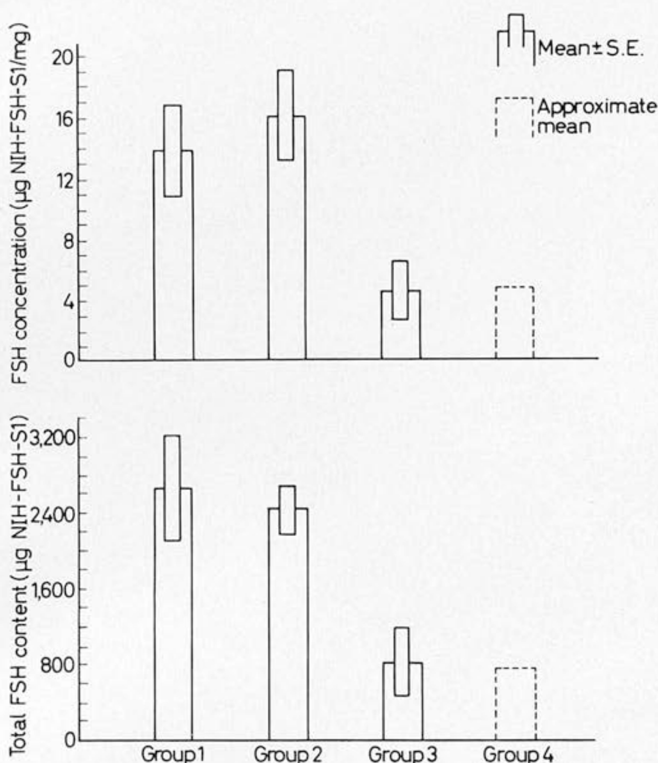

Figure 3. Changes in the anterior pituitary FSH content of sows between late lactation and the post-weaning ovulation

The mean results calculated for LH in the first experiment are shown in *Figure 4*. The data presented in the form of the mean relative potencies per milligramme for each group show a significant rise ($P<0{\cdot}01$) between late lactation and the post-weaning period prior to oestrus, followed by a significant fall ($P<0{\cdot}01$) with the onset of oestrus. When the results are converted to compare the total anterior pituitary gland contents of LH, the same picture is seen.

Thus the follicular changes occurring in the ovaries of sows over the period covered by the first experiment are accompanied by

changes in the anterior pituitary gland content of FSH and LH. With regard to FSH, no effect of weaning on the pituitary could be demonstrated but in the case of LH there was a marked rise initially after weaning. This increase was interpreted as representing a

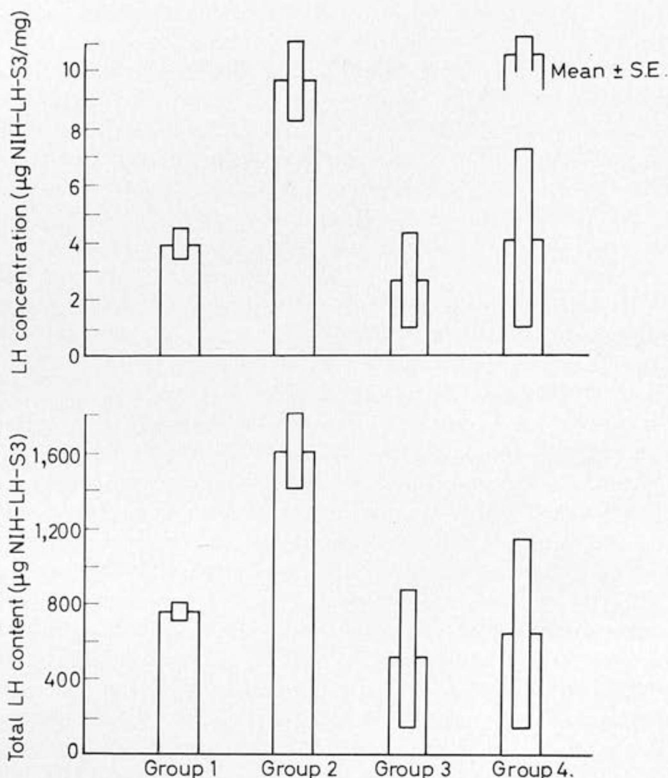

Figure 4. Changes in the anterior pituitary LH content of sows between late lactation and the post-weaning ovulation

blockage of the synthesis of LH during lactation which was relieved by weaning. The subsequent fall in both FSH and LH in the anterior pituitary gland with the onset of oestrus was interpreted, as it has been by many workers in several species, as representing a massive release of these hormones associated with rapid follicular development and ovulation. The results suggest that FSH release rather than synthesis may be inhibited during lactation, since the level was uniform and quite high before and after weaning. Alter-

natively, it is possible that a post-weaning increase in FSH synthesis comparable to that of LH occurred but was not demonstrable because it was balanced by a greater release of FSH at this time, resulting in rapid growth of follicles.

It was decided to test the hypothesis that LH synthesis and release are inhibited during lactation and the second experiment was designed for this purpose. Ovariectomy in the rat is known to cause a marked rise in the synthesis and release of LH as judged by increases in pituitary and blood levels of this hormone (Taleisnik and McCann, 1961; Ramirez and McCann, 1963; McCann and Ramirez, 1964). Increased pituitary levels after ovariectomy in the sow were found by Parlow, Anderson and Melampy (1964). Thus the second experiment was carried out and anterior pituitary and serum LH levels were examined.

The mean results calculated for anterior pituitary LH in the second experiment are shown in *Figure 5*. The data presented in the form of the mean relative potencies per milligramme for each group demonstrate that the level in lactating sows showed no significant change as a result of ovariectomy. There was, however, a significant rise ($P < 0{\cdot}001$) in anterior pituitary LH potency after ovariectomy during the cycle. The value for sows ovariectomized during the cycle was also significantly higher ($P < 0{\cdot}01$) than that for sows ovariectomized during lactation. When the results are converted to compare total anterior pituitary gland contents of LH, the same picture is seen.

The finding that the low anterior pituitary content characteristic of lactation failed to increase during the 25 day period after ovariectomy whereas the low level characteristic of day 5 of the oestrous cycle increased approximately fivefold is striking. It demonstrates an inability of the pituitary gland of the lactating sow to increase the synthesis of LH under circumstances where that of the cycling animal is capable of doing so. It also shows that the ovaries play no part in the maintenance of the low level of pituitary LH in the lactating sow.

In order to determine whether the serum samples might provide information supplementing the pituitary assay work, the 1 h modification of the Ovarian Ascorbic Acid Depletion assay was employed. Aliquots of 2·0 ml of serum were injected intravenously into the assay rats, the serum of each individual sow being injected into 6 rats. In addition, 10 rats were injected with 2·0 ml isotonic saline. The injection of saline produced a negative depletion (increase) in ascorbic acid ($-1{\cdot}1$ per cent) whereas the mean

depletions produced by the sow treatment groups were at least 44 per cent (Table 3). The differences between the means of the groups were not significant.

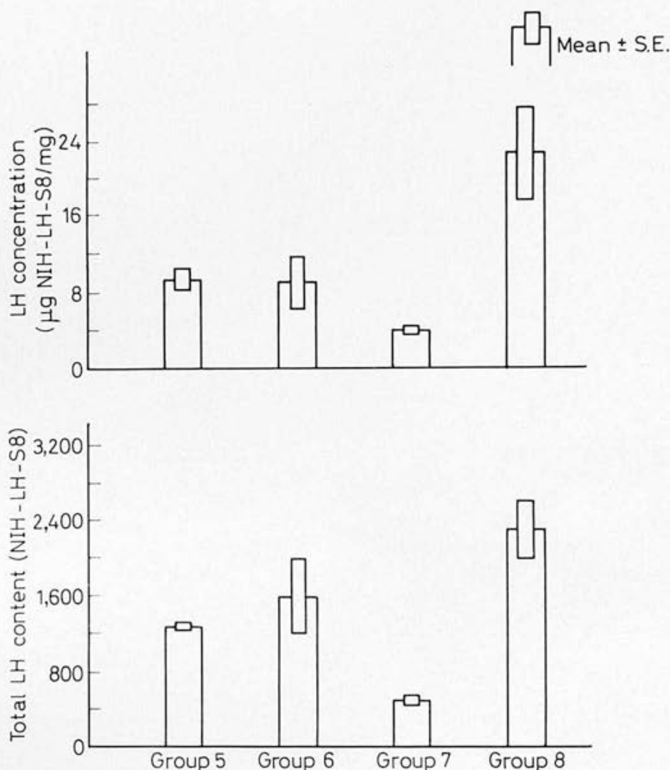

Figure 5. Anterior pituitary LH contents of intact and ovariectomized lactating and cycling sows

The consistency of the large ovarian ascorbic acid depletions produced by samples from sows of such widely differing reproductive status and anterior pituitary LH content is surprising. It suggests strongly that the depletions observed do not represent the levels of LH circulating in the blood during life. It is therefore pertinent to examine the possible reasons for the effect.

Firstly, it is possible that the depletions may be due to an artificial elevation of serum LH levels, by some feature in the treatment at slaughter, after the sows had been stunned with a captive bolt pistol.

This treatment may be responsible for releasing LH into the blood from the pituitary glands of the sows although the apparent contents of LH indicated by the depletions would seem to be so massive as to make this hypothesis unlikely. Such a situation would seriously undermine the value of the pituitary LH assays carried out in both our experiments, as measures of anterior pituitary LH during life, and would therefore render suspect the interpretation placed upon these results.

Table 3

Depletions of rat ovarian ascorbic acid produced by the serum of lactating and cycling sows

Group	*Reproductive status at slaughter*	*Mean depletion of rat ovarian ascorbic acid* ($\% \pm$ *S.E.*)
5	Lactating, 45–46 days, anoestrous	$44{\cdot}8 \pm 3{\cdot}6$
6	Lactating, 45–46 days, ovariectomized 25 days previously	$48{\cdot}9 \pm 10{\cdot}4$
7	Cycling, day 5 of cycle	$48{\cdot}1 \pm 2{\cdot}0$
8	Ovariectomized 25 days previously on day 5 of cycle	$50{\cdot}8 \pm 5{\cdot}2$

Secondly, the depletions may be due to some agent in serum other than LH. Other hormones are capable of depleting ovarian ascorbic acid when present in very large quantities. Also a LH releasing factor is known to be present in the hypothalamus of the rat and may perhaps exist in the sow. This factor may be responsible for releasing LH from the pituitary glands of the assay rats resulting in ovarian ascorbic acid depletion.

Finally, it is possible that a non-specific effect may occur when large quantities of serum from the sow are injected into the rat, resulting in the release of the pituitary LH of the latter or acting directly on the ovaries to produce ascorbic acid depletion.

Thus at present no interpretation is placed upon the results of the serum assays. Further experiments are planned to investigate the nature of the ovarian ascorbic acid depleting agent(s) present in the serum of the sow.

SUMMARY

These results confirm the existence of a state of ovarian follicular quiescence during lactation in the sow, resulting in a low level of circulating ovarian hormones as indicated by the lack of uterine development. Weaning resulted in a rapid growth of ovarian follicles followed by oestrus and ovulation.

The level of pituitary FSH was high during lactation and was unaffected by weaning suggesting that the lack of follicular growth was due to a failure of FSH release rather than lack of FSH synthesis.

The level of pituitary LH rose significantly as a result of weaning and declined prior to ovulation. The LH content of the lactating sow failed to rise after ovariectomy. These findings suggest that lactation inhibits LH synthesis and FSH release.

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DISCUSSION

B. T. Donovan (*London*)

In view of the large litter size in the pig, would you imagine that this stimulation of lactation is the cause of the delay in the onset of oestrus? Have experiments been done where the litters have been reduced to half or less of the original size and the delay in oestrus measured?

Crighton

As far as I know no variation of the suckling stimulus has been studied in the pig but it is commonly observed that pigs suckling 2–4 piglets return to oestrus early in lactation.

I. Rothchild (*Cleveland, Ohio, U.S.A.*)

The early return to oestrus in sows suckling small litters is similar to the observations that I made in rats and in the mouse, the interval to post-partum oestrus is in inverse proportion to litter size. The castration changes in pituitary LH content conforms with work of McCann in the rat and the work I did using mouse uterine weight as an end-point of gonadotrophin potency in the pituitary. There appears to be an inverse relationship between the size of litter and response of the pituitary to castration in the rat and, from your results, the same appears true of the pig.

The content of LH in the blood should be measurable but as you have shown, it is not possible to interpret results obtained with the OAAD method. Could you examine this in another way by treating an animal during lactation with known amounts of LH or FSH or combinations and observe the response of the ovary? If you could stimulate the ovary directly and get oestrogen secretion then you would know that it is not an inability of the ovary to respond but a lack of release of gonadotrophins from the pituitary.

Crighton

One situation does have a bearing on the reduction of the suckling stimulus under certain conditions, for the separation of a sow from its litter for 12 h/day for 4–5 days will cause the sow to return to oestrus. Additional factors in the expression of oestrus involves the presence or absence of a boar and the use of PMS injections during the period of partial weaning. Several years ago Cole and his colleagues injected up to 1,600 I.U. PMS into sows in early lactation and obtained a comparatively poor response. However, after about 40 days of lactation the sows responded well with oestrus, ovulation and pregnancy. We have examined, in a series of small scale experiments the effects of injecting PMS on day 21 of lactation

compared with the effect of separating sows for 2 days and then injecting with PMS on day 23. The sows responded poorly to direct injections but well to PMS given in combination with partial weaning.

H. Karg (*Munich, Germany*)

To prove these doubts of the specificity of LH in the serum, did you not check the serum removed from the animals before slaughter for comparison?

Crighton

We plan to withdraw blood samples from sows before slaughter to make these comparisons.

H. A. Robertson (*Aberdeen*)

We have killed animals both by stunning and by exsanguination and with neither have we observed an LH response.

J. A. Lorraine (*Edinburgh*)

I would like to make a comment on the specificity of the OAAD test. There is a feeling now that the method was not as specific as the originator believed and that the work of Albert and Rosemberg has tended to show that where large amounts of FSH are given this may be capable of producing ascorbic acid depletion.

In the work we did in relation to the cholesterol method we found that very large amounts of Growth Hormone which we regarded as being unphysiological, as far as the application of the assay is concerned, might produce a depletion in ovarian cholesterol. So I think there are two hormones which may have affected your results in relation to serum. In studies on human subjects we have looked at the effect of operative stress on gonadotrophic activity in the urine, and have found that in a proportion of these subjects there is a marked rise in urinary gonadotrophin excretion associated with operation.

R. Pinot (*France*)

I have frequently found that the injection of plasma from hypophysectomized ewes depresses ovarian ascorbic acid. This is a non-specific effect. I have also found that egg albumin gave a depression of ascorbic acid. With low doses, egg albumin gave a depletion of ascorbic acid in normal rats, but not in the hypophysectomized rats. In these experiments it would have been difficult to distinguish between egg albumin and LH.

K. Yoshinaga (*Cambridge*)

I have found that the effect of lactation on concurrent pregnancy in the rat was the same as you report in the sow. If the litter size is small there is no delay in implantation, but if the litter size is six or above then implantation is delayed. If oestrogen or LH is given to these rats, implantation occurs at the normal time. Thus I believe that the endocrine background is the same as for the pig.

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LACTATIONAL ANOESTRUS AND THE EFFECTS ON LACTATION OF THE INDUCTION OF VARYING LEVELS OF OVARIAN AND UTERINE ACTIVITY

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Observations have been made on the patterns of oestrous behaviour and ovulation as these are affected by lactation in a number of species. Lactation is generally associated with some degree of inhibition of oestrus and ovulation although there is considerable variation between species. Two species only will be considered here, the rat and the pig. In both cases, investigations into the mechanisms underlying the behavioural pattern have been carried out and sufficient information is available to allow comparisons to be made.

NATURAL PATTERNS OF REPRODUCTION DURING LACTATION

NATURAL PATTERN IN THE RAT

In the rat, within 48h of parturition there is a postpartum oestrus accompanied by ovulation (Kirkham and Burr, 1913). Blandau and Soderwall (1941) found that in a small percentage of cases postpartum ovulation occurred without oestrus. After the oestrus and ovulation postpartum, both these events are suppressed during lactation for a period of approximately three weeks (Long and Evans, 1922) or until the young are weaned, when oestrus and ovulation recur within a few days at whatever time during lactation the young are removed (Long and Evans, 1922; McKeown and Zuckerman, 1938). In the case of the rat colony of Wistar origin housed at the School of Agriculture, observations made in 15 animals with standard

litter size (nine to eleven young) have confirmed this pattern, there being no oestrus detectable by vaginal smearing during lactation after the postpartum oestrus. When weaning was carried out at day 20 of lactation, the time required to return to oestrus (mean ± S.E.) was 2·4 ± 0·3 days.

NATURAL PATTERN IN THE PIG

In the pig, a postpartum oestrus occurs in a variable proportion of animals, apparently depending upon breed and strain. This occurs within two or three days of parturition but is not accompanied by ovulation (Warnick *et al.*, 1950; Burger, 1952). Thereafter there is no oestrus or ovulation until the young are weaned, when oestrus and ovulation recur within a few days (Marshall and Hammond, 1937). In the case of the herd of Essex sows in which a number of our experiments have been carried out, testing 11 animals with a boar daily from day 10 of lactation to day 10 after weaning has shown that in no case did oestrus occur prior to weaning but when weaning took place on day 49 of lactation the time required to return to oestrus (mean ± S.E.) was 4·1 ± 0·4 days.

COMPARATIVE ASPECTS IN THE RAT AND PIG

Thus although the pattern appears essentially the same in both species in that the remainder of lactation after postpartum oestrus is characterised by anoestrus and anovulation, one clear difference is that in the rat there are corpora lutea of lactation whereas there are none in the pig. It seems clear, however, from the evidence available that the corpora lutea play a part in the inhibition of oestrus and ovulation only in exceptional circumstances. McKeown and Zuckerman (1938) used electro-cautery on the second and third days of lactation to destroy the corpora lutea resulting from the postpartum ovulation. None of the rats treated in this way returned to oestrus when lactation was continued whereas rats whose litters were weaned after electro-cautery returned to oestrus on the fifth or sixth day after weaning. This demonstrated a mechanism of inhibition of oestrus and ovulation not involving the corpora lutea. The imposition of the same treatment in pseudopregnancy and early pregnancy led to the almost immediate development of ovarian follicles and oestrus. These results have been confirmed by Rothchild (1960) using autotransplantation of ovarian tissue devoid of viable corpora lutea.

Apart from the presence of viable corpora lutea, the ovaries of the rat are normally in an inactive state during lactation. Stotsenburg (1923) found that lactation was accompanied by a steady decline in the weight of the ovaries. The degree of weight loss has been shown by Rothchild and Dickey (1960) to be dependent on litter size. The decline in weight is accompanied by a stasis in follicular growth.

Desclin and Grégoire (1937) found that the ovaries of immature rats transplanted beneath the kidney capsule in ovariectomised lactating rats developed only very small follicles and no corpora lutea. The contralateral ovaries of the same donors transplanted similarly into ovariectomised postparturient rats deprived of their young grew rapidly and produced large numbers of follicles and corpora lutea. Thus removal of the young relieved the inhibition on ovarian growth and follicle development.

In the pig, similar changes to those recorded in the rat are observed at weaning. In an experiment designed to examine the endocrine effects of weaning, 13 Large White sows from the School of Agriculture herd were used. These sows entered the experiment after approximately 45 days of lactation, at which time they were nursing litters ranging in size from 4 to 11 young. They were divided into four groups on the bases of time of slaughter relative to a 56-day lactation, presence or absence of oestrous behaviour immediately prior to slaughter and the presence or absence of recent ovulations on the ovaries examined after slaughter. All were tested for oestrus with a boar from approximately 45 days of lactation until slaughter. The final determination of sexual receptivity took place not more than five hours prior to slaughter. The effects of weaning on three parameters of ovarian function in these animals are shown in *Table 1*.

Table 1 EFFECTS OF WEANING ON OVARIAN DEVELOPMENT IN THE PIG

Reproductive status	*No. of sows*	*Mean total ovarian weight*	*Mean diameter of largest follicle*	*Mean total number of follicles over* 5·0 mm *diameter*
		(g ± S.E.)	(mm ± S.E.)	(no ± S.E.)
Lactating, anoestrous	4	13·60 ± 1·16	6·4 ± 0·4	6·8 ± 2·1
Post-weaning, anoestrous	3	13·72 ± 1·13	7·8 ± 0·5	23·7 ± 1·5
Post-weaning, oestrous, no ovulation	3	14·71 ± 2·04	8·3 ± 1·0	20·3 ± 3·7
Post-weaning, oestrous, ovulated	3	11·98 ± 0·53	5·3 ± 1·2	1·3 ± 1·2

The results show a trend of increasing ovarian weight from late lactation until the occurrence of the post-weaning oestrus, followed by a decrease at ovulation. While these changes fail to reach significance, the work of Lauderdale *et al.* (1965) suggests that they represent real changes contributed to by the total weight of follicular fluid present. Of the other parameters, the diameter of the largest follicle present showed an increase after weaning, reaching a maximum at oestrus and followed by a decrease at ovulation. These changes approached significance at $P = 0{\cdot}05$. The total number of large follicles (diameter over 5·0 mm) present in both ovaries showed an increase ($P < 0{\cdot}001$) after weaning. There was no further change after the onset of oestrus until ovulation when a decrease occurred ($P < 0{\cdot}001$).

There is good evidence that in the rat the effects of lactation on ovarian function are mediated via the anterior pituitary gland and specifically that gonadotrophin synthesis and release are suppressed during lactation (Desclin, 1936, 1947; Rothchild, 1960; Rothchild and Parlow, 1960; McCann *et al.*, 1961). Rothchild (1960) and Rothchild and Parlow (1960) demonstrated that lactation in the rat is characterised by low levels of pituitary 'total gonadotrophin' and luteinising hormone (LH) respectively which fail to rise after ovariectomy in contrast to the rise which occurs when ovariectomy is performed during the oestrous cycle. Thus synthesis of gonadotrophins (or at least LH) is apparently suppressed by some influence not arising from the ovaries, probably the stimulus of suckling acting via the central nervous system (Desclin, 1936, 1947; McKeown and Zuckerman, 1938; Rothchild, 1960). Thus the gonadotrophin-inhibiting powers of the corpora lutea are only potential during lactation since gonadotrophin synthesis is suppressed by other means. Under certain circumstances, the powers of the corpora lutea can be exercised, i.e. when a very small litter is being nursed. Rothchild (1960) showed this when he demonstrated that intact rats nursing small litters exhibited no suppression of pituitary 'total gonadotrophin' levels during lactation but showed an average interval from parturition to the return of oestrus (other than postpartum oestrus) of 16 days. However, ovariectomy on the second day of lactation and autotransplantation with ovarian tissue devoid of viable corpora lutea resulted in a return to oestrus within seven days.

In the pig, experiments comparable to those of Rothchild and Parlow (1960) have been carried out by Crighton and Lamming (1969) and it has been possible to show that lactation in the pig is also characterised by a low level of pituitary LH (*see* also Parlow *et al.*, 1964) which fails to rise after ovariectomy in contrast to the rise which occurs when ovariectomy is performed during the oestrous cycle.

This suggests that, as in the rat, the synthesis of LH is inhibited during lactation and that the ovaries play no part in this inhibition.

If it is postulated that the mechanisms underlying the anoestrus of lactation are similar in the rat and the pig, it should be possible to demonstrate similar changes in pituitary gonadotrophin levels in relation to weaning in both species even in the absence of corpora lutea of lactation from the ovaries of the rat. Such a comparison has been made. In the case of the pig, the animals used were the 13 Large White sows referred to previously. In the case of the rat, 22 animals from the School of Agriculture colony were used. In the rat, it was decided to destroy the corpora lutea of lactation by electro-cautery shortly after their formation. This was done on the third to sixth day of lactation and weaning was carried out on day 20. Litter size was standardised within the range of 9–13 young. Animals were allocated to one of three groups, (*a*) lactating, anoestrous (day 18), (*b*) post-weaning, anoestrous and (*c*) post-weaning, oestrous, and were killed at these stages. Oestrus was detected by the vaginal smear technique.

In both species, the anterior pituitary glands were removed shortly after the animals were killed. In the case of the pig, the individual glands were dried and were stored at −20°C until required for biological assay. In the case of the rat, the glands were ground up in isotonic saline and again stored at −20°C. The LH assay used was the Ovarian Ascorbic Acid Depletion (OAAD) Assay of Parlow (1958) with modifications. In the case of the rat, the pituitary glands from each group were pooled to provide suitable amounts of material for assay whereas in the case of the pig each pituitary gland was assayed individually. In all but one case a four-point biological assay design was employed, the remaining assay being of the three-point

Table 2 COMPARISON OF PITUITARY LH LEVELS IN THE RAT (CORPORA LUTEA OF LACTATION DESTROYED) AND PIG DURING LACTATION AND AFTER WEANING

Reproductive status	*Pituitary LH content*	
	*Rat** µg NIH–LH–S14/mg *anterior pituitary wet weight*	*Pig†* µg NIH–LH–S3/mg *anterior pituitary dry weight*
Lactating, anoestrous	3·00 (1·93–4·45)	3·96 ± 0·65
Post-weaning, anoestrous	5·44 (3·73–7·71)	9·68 ± 1·38
Post-weaning, oestrous	2·29 (1·44–3·84)	3·43 ± 1·65

* Assay of pooled material from each group.
Relative potency with confidence limits at P = 0·95 in parentheses.
Mean λ of assays = 0·18.
† Assay of individual pituitary glands. Arithmetic mean relative potency ± S.E.
Mean λ of assays = 0·43.

design. The standard preparation used was NIH-LH (National Institutes of Health, U.S.A.) and accepted statistical methods for testing validity and for calculating the results were employed (Gaddum, 1953; Sakiz and Guillemin, 1963). The results were expressed as μg NIH-LH per mg anterior pituitary material and are shown in *Table 2*.

In both species, there was an increase ($P < 0{\cdot}05$) in the LH content of the pituitary after weaning and before the onset of oestrus and a decrease ($P < 0{\cdot}05$) with the onset of oestrus. In both cases, the pituitary LH content during late lactation was similar to that at oestrus. The LH content characteristic of oestrus is known to be the lowest reached during the oestrous cycle in the rat (e.g. Schwartz and Bartosik, 1962) and in the pig (Parlow *et al.*, 1964). These results emphasise the similarity between the two species with respect to the suppression of pituitary LH content during lactation and the relief of the suppression on weaning. They add weight to the concept that similar mechanisms control the lactational anoestrus in both species.

THE INDUCTION OF VARYING LEVELS OF OVARIAN AND UTERINE ACTIVITY DURING LACTATION

With a control mechanism operating through the suppression of pituitary gonadotrophin secretion, one would expect the ovaries to be capable of responding to gonadotrophin supplied during lactation and it should be possible to induce follicle growth and ovulation and possibly establish pregnancy during lactational anoestrus. This is potentially of considerable commercial importance in the pig, in which lactation and the period after weaning prior to oestrus represent together some 25–35 per cent of the interval between successive parturitions under normal husbandry conditions and in which attempts have been made rather unsuccessfully to reduce this interval by 'early weaning' techniques.

In a successful system involving the induction of pregnancy concurrent with lactation in the pig it would be necessary to raise the ovarian follicles from their quiescent state through a phase of rapid development and oestrogen secretion to ovulation and, after mating, cause pregnancy to be established and maintained without severely adverse effects on lactation. If the method employed had such adverse effects, any advantages over 'early weaning' systems would be lost. The earlier in lactation that such a treatment was applied, the more important would be this restriction.

There is some information available on the effects on lactation of both extremes of ovarian and uterine activity. Experiments have

been carried out in the pig to examine, at the one extreme, complete absence of ovarian activity during lactation and at the other the effects of follicle growth, ovulation and early pregnancy.

OVARIECTOMY DURING LACTATION

For an experiment to examine the effects of ovariectomy, six Large White sows nursing litters of 6–11 piglets were used. Three were subjected to ovariectomy on day 20 or 21 of lactation, and the other

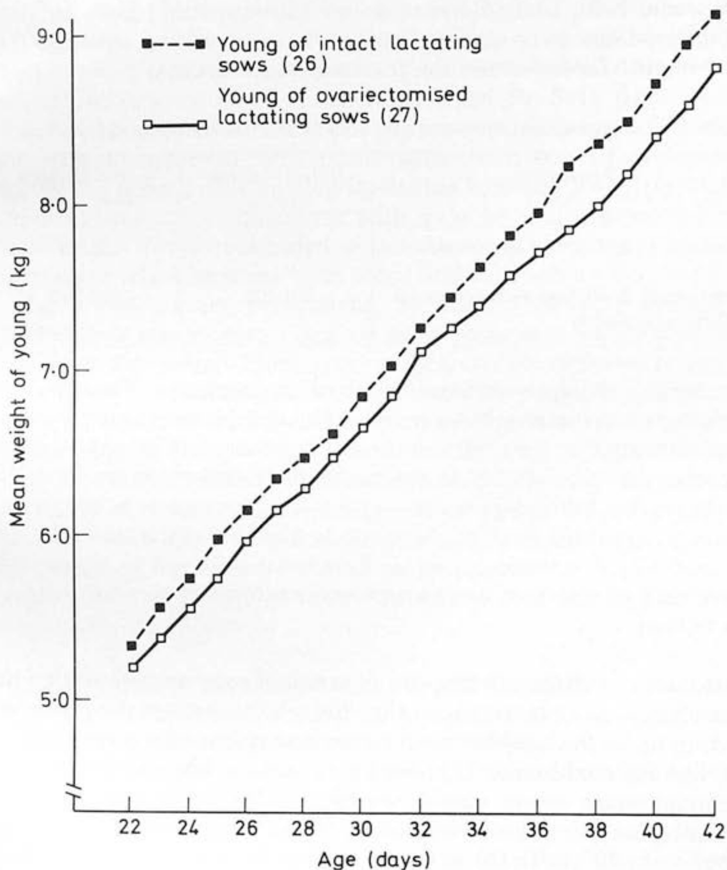

Figure 1. Growth of young of intact and ovariectomised sows. There was no significant difference between the growth rates of the two groups. Numbers of young in each group in parentheses. (From Crighton and Lamming, 1969)

three were subjected to laparotomy at the same stage. From 22 to 42 days of age each piglet was weighed every day. The piglets received no supplementary feeding during this period and thus the mean growth curve of each litter reflected the milk production of the sow. The sows were slaughtered 25 days after operation. The mean growth curves of the young of intact and ovariectomised lactating sows are shown in *Figure 1*. There was no significant difference in growth rate between the two groups, showing that lactation was unaffected by absence of the ovaries. Although no other reports have been found on lactation after ovariectomy in the sow, ovariectomy is known not to affect lactation in the rat (Long and Evans, 1922; Folley and Kon, 1938; Barsantini and Masson, 1947; Flux, 1955).

Observations were carried out on the uteri of the sows on this experiment. *Table 3* shows the results of considering two parameters

Table 3 WEIGHTS AND LENGTHS OF UTERINE HORNS OF LACTATING SOWS (MEAN $\pm$ S.E.)

Reproductive status	*Weight of uterine horns* (g)	*Length of uterine horns* (mm)
Lactating, 45 or 46 days, anoestrous	204 ± 44	139 ± 4
Lactating, 45 or 46 days, ovariectomised 25 days previously	201 ± 41	132 ± 22

of uterine activity, weight and length of uterine horns. There was no decline in uterine weight or length after ovariectomy during lactation, showing that the uterus of the sow is in a state of atrophy during lactation due presumably to absence of oestrogen secretion from the small ovarian follicles present.

INDUCTION OF OESTRUS, OVULATION AND PREGNANCY DURING LACTATION

Various methods for altering the situation of ovarian and uterine inactivity typical of lactation so that the sow undergoes the processes leading up to the establishment of pregnancy have been reported in the literature although the degree of success has not been high. Pregnant mare serum gonadotrophin (PMSG) has been used for this purpose (Cole and Hughes, 1946; Heitman and Cole, 1956; Allen *et al.*, 1957). In the first two studies, the majority of sows were injected during excessively extended lactations but there was failure to obtain satisfactory oestrous responses to PMSG when using a wide range of doses of up to 3400 IU per sow in the first four weeks of

lactation. Allen *et al.* (1957) obtained an oestrous response of only 16·7 per cent in sows treated with 1000 IU at approximately 20 days postpartum although the response increased to 65·4 per cent when the treatment was carried out at day 40. The lack of success achieved with PMSG in this particular situation may be due to the complex mode of action of this hormone (Quinn and Zarrow, 1965).

The technique of separating the sow from the young for a period each day was mentioned by Marshall and Hammond (1937) as a method for inducing oestrus in the lactating sow but no experimental evidence was offered as a basis for this. Burger (1952) was unable to confirm the efficacy of this treatment even when applied for extensive periods as a 12h separation in each 24h. Smith (1961), however, was able to induce oestrus in small numbers of animals by 12h periods of separation beginning on the twenty-first or thirty-first to thirty-fifth days of lactation and continued for 5–16 days. It was therefore clear at the outset of our experiments that, regardless of the success rate achieved with these methods, it was physiologically possible to induce ovarian activity during lactation in the pig and to establish pregnancy concurrent with some level of continued lactation. It was therefore decided to investigate the treatments already referred to when imposed both alone and in combination.

For a preliminary experiment, 24 Essex sows were divided at random into four groups. The litter size of these sows was standardised at nine or ten young. Three treatments were assessed with regard to their efficacy in inducing oestrus and permitting the establishment of pregnancy during lactation. The treatment groups were compared with a control group of lactating sows. All sows were tested once daily for oestrus with a boar from the tenth day of lactation to the tenth day after weaning. Weaning was carried out in all groups on the forty-ninth day of lactation. Sows which came into oestrus during the period of testing were mated on two successive days whenever possible, and, if lactating, were returned to their litters. The treatments were as follows:

(1) Separation of each sow and litter for 12h each day beginning on the twenty-first day of lactation and continued until oestrus was induced or ten days had elapsed.

(2) Injection of each sow with 1500 IU PMSG on the twenty-first day of lactation.

(3) Separation of each sow and litter for 12h each day on the twenty-first, twenty-second, and twenty-third days of lactation, followed by injection of the sow with 1500 IU PMSG at the end of the period of separation on the twenty-third day of lactation.

As an extension of the preliminary study, a further experiment was carried out in which the treatment combining separation and PMSG injection was evaluated in a total of 29 Essex sows (11 control, 18 treated) including the 13 sows (6 control, 7 treated) from the preliminary experiment and in 32 Large White and Large White × Landrace sows (10 control, 22 treated). In the latter sows, the treatment was repeated for up to four successive lactations. Litter size in the Essex sows was restricted within the range 8–12 young. No restriction was imposed in the Large White/Large White × Landrace herd. In the Essex herd, weaning was carried out on the forty-ninth day of lactation and in the Large White/Large White × Landrace herd on the forty-second day of lactation. In both herds the procedure was as in the preliminary experiment except that in the case of the Large White/Large White × Landrace herd, control sows were not tested daily for oestrus during lactation as it had been established previously that these sows do not normally exhibit oestrus during lactation (Crighton, unpublished data; Crighton and Lamming, 1969). Data were recorded from 26 lactations from the 10 control sows in this herd.

In the case of the Essex herd, data were obtained on the growth and supplementary food consumption of the young from 9 sows which were lactating and pregnant concurrently and from 11 controls. The young were numbered individually, males were castrated and a suitable creep feed was offered on day 10 of lactation. Litter size was within the range 9–12 young on day 14 of lactation. The mean litter size on day 14 was $10{\cdot}0 \pm 0{\cdot}3$ for both control and treated groups, and on day 48 was $9{\cdot}9 \pm 0{\cdot}3$ for controls and $9{\cdot}7 \pm 0{\cdot}5$ for treated sows. The young were weighed individually daily from day 14 of lactation to day 42 and on days 45 and 48. The consumption of creep feed, which was offered *ad libitum* was determined daily from day 14 to day 48.

The oestrous response and pregnancy data from the preliminary experiment are shown in *Table 4*. None of the control sows exhibited oestrus during lactation but all returned to oestrus at a mean interval of 3·8 days after weaning. In those cases in which separation was imposed, suckling behaviour was resumed immediately on re-uniting sow and litter. Normal suckling behaviour continued throughout the period of lactational oestrus where this occurred and in early pregnancy. Of the treatments employed, the combination of separation and PMSG injection was clearly the most effective in inducing oestrus during lactation. Only one of the sows which exhibited lactational oestrus on any of the treatments failed to carry pregnancy to term after mating during lactation. One sow which mated on the twenty-sixth day of lactation returned to oestrus 21 days later and

Table 4. OESTROUS RESPONSE AND PREGNANCY RESPONSE DATA (PRELIMINARY EXPERIMENT, ESSEX SOWS)

Group	*Sow No.*	*Litter size on the 21st day of lactation*	*Day of lactation on which oestrus exhibited*	*Day after weaning on which oestrus exhibited*	*No. of young born alive at subsequent parturition*	*Interval between successive parturitions* (days)
Control	1	10	—	5	12	168
	2	9	—	4	10	167
	3	9	—	4	11	168
	4	10	—	5	12	169
	5	9	—	4	5	168
	6	10	—	1	10	165
Separation	7	10	—	5	11	169
	8	9	—	4	9	168
	9	9	—	1	9	167
	10	10	—	6	13	171
	11	9	25	—	11	140
PMSG injection	12	9	25	—	9	140
	13	10	26	—	9	144
	14	9	—	3	12	169
	15	10	—	4	13	168
	16	10	—	4	11	167
	17	9	—	3	11	169
Separation + PMSG injection	18	10	26 and 47	—	10	163
	19	9	30	—	12	145
	20	9	27	—	13	143
	21	9	28	5	5	171
	22	10	—	4	5	169
	23	10	—	5	11	168
	24	9	27	—	8	146

Plate 20

Nerve tissue in the mammary gland. (Reproduced from the Histochemical Journal 1970 vol. 2, by permission of the publisher Chapman and Hall Ltd.)

(a and b) Adjacent sections of an artery from a lactating rabbit mammary gland (freeze dried). (a) Falch's technique for catecholamines. (b) Butyrylcholinterase stain (BuChE).

(c) Whole mount of same vessel as (a) and (b), stained for butyrylcholinesterase, showing nerves following the artery (A) *and fine nervous network in the arterial wall. There are no nerves by the vein* (V). *Nerves accompany the arteriole lying between the artery and vein but note that there are no nerves in the secretory tissue below.*

(d) Innervation of a mammary gland from a non-lactating rabbit. The nerves follow the arteries closely and veins, containing blood (grey), are not innervated. BuChE. Whole mount.

(e) Nerves near a mammary duct. Examination by phase contrast showed that only those marked (A) *accompany arteries; the rest are probably sensory. BuChE. Whole mount.*

conceived at the latter oestrus.

The oestrus response and pregnancy data from evaluation in the two herds of the treatment combining separation and PMSG injection are shown in *Table 5*. None of the control Essex sows which were tested daily exhibited oestrus during lactation but all returned to oestrus at a mean interval of 4·1 days after weaning. Considering the first lactation in which this treatment was imposed, of a total of 40 sows of both breeds treated, 33 (83 per cent) exhibited oestrus during lactation. Of these, 24 farrowed as a result of mating at the induced oestrus (73 per cent of lactationally oestrous sows; 60 per cent of all treated sows). The mean number of young born alive as a result of mating during lactation was not significantly different from the mean number born alive to control sows mated at the oestrus after weaning when the results were compared either on a herd basis or overall. The mean interval between successive parturitions in sows becoming pregnant during lactation was reduced in both herds by approximately 25 days when compared with controls.

Considering the repetitive application of the treatment, the oestrous response and pregnancy data from the Large White/Large White × Landrace herd are also shown in *Table 5*. The percentages of treated sows showing oestrus during lactation were 77·3, 80·0, 75·0 and 85·7 per cent in the first, second, third and fourth lactations respectively. There were no significant differences between the responses. When the numbers of sows carrying pregnancy to term as a result of lactational mating (as a percentage of sows showing oestrus during lactation) were compared in successive lactations, the percentages were 76·5, 56·3, 58·3 and 66·7 respectively. There were no significant differences between the responses. When the mean numbers of young born alive to treated sows were compared, there were no significant differences with successive lactations.

Plate 27

Histological structure of the mammary gland of the goat during the second half of pregnancy.

(a) day 82 —lobules of small alveoli are forming, alveoli contain some secretion.

(b) day 101—lobules of alveoli have replaced most of the stroma, alveoli have increased in size and contain more secretion.

(c) day 120 } alveoli are distended with secretion which is very
(d) day 140 } rich in fat-globules.

Plates are at the same magnification as in Plate 26.

Table 5. OESTROUS RESPONSE AND PREGNANCY DATA (BREED COMPARISON AND REPETITION OF TREATMENTS)

Breed	*Treatment*	*Lactation*	*No. of sows*	*Litter size on the 21st day of lactation* (Mean ± S.E.)	*No. of sows exhibiting oestrus during lactation*	*Interval between PMSG injection and oestrus* (days) (Mean ± S.E.)	*No. of sows carrying pregnancy to term as result of lactational mating*	*No. of young born alive* (Mean ± S.E.)	*Interval between successive parturitions* (days) (Mean ± S.E.)
	Control		11	9·8 ± 0·3	0	—	0	10·5 ± 1·1	169·6 ± 1·1
Essex	Separation + PMSG injection		18	9·6 ± 0·3	16	4·2 ± 0·3	11	9·7 ± 0·6*	143·8 ± 1·8* (154·0 ± 3·5)†
	Control		10 (26 lactations)	8·2 ± 0·5	—	—	—	9·7 ± 0·5	168·1 ± 2·0
Large White/ Large White × Landrace	Separation + PMSG injection	1	22	8·4+0·4	17	4·5+0·3	13	9·5 ± 0·8*	143·3 ± 2·7* (154·1 ± 3·1)†
		2	20	7·8 ± 0·8	16	4·8 ± 0·2	9	9·2 ± 1·0*	144·2 ± 0·5* (157·9 ± 3·2)†
		3	16	7·9 ± 0·1	12	4·7 ± 0·3	7	8·3 ± 1·2*	144·4 ± 0·9* (156·1 ± 1·3)†
		4	14	8·4 ± 0·4	12	5·0 ± 0·4	8	7·8 ± 1·5*	144·3 ± 1·1* (157·2 ± 5·3)†

* Mean of sows carrying pregnancy to term as result of lactational mating
† Mean of all sows treated.

The piglet growth and supplementary food consumption data from the Essex herd are shown in *Figure 2*. The growth of the young of treated sows was faster than that of controls. The growth curves were clearly different, both in average live weight ($P < 0{\cdot}001$) and in regression coefficients ($P < 0{\cdot}001$), the former being the greater

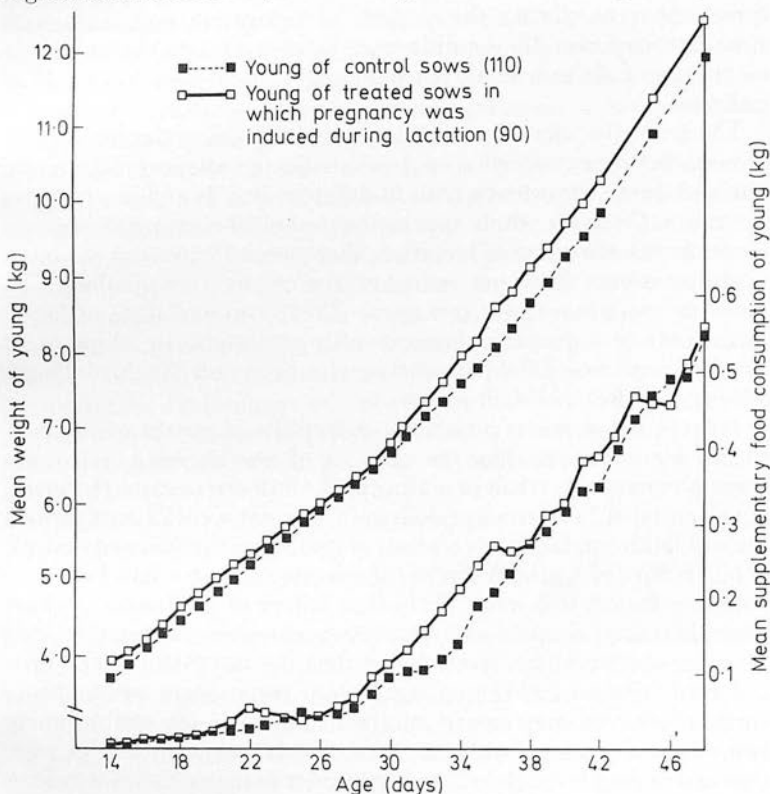

Figure 2. Growth and supplementary food consumption of young of control and treated sows. There were no significant differences between the growth rates and consumptions of the two groups. Numbers of young in each group in parentheses

effect. The supplementary food consumption curves were also markedly different, most of the difference being due to the higher average level of consumption of the treated group ($P < 0{\cdot}001$). The difference in the regression coefficients was, however, also significant ($P < 0{\cdot}05$). The period of separation and injection of the sow and the induced oestrus were not accompanied by an appreciable check in the growth of the young. The establishment of pregnancy was followed by a divergence of the growth curves, the young of treated

sows increasing from a mean of about 0·09 kg heavier on day 29 to about 0·55 kg heavier on day 36. A mean difference of about 0·5 kg per piglet was maintained until weaning. The supplementary food consumption was significantly elevated in the young of treated sows from day 20 to day 48 of lactation. An increase in consumption became apparent during the periods of separation and was again apparent from day 27, the difference relative to controls reaching a maximum of about 0·07 kg per piglet per day on days 34 and 37 of lactation.

These results demonstrate the possibility of inducing follicle growth, oestrogen secretion and oestrus in the anoestrous lactating sow with some consistency both in different breeds and in successive lactations. Over the whole experiment out of 90 treatments imposed on the twenty-first day of lactation, there were 73 oestrous responses to the treatment (81·8 per cent) and the results were unaffected by breed or repetition of the treatment. There was also little variation in the time of expression of oestrus after treatment. In 59 out of 73 oestrous responses (80·8 per cent) oestrus began on the third, fourth or fifth day after PMSG injection.

It is clear that in the present work lactational oestrus was accompanied by ovulation, since the majority of sows showing oestrus became pregnant as a result of mating at the induced oestrus. However, out of a total of 73 oestrous periods induced there were 23 unexplained cases of failure to farrow as a result of mating at the induced oestrus. While failure of a proportion of the sows to ovulate might be a contributing factor, it is more likely that failure of fertilisation, failure of implantation or early embryonic mortality were responsible. Preliminary investigations have shown that the last factor is certainly involved. The present results suggest that two to three weeks of concurrent lactation may create conditions hostile to the establishment and/or maintenance of pregnancy but which are insufficiently severe to result in the prevention of these processes in the majority of sows or indeed to reduce the number of young surviving to term compared with controls in pregnancies which are established and maintained.

The data in *Figure 2* showed a more rapid rate of growth from day 20 to day 48 of lactation of the young of treated sows which were concurrently pregnant and lactating from about day 29 of lactation. Since supplementary food consumption is known to be inversely related to sow milk yield (Smith, 1952, 1961; Barber *et al.*, 1955), the fact that the mean creep feed consumption per day was greater in the litters of concurrently pregnant and lactating animals would by itself suggest that milk yield and/or quality was reduced by concurrent pregnancy. However, the improved growth of the young of concurrently pregnant and lactating sows on the increased supple-

mentary food consumption suggests that milk yield and composition were not affected detrimentally to any extent by concurrent early pregnancy. The increased mean daily supplementary food consumption may be explained by the early introduction of the young of treated sows to the necessity of eating solid food during the daily periods of separation prior to PMSG injection (*Figure 1*). Increased supplementary food consumption by the young of separated sows has also been observed by Smith (1961). This may have resulted in a greater readiness to increase consumption again in later lactation as milk yield declined.

Further experiments will be required to determine whether a treatment such as has been described may be employed successfully earlier in lactation than in the present study. While preliminary results suggest that oestrus and ovulation can be induced to occur consistently as early as day 14 of lactation, the effects on the establishment of pregnancy of such factors as incomplete uterine regression after farrowing or of longer periods than two to three weeks of concurrent pregnancy and lactation on the maintenance of either are as yet uncertain. Preliminary studies suggest that there is greater interference with the course of pregnancy under these circumstances. It is not yet possible to state what are the effects on the concurrent lactation.

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I am indebted to Miss Katherine Barker for permission to quote results obtained in a co-operative study on the rat. The work was supported by a grant from the Pig Industry Development Authority. Mr G. H. Macdonald of Burroughs Wellcome and Company and the National Institutes of Health, U.S.A. kindly provided gifts of purified hormone preparations. The co-operation of Mr A. Goddard of East Leake, Nottinghamshire who made his herd of Essex sows available for these studies is gratefully acknowledged.

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PART III

THE NATURAL CONTROL OF OVULATION IN THE SHEEP

D. B. Crighton

In animals such as the sheep which possess an oestrous cycle, ovulation usually occurs in close relation to a period of sexual receptivity known as oestrus or "heat". This ensures that deposition of spermatozoa in the female tract is synchronised with ovulation thus making the chances of conception high. The seasonal non-breeding period (anoestrus and anovulation) which may last from early March to early September in a breed such as the Clun Forest which we have at Sutton Bonington, together with the inhibitory effects on breeding associated with the post-partum period, limit the annual production of lambs. This restricts not only the profitability of sheep enterprises but also the rôle which the species plays in the overall production of protein for human consumption.

The natural control of the timing of the breeding season and the events of the oestrous cycle in the sheep has been the subject of much interest since the pioneering studies of Marshall [1] in 1903. The accumulation of information was very slow at first but has accelerated since about 1965, largely because of the effort devoted to the development of hormone measurement techniques, together with a realisation that the central nervous system plays a key rôle in controlling these events and is capable itself of secreting hormones. This revolutionary idea owes a great deal to the work of the late Professor Geoffrey Harris [2] whose studies may be said to have formed the foundation of modern concepts of neuro-endocrine control mechanisms.

Studies of these aspects in the sheep are contributing not only to a more complete understanding of reproduction in this species but in the wider field of neuro-endocrinology, the sheep is a valuable experimental animal, results from which may be extrapolated to other species including the human. Ultimately, knowledge gained from physiological experiments on the sheep may help us to devise reliable means of controlling reproduction artificially. Such detailed knowledge is, I believe, vital to provide a sound basis for manipulation of the natural reproductive patterns of animals for productive purposes.

Over the years, various empirical treatments have been used in the hope of inducing ovulation, oestrus and fertility in the sheep out of season. Until recently, investigators could not be blamed for making such attempts, since the potential returns are great and the complexity of the control mechanisms was not understood. Some of these methods were sometimes successful to a degree, in the same way that kicking a faulty television set may sometimes bring back the picture. As with the television set, such treatments have little chance of consistent success. Now that the

complexity of reproductive control mechanisms is apparent and many of the tools are to hand to enable detailed and logical investigation, we may hope to arrive at a situation in which we can make with some confidence the delicate adjustments needed to obtain consistent optimal performance from the reproductive apparatus. When this is possible, the results may have far-reaching effects in the livestock industry.

Timing of Ovulation in the Sheep

Ovulation can be observed at laparotomy, that is surgical intervention in which the abdomen is opened under general anaesthesia and the ovaries are observed directly. In a more recently developed technique, laparoscopy, the ovaries are observed using an optical instrument inserted through a puncture incision in the abdomen, again under general anaesthesia, a procedure which can be repeated with minimal risk of trauma and haemorrhage. There is considerable variation within a single sheep and between sheep in the length of the oestrous period and also in the timing of ovulation in relation to the onset and termination of sexual receptivity, although in most individuals ovulation occurs late in the oestrous period. Part of the variation in ovulation timing is due to variation in the timing relative to the onset of oestrus of a pre-ovulatory surge of release from the pituitary gland of one of the gonadotrophic hormones, luteinizing hormone (LH). This surge of LH into the bloodstream is believed to be responsible alone, or in conjunction with a concurrent surge of another pituitary gonadotrophin, follicle-stimulating hormone (FSH), for acting on the gonad (ovary) causing rupture of the ovarian follicle and shedding of the ovum (ovulation).

Timing of the Pre-ovulatory LH Surge

Blood concentrations of LH are measured by radio-immunoassay. Such assays depend on *in vitro* competition between LH in the sample of blood plasma and radioactively-labelled LH for binding sites on a specific antibody developed against LH. The details of the method are inappropriate here; suffice it to say that such a method has been developed at Sutton Bonington [3] with valuable help in the early hormone purification stages from Dr. Anne Hartree of the Department of Biochemistry, University of Cambridge, with whom we have carried out several joint projects since 1968. Blood samples for assay can be collected from experimental sheep via indwelling jugular vein cannulae. The interval between the taking of successive blood samples depends on many factors but is never more than thirty minutes and often as little as four minutes. Blood samples are centrifuged and the supernatant plasma is retained frozen at -20° C and later subjected to assay for LH.

The variability of timing of the pre-ovulatory LH surge in relation to the onset of oestrus causes practical problems in characterising the surge completely. In the majority of animals the surge will have begun by four

hours after the onset of oestrus and in the majority it will be completed by 20 hours after onset. It is therefore essential to take blood samples for a period of 20 hours from onset of oestrus. The only practical means available to the experimenter of determining onset of oestrus is to put a ram in the pen with the ewes and observe the group continuously for signs of sexual activity in the period when oestrus is expected. Since successive cycle lengths in an individual may vary by two days and since the onset of oestrus is rarely a sudden phenomenon, it is only too easy to begin blood sampling too soon or to delay too long before starting, both of which errors result in incomplete "capture" of the LH surge in the blood samples. In spite of these difficulties, we have been able to show [3] that in the Clun Forest ewe the pre-ovulatory LH surge lasts for approximately 8–10 hours and shows a peak of approximately 180–260 ng/ml from a baseline of 1–18 ng/ml (see examples in Fig. 1). In these animals laparotomy after blood sampling showed that ovulation had occurred at about the expected time.

L'Hermite, Niswender, Reichert and Midgley [4] in the U.S.A. have been able to demonstrate that there is a pre-ovulatory peak of FSH, coincident with that of LH although it is less marked. At Sutton Bonington, as a result of work by a graduate technician, Brenda Siddall, we have recently been able to overcome most of the severe technical problems involved in the development of a radio-immunoassay for ovine FSH. Although our data on this hormone are as yet very limited, the results do show that when blood concentrations of LH rise, FSH rises also in all the circumstances we have so far examined. Experiments involving the assay of the same plasma samples for LH and FSH are important to carry out since they may help to answer one of the burning questions in reproductive endocrinology: are the two gonadotrophic hormones controlled by a common releasing hormone produced in the central nervous system (neuro-hormone) or by two different releasing hormones? The question can be posed in this form because there is now no dispute that gonadotrophin release is controlled neuro-hormonally. It is also clearly established that the main area of the central nervous system involved is the hypothalamus which directly overlies the pituitary gland and is connected to it by a pituitary stalk.

Timing of Hypothalamic Stimulation Causing Pre-ovulatory Gonadotrophin Release

That the part of the brain known as the hypothalamus plays a rôle in controlling the release of hormones from the pituitary gland has been realised since the early studies on the close anatomical relationship of these two areas. The nature of this control as far as the gonadotrophins are concerned was clarified somewhat by the discovery in 1960 in the neural tissue of the hypothalamus of an LH-releasing factor [5] and four years later of an FSH-releasing factor [6]. Much of the effort of researchers in this field since then has been devoted to attempts to establish whether these

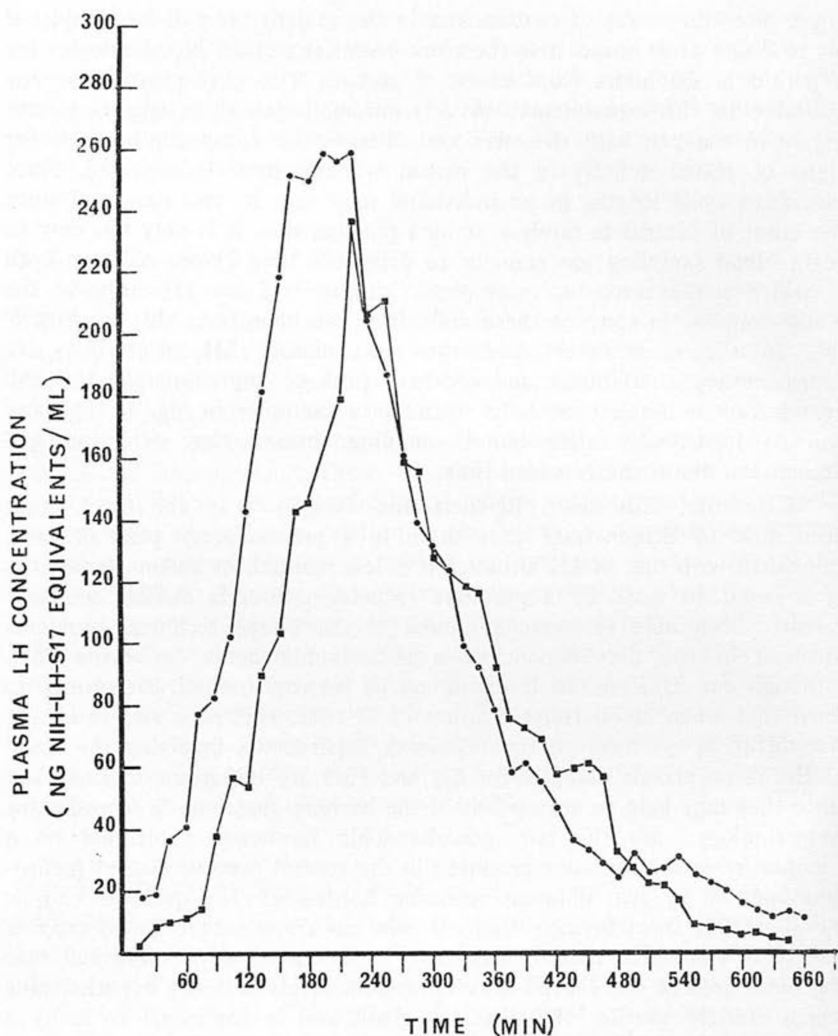

FIG. 1. Luteinizing hormone (LH) concentrations in the jugular venous plasma of two Clun Forest ewes at oestrus.

activities reside in the same neuro-hormone molecule or in separate but closely-related molecules. Since until very recently the chemical investigation of neuro-hormones lagged behind their physiological investigation, all of the early studies carried out on gonadotrophin-releasing activity were based on biological assay methods using animals or pituitary gland tissue to measure concentrations of releasing activity in hypothalamic tissue. Changes in the concentration of hypothalamic releasing activity could be correlated with changes in pituitary hormone concentrations in experimental animals. The results of such an experiment carried out at Sutton Bonington

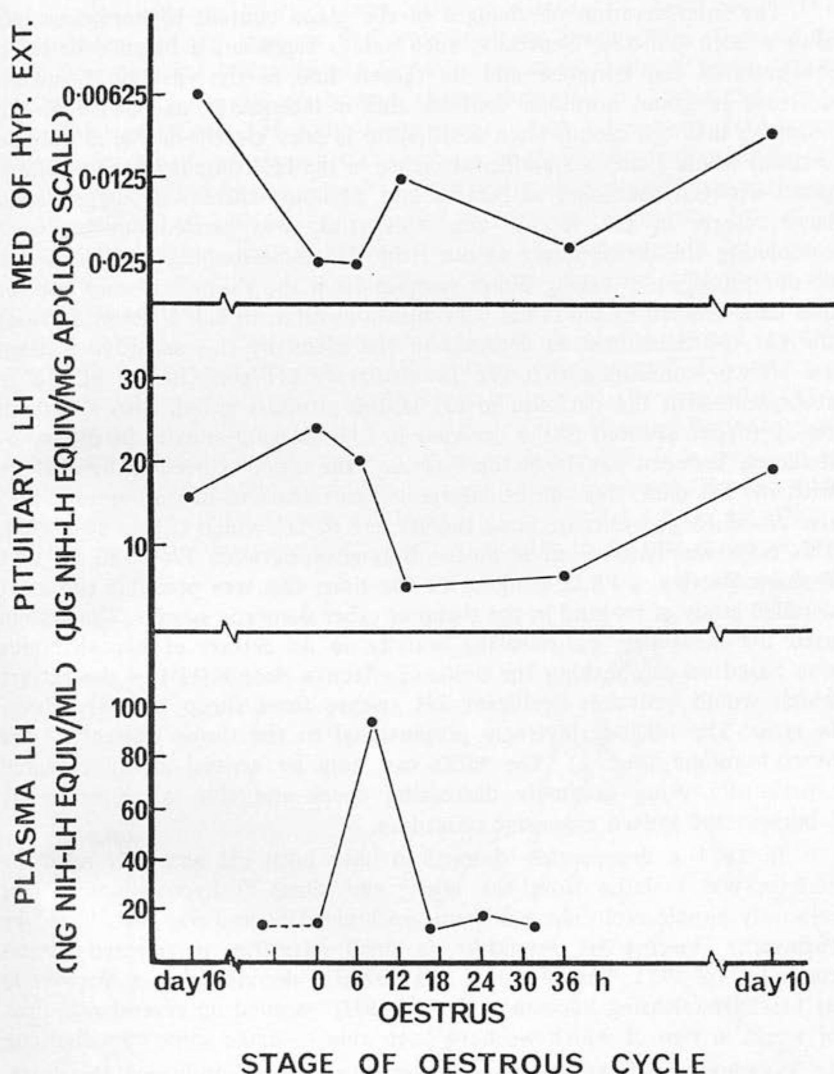

FIG. 2. Changes in the luteinizing hormone (LH) releasing activity of the hypothalamus and in LH in the pituitary gland and plasma during the oestrous cycle of the sheep. Means are shown. MED = minimal effective dose.

on the sheep are shown in Fig. 2 [7]. In this case a sheep pituitary tissue incubation system [8] was used to estimate LH-releasing activity in the hypothalami of sheep slaughtered at known stages of the oestrous cycle. LH concentrations in the pituitary glands and plasma of the same sheep were determined by biological assay and radio-immunological assay respectively.

The interpretation of changes in the gland content of hormones has always been difficult. Generally, such values represent a balance between synthesis of the hormone and its release and in the case of a sudden decrease in gland hormone content, this is interpreted as release of the hormone into the blood. Such a situation is seen clearly in Fig. 2 (middle section) where there is a sudden decrease in the LH content of the pituitary gland between the onset of oestrus and 12 hours thereafter, suggesting a large release of LH. At the time this study was carried out we were completing the development of our ovine LH radio-immunoassay, and due to our foresight in taking blood samples from the sheep, we were able to add data concerning blood LH concentrations later. In Fig. 2 (lower section) the LH concentrations as detected in the blood by this sensitive method are shown, confirming that the pre-ovulatory LH peak in the plasma is coincident with the decrease in LH in the pituitary gland. Also shown in Fig. 2 (upper section) is the decrease in LH-releasing activity in the hypothalamus between day 16 of the cycle and the onset of oestrus. By analogy with the LH data, this can be interpreted as release of neuro-hormone into the blood to provoke, in turn, the release of LH which causes ovulation. This work was carried out at Sutton Bonington between 1968 and 1971 by Barbara Hartley, a Ph.D. student. At the time, this was probably the most detailed study of its kind in the sheep or other domestic species. The system used for measuring LH-releasing activity in an extract of hypothalamus was based on establishing the minimal effective dose (MED) of the extract which would provoke significant LH release from sheep pituitary tissue *in vitro*. The MED is inversely proportional to the tissue content of the neuro-hormone (Fig. 2). The MED can only be arrived at by repeated experiments using gradually decreasing doses and this is an extremely laborious and indeed expensive procedure.

In 1971 a decapeptide claimed to have both LH and FSH releasing activity was isolated from the pig [9] and sheep [10] hypothalamus. This relatively simple molecule was soon synthesised [11] and was distributed by Farbwerke Hoechst AG, Frankfurt, in small quantities to selected laboratories in late 1971. The advent of this material, described by its discoverers as LH/FSH releasing hormone (LH/FSH–RH)* opened up several new lines of work, in two of which we have been able to make some contribution:

1. Examination of the effects of the synthetic decapeptide on LH release, FSH release and ovulation in the sheep.
2. Development of a radio-immunoassay technique for LH–RH and attempts to measure possible changes in concentrations in the blood of the sheep in relation to the pre-ovulatory LH peak and ovulation.

1. Initially, we used the sheep pituitary tissue incubation system referred to previously to determine whether the synthetic decapeptide would release both LH and FSH *in vitro* as claimed. Release of both hormones was significantly augmented by the decapeptide [12] suggesting that it might

* Throughout the article this material will be referred to as "the decapeptide" or "LH-RH".

have some value in inducing gonadotrophin release and ovulation *in vivo*. Peter Foster (at the time an SRC Post-graduate Student and currently an ARC Post-doctoral Fellow in the Department) and I designed some simple preliminary experiments to extend these observations. Using the blood sampling procedure, LH radio-immunoassay and laparotomy techniques already described, we examined the effects of single intravenous injections of the decapeptide in the sheep [(3)]. After preliminary dose-response experiments, a dose of 150 μg was selected and injected into ewes in various reproductive states. The results showed that LH release could be induced during the reproductive cycle, during seasonal anoestrus and during the early post-partum period. In all situations the LH release patterns were similar (see Fig. 3 for examples) and almost all of the treated animals ovulated shortly after the peak in LH concentration occurred. This evidence when considered superficially would seem to support the suggestion from our previous study (Fig. 2) that a single large surge of neuro-hormone with LH-releasing ability could trigger the pre-ovulatory LH peak and lead to ovulation. Unfortunately, although ovulation was produced in almost all cases examined, the LH peak resulting from a single injection of 150 μg of the synthetic decapeptide was much smaller in height, duration and

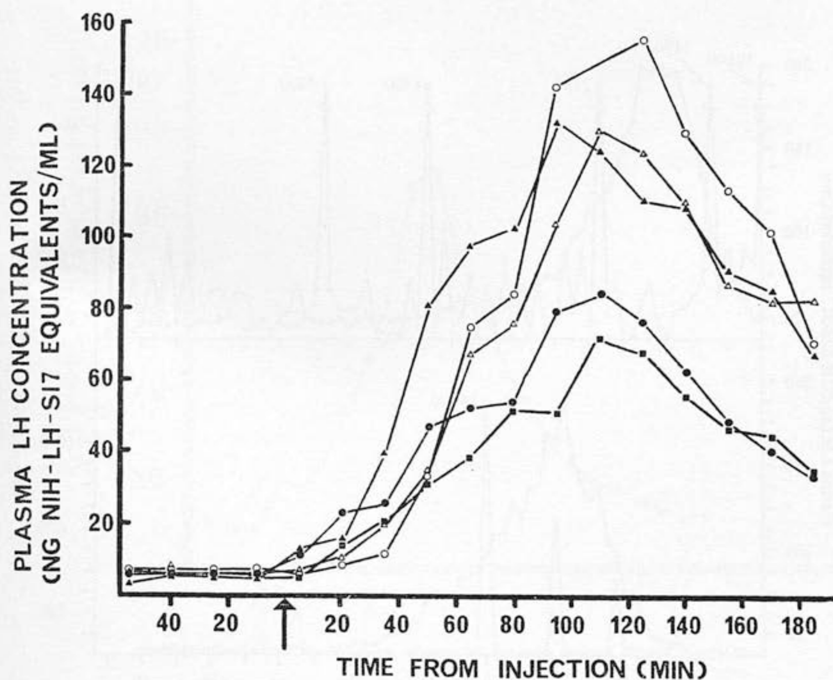

FIG. 3. Luteinizing hormone (LH) concentrations in the jugular venous plasma of five Clun Forest ewes injected intravenously with 150 μg synthetic decapeptide on day 16 post-partum. The arrow indicates the time of injection. Similar results were obtained on day 12 of the oestrous cycle and in mid-seasonal anoestrus.

area included by the peak than the natural pre-ovulatory surge. Area included by the peak is generally accepted as the best indicator of amount of hormone released and in this case the induced release was only about 20% of the natural release. This situation was not improved by increasing the dose of decapeptide administered as a single injection to 300 μg. It seemed likely, therefore, that factors other than a single discharge of neuro-hormone were involved.

2. In 1973 Jeffcoate, Fraser, Gunn and Holland[13] reported the development of a radio-immunoassay for LH–RH. A co-operative study was begun with this group (working at St. Thomas's Hospital, London) to examine plasma concentrations of LH–RH in relation to the pre-ovulatory LH peak in the sheep. Blood samples were obtained at 15-minute intervals from the onset of oestrus from two ewes and the results are shown in Fig. 4[14]. An apparent series of discharges of LH–RH occurred rather than a single discharge. The qualification "apparent" is used because from chemical studies we now know that the material present in sheep blood is not the decapeptide itself but appears to be a large fragment similar in some respects to the C-terminal nonapeptide which can be derived from the decapeptide molecule and which is presumably a metabolite. Thus

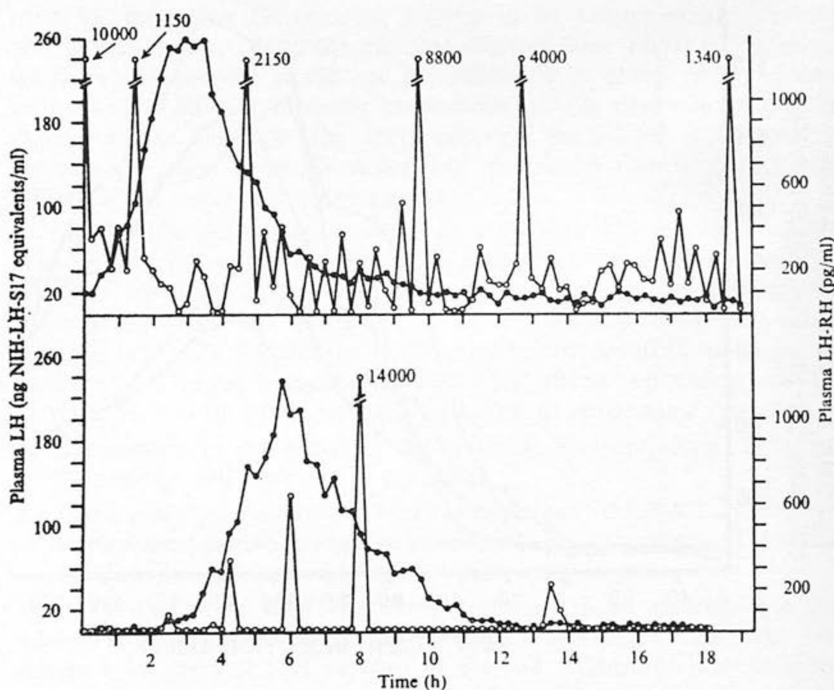

FIG. 4. Luteinizing hormone (LH) (●——●) and LH-releasing hormone (LH–RH)—like immunoactivity (o——o) concentrations in the jugular venous plasma of two Clun Forest ewes at oestrus.

the assay is not completely specific for the decapeptide but this may not be a serious disadvantage if the peaks seen reflect the discharge pattern of the decapeptide from the hypothalamus. The results suggest that the pituitary gland may be stimulated to action by a fusillade of shots from the hypothalamus rather than by a single detonation.

In order to throw some light on this, we decided to test the response of the pituitary gland of the seasonally anoestrous sheep in a multiple injection schedule of the synthetic decapeptide and to compare this with a single injection as given previously, the total dose in each case being 150 μg. The multiple injection schedule (5 x 30 μg) was designed to mimic approximately the sequence of natural discharges shown in Fig 4. It was obvious that the natural situation could not be reproduced with absolute accuracy since the concentrations of neuro-hormone impinging upon the pituitary gland in either the natural or artificial situation could not be calculated. Measurement of these concentrations was also impossible because it would have required taking blood samples close to the pituitary gland and this could not be achieved without creating highly artificial circumstances

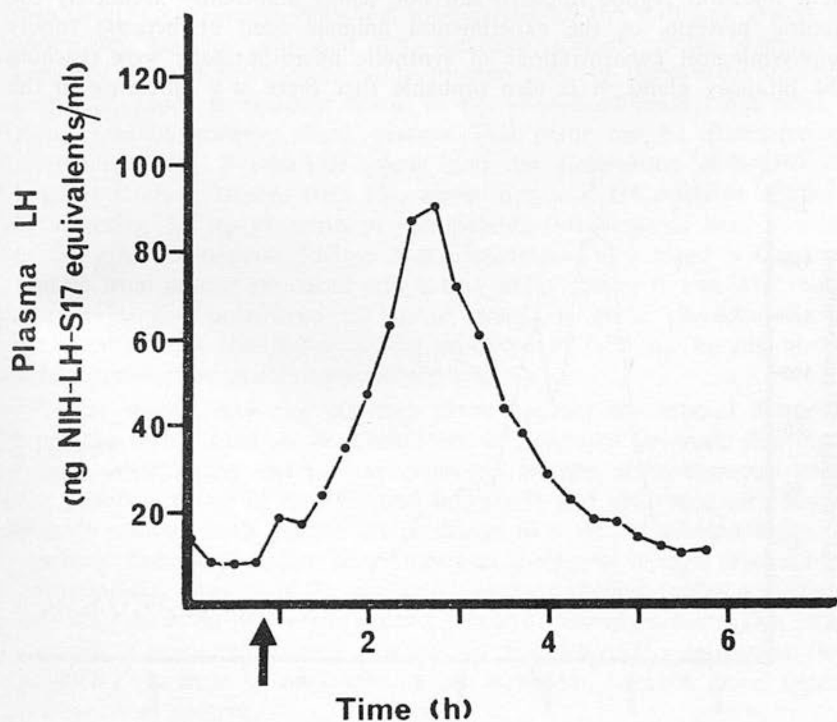

Fig. 5. Mean luteinizing hormone (LH) concentrations in the jugular venous plasma of five seasonally anoestrous Clun Forest ewes given a single intravenous injection of 150 μg synthetic decapeptide. The arrow indicates the time of injection.

due to anaesthesia and surgical intervention. However, the experiment was considered worthwhile and was taken up as an honours research project in 1973-74 by Susan Scott. The mean LH assay results from the single injection and multiple injection groups are shown in Figs. 5 and 6 respectively and the findings are summarised in Table 1 [15]. Since peak concentrations were not reached at exactly the same time after injection in different animals, the mean heights of the LH peaks in Table 1 are different from the maxima shown in Fig. 5 and 6. Using the multiple injection schedule, the same total dose of synthetic decapeptide produced a significantly greater response than if single injection was used, this in spite of the fact that the fourth and fifth injections made little, if any, contribution to the size of the LH peak, a finding which shows a striking similarity to the natural situation upon which the injection schedule was based (Fig. 4).

The LH peak produced by multiple injection was much closer in form to the natural pre-ovulatory surge, certainly within the physiological range we have observed, although examination of Table 1 shows that it still does not equal the mean values for the natural surge in two ewes in the same year, in duration or area included by the peak. This may be because the rigid injection regime imposed did not reflect sufficiently accurately the natural patterns of the experimental animals used or because totally unphysiological concentrations of synthetic neuro-hormone were reaching the pituitary gland. It is also probable that there is a difference in the

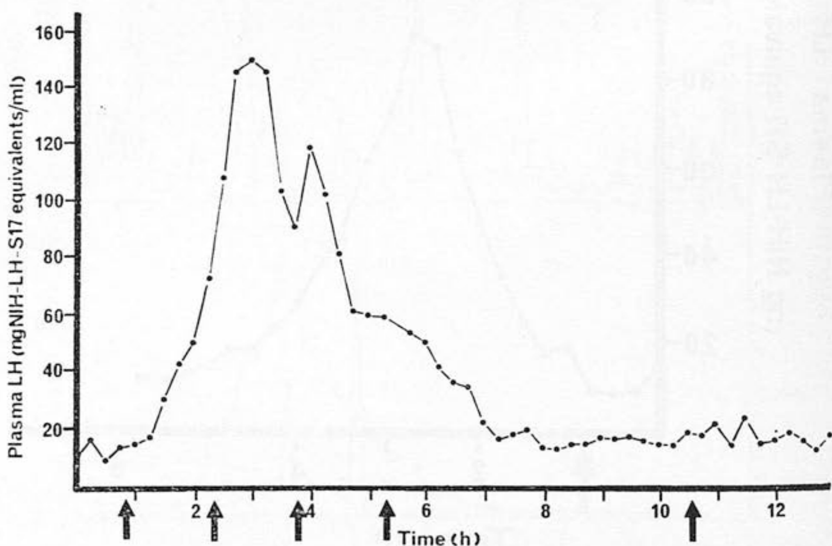

FIG. 6. Mean luteinizing hormone (LH) concentrations in the jugular venous plasma of four seasonally anoestrous Clun Forest ewes given multiple intravenous injections of synthetic decapeptide. The arrows indicate the times of injection of 30 μg decapeptide (total dose 150 μg).

Table 1 COMPARISON OF PLASMA PEAKS OF LUTEINIZING HORMONE (LH) INDUCED BY SINGLE AND MULTIPLE INJECTIONS OF SYNTHETIC DECAPEPTIDE (means ± SEM)

Treatment	Height (ng NIH–LH/ml)	Duration (hours)	Area (mm^2)
Single injection	98±12	4.30±0.22	2477±279
Significance of difference	$P<0.01$	$P<0.05$	$P<0.001$
Multiple injection	172±16	6.87±0.87	7442±815
Natural pre-ovulatory peak	159±24	8.80±0.62	12119±2719

responsiveness to neuro-hormonal stimulation of the pituitary glands of anoestrous animals compared with oestrous animals. Such changes in pituitary gland responsiveness can be brought about by the ovarian hormone oestrogen which is virtually absent in the anoestrous animal but plays a major role in bringing about oestrus. This point can be illustrated by reference to Fig. 7 which is taken from the dissertation of a 1974–75 honours student, George Ball. This shows a typical LH response obtained by injecting 50 μg of synthetic decapeptide intravenously into a sheep at the onset of oestrus. Such a dose administered at a stage of the cycle remote from oestrus produced only a very slight change in the LH concentration [16]. The peak seen in Fig. 7, which is quite characteristic, is comparable with the largest peaks produced by 150 μg in any of the other reproductive situations examined (Fig. 3).

The reason why the pituitary gland appears to respond better to repetitive rather than single stimulation is suggested by work showing a second sensitisation effect, that produced by the neuro-hormone itself. Rat pituitary tissue *in vitro* [17] and in *vivo* [18] and the ovine pituitary *in vivo* [19] respond with greater LH discharge to a second administration of synthetic decapeptide when this is given at a suitable interval after a first, priming dose. Thus what the multiple injection schedule in the anoestrous ewe may do is to utilise the second (neuro-hormonal) sensitisation effect to produce a greater LH peak but not the first (steroid) sensitisation thus producing the type of peak seen, which is almost, but not quite, typical pre-ovulatory pattern.

Such conjectures are interesting, but it might be asked: "Why is it possible to produce ovulation consistently in anoestrous and cycling ewes with a single injection of synthetic decapeptide which releases only a small amount of LH?" or to reverse the question: "Why does the sheep produce

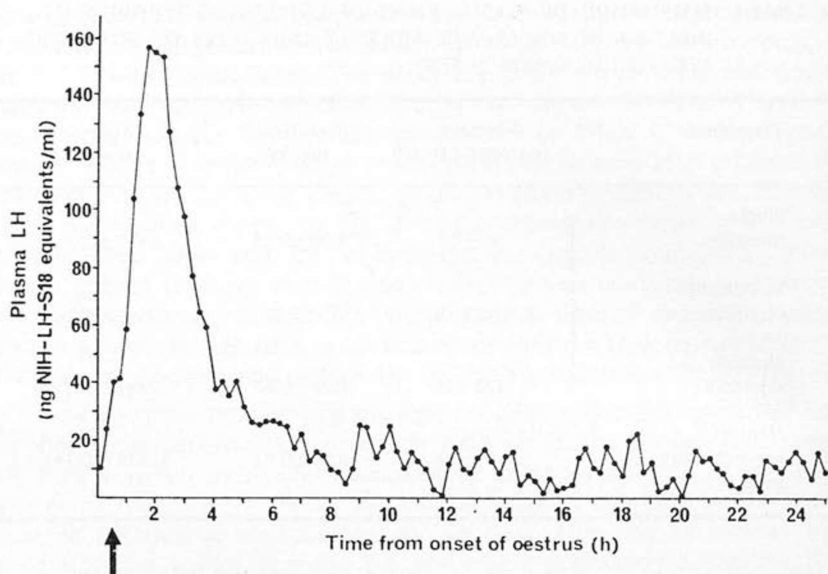

FIG. 7. Luteinizing hormone (LH) concentration in the jugular venous plasma of a Clun Forest ewe injected intravenously with 50 μg synthetic decapeptide at onset of oestrus. The arrow indicates the time of injection.

much more LH in the pre-ovulatory surge than it apparently requires to provoke ovulation?" No answer can be given at present. What is clear is that by administering synthetic decapeptide to the anoestrous ewe, whether in single or multiple doses, although we cause ovulation, we do not set in train the natural series of events which follows ovulation and leads to the typical secretion of progesterone by the corpus luteum of the ovary.[20] These events normally lead to the determination of the cycle length characteristic of the species when fertilisation does not occur and to the ability of the fertilised ovum to implant in a receptive uterus if the animal becomes pregnant. To consider these further problems is outside the scope of this article. Solutions will undoubtedly be found if the physiological approach is continued. Progress may be slow but the knowledge obtained will be invaluable as a sound basis for treatments designed to manipulate normal reproductive patterns for purposes of animal production.

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With 259 figures
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Control of FSH and LH Secretion in Animals

D. B. Crighton, G. E. Lamming

It is generally agreed that plasma levels of LH are basal during most of the oestrous cycle with well-defined peaks occurring at oestrus [sheep: (9); cow: (17)]. When blood sampling has been sufficiently frequent, subsidiary peaks have been observed at other stages of the cycle in some individuals [sheep: Foster and Crighton, unpublished data; cow: (11, 17)]. Recent evidence shows that the release of FSH occurs simultaneously with that of LH [sheep: (13); cow: (12)]. These findings seem at variance with the data of Robertson and Rakha (16) in the sheep at oestrus, showing a decline in the pituitary content of FSH preceding that of LH by some hours.

The taking of blood samples from a jugular vein cannula at intervals of 15 mins. from the onset of oestrus has allowed characterization of the pre-ovulatory LH peak in the sheep. In the Clun Forest ewe the height of the peak is approximately 250 ng LH/ml plasma compared with a baseline of <10 ng/ml and the duration of the elevated level of LH is about 10 hours (Foster and Crighton, unpublished data).

The control which the hypothalamus exerts over the release of FSH and LH is influenced by the level of ovarian steroid hormones. The development of radioimmunoassay procedures has aided work on the influence of changes in plasma steroid hormone levels on plasma FSH and LH, although the difficulties involved in the development of specific radioimmunoassays for FSH have so far limited evidence concerning FSH levels. Notwithstanding the differences in cycle length the changes in plasma levels of steroid hormones show similar patterns in the sheep and cow. In both species, plasma levels of progesterone are low at base values of less than 0.5 ng/ml for the first 3 days of the cycle rising slowly from day 4 reaching a peak on approximately days 10 to 12. In the cow the progesterone level declines sharply approximately four days before oestrus and in the ewe at approximately day 14 of the 17-day cycle (18, 19).

During the oestrous cycles of the sheep and cow there is a peak of plasma oestradiol either just before or just at the onset of oestrus [sheep: (2); cow: (20)]. This is thought of as an early link in the chain of events leading to LH release and ovulation. It has been shown that the injection of oestradiol into anoestrous ewes will

cause LH release (10). A similar observation has been made in ovariectomized cows (7).

In the sheep, levels of hypothalamic LH-releasing activity are high at day 16 of the cycle and fall at the onset of oestrus, there being a gradual recovery by mid-cycle. These changes precede by some hours the decline in pituitary LH and coincident peak of LH in the plasma which occur within the first 18 hours of oestrus (5). Preliminary observations using a specific radioimmunoassay show that LH-RF is present in the jugular venous plasma of the ewe as intermittent peaks both before and during the pre-ovulatory LH peak (Crighton, Foster, Jeffcoate, Holland, unpublished data).

The synthetic decapeptide produced by Geiger, König, Wissman, Geisen and Enzmann, (8) released both LH and FSH from the sheep pituitary *in vitro* (3). Administration of 150 μg of the decapeptide as a single jugular intravenous injection to both cycling and anoestrous ewes resulted in a peak of plasma LH (4). In both types of ewe the pattern of LH release was similar. There was generally a slow increase in plasma LH for 20–40 mins. after administration followed by a steeper rise until a peak was reached between 65 and 120 mins. after injection. Plasma LH then fell at about the same rate as the second phase of the rise. This pattern of response has also been observed in anoestrous ewes by Arimura, Debeljuk, Matsuo and Schally (1) in response to intra-carotid injection of 250 μg decapeptide. A much more rapid response is seen in ovariectomized ewes (15) and in castrate males (Foster and Crighton, unpublished data).

In anoestrous ewes ovulation occurs in a high percentage of animals in response to decapeptide injection, although the occurrence of ovulation is unrelated to the height of the LH peak. Observations on progesterone levels in these ewes, however, showed that progesterone secretion by induced corpora lutea was abnormal, levels being basal or only slightly elevated when compared with those of the cycle (6). Further work is required to elucidate the reasons for these abnormalities.

Summary

Two aspects of the subject are considered briefly:

1. The current state of knowledge of the natural patterns of FSH and LH secretion during the oestrous cycle and their hypothalamic control and
2. experimental effects of the administration of gonadotrophin releasing factor (13) in the synthetic form on gonadotrophin release, ovulation and corpus luteum formation. An exhaustive review of the field is not attempted and special reference is made to the sheep.

Zusammenfassung

Zwei Aspekte der Regulation der FSH- und LH-Sekretion bei Tieren wurden kurz gestreift:

1. Das derzeitige Wissen über den natürlichen Ausscheidungsmodus von FSH und LH während des Menstruationszyklus' und die Regulation dieser Vorgänge durch den Hypothalamus.
2. Untersuchungen der Wirkung vom synthetischen Gonadotropin-Releasing-Faktor auf die Gonadotropin-Sekretion, Ovulation und Corpus-luteum-Bildung. Eine erschöpfende Übersicht wurde nicht gegeben. Es wurde speziell über Versuche beim Schaf berichtet.

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CHAPTER 3. ARTIFICIAL CONTROL OF REPRODUCTION IN DOMESTIC ANIMALS

Induction of reproductive activity in seasonal and lactational anoestrus. Inhibition of the hypothalamo-pituitary-ovarian system. Endocrinology of meat production.

Papers in scientific journals (refereed)

J. Reprod. Fert. (1970) **22,** 223–231

INDUCTION OF PREGNANCY DURING LACTATION IN THE SOW

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(*Received* 14*th May* 1969)

Summary. In a preliminary experiment carried out in Essex sows, three treatments were compared with a control group of lactating sows. The treatments were: (1) intermittent separation of the sow from the young, (2) injection of pregnant mare serum gonadotrophin (PMSG), and (3) a combination of treatments 1 and 2.

None of the control sows exhibited oestrus during lactation. Of the oestrus-induction methods used, only the combination of separation and PMSG injection induced oestrus consistently.

In an extension of the preliminary experiment, the combined treatment was tested in Essex, Large White and Large White × Landrace sows. Of forty sows treated overall, thirty-three exhibited oestrus during lactation and twenty-four carried pregnancies to term as a result of mating at the induced oestrus. The mean interval between parturitions was reduced by approximately 25 days in sows becoming pregnant during lactation. There appeared to be no detrimental effects of 2 or 3 weeks of concurrent lactation on pregnancies established during lactation.

The results are discussed in relation to the possible modes of action of separation and PMSG injection in the lactating sow.

INTRODUCTION

The regular oestrous cycles of the pig are normally interrupted only by pregnancy and lactation. There is agreement that *post-partum* oestrus occurs in the sow within 60 hr of parturition but the percentage of sows exhibiting this has varied widely in different studies (Warnick, Casida & Grummer, 1950; Burger, 1952; Heitman & Cole, 1956; Self & Grummer, 1958). *Post-partum* ovulation does not occur, regardless of whether or not there is *post-partum* oestrus. This has been established by examining the ovaries of sows slaughtered shortly after *post-partum* oestrus (Warnick *et al.*, 1950; Burger, 1952) and about 10 days after parturition when *post-partum* oestrus had not occurred (Warnick *et al.*, 1950).

Throughout the remainder of lactation, the sow does not normally exhibit oestrus or ovulation (Marshall & Hammond, 1937; Burger, 1952; Heitman &

Cole, 1956; Allen, Lasley & Uren, 1957; Self & Grummer, 1958; Palmer, Teague & Venzke, 1965). When lactation is terminated by removal of the young some 8 weeks after parturition, oestrus and ovulation occur generally within about 4 to 7 days (Marshall & Hammond, 1937; Burger, 1952; Self & Grummer, 1958; Smidt, Scheven & Steinbach, 1965). In practice, weaning is now commonly carried out as early as 6 weeks after parturition. Since the duration of pregnancy in the pig is about 115 days, lactation and the period after weaning before oestrus represent together some 25 to 35% of the interval between successive parturitions.

The work reported here was designed to assess methods of inducing oestrus and ovulation in the sow during lactation with a view to reducing the interval between successive parturitions by causing pregnancy concurrent with lactation. A preliminary report of certain of the results has been presented previously (Crighton, 1968).

MATERIALS AND METHODS

Animals

Sows from two herds were used. The first herd was a commercial one consisting of Essex sows mated with a Large White boar, and the second was the experimental herd of the School of Agriculture consisting of Large White and Large White × Landrace sows mated with one of several Landrace boars. All sows had had at least one previous litter when they entered the experiment.

Experimental treatments

For a preliminary experiment, twenty-four Essex sows were divided at random into four groups. The litter size of these sows was standardized at nine or ten young. Three treatments were assessed with regard to their efficacy in inducing oestrus and permitting the establishment of pregnancy during lactation. The treatment groups were compared with a control group of lactating sows. All sows were tested once daily for oestrus with a boar from the 10th day of lactation to the 10th day after weaning. Weaning was carried out in all groups on the 49th day of lactation. Sows which came into oestrus during the period of testing were mated on 2 successive days whenever possible and, if lactating, were returned to their litters. The treatments were as follows:

1. Separation of each sow and litter for 12 hr each day, beginning on the 21st day of lactation and continuing until oestrus was induced or 10 days had elapsed.

2. Injection of each sow with 1500 i.u. pregnant mare serum gonadotrophin (PMSG) on the 21st day of lactation.

3. Separation of each sow and litter for 12 hr each day on the 21st, 22nd and 23rd days of lactation, followed by injection of the sow with 1500 i.u. PMSG at the end of the period of separation on the 23rd day of lactation.

As an extension of the preliminary study, a further experiment was carried out in which the treatment combining separation and PMSG injection was evaluated in a total of twenty-nine Essex sows (eleven control, eighteen treated) including the thirteen sows (six control, seven treated) from the preliminary experiment and in thirty-two Large White and Large White × Landrace sows

(ten control, twenty-two treated). Litter size in the Essex sows was restricted within the range of eight to twelve young. No restriction was imposed in the Large White/Large White × Landrace herd. In the Essex herd, weaning was carried out on the 49th day of lactation and in the Large White/Large White × Landrace herd, on the 42nd day of lactation. In both herds, the procedure was as in the preliminary experiment except that, in the case of the Large White/ Large White × Landrace herd, control sows were not tested daily for oestrus

Table 1

OESTROUS RESPONSE AND PREGNANCY DATA (PRELIMINARY EXPERIMENT, ESSEX SOWS)

Group	*Sow no.*	*Litter size on the 21st day of lactation*	*Day of lactation on which oestrus exhibited*	*Day after weaning on which oestrus exhibited*	*No. of young born alive at subsequent parturition*	*Interval between successive parturitions (days)*
Control	1	10	—	5	12	168
	2	9	—	4	10	167
	3	9	—	4	11	168
	4	10	—	5	12	169
	5	9	—	4	5	168
	6	10	—	1	10	165
Separation	7	10	—	5	11	169
	8	9	—	4	9	168
	9	9	—	1	9	167
	10	10	—	6	13	171
	11	9	25	—	11	140
PMSG injection	12	9	25	—	9	140
	13	10	26	—	9	144
	14	9	—	3	12	169
	15	10	—	4	13	168
	16	10	—	4	11	167
	17	9	—	3	11	169
Separation + PMSG injection	18	10	26 and 47	—	10	163
	19	9	30	—	12	145
	20	9	27	—	13	143
	21	9	28	5	5	171
	22	10	—	4	5	169
	23	10	—	5	11	168
	24	9	27	—	8	146

during lactation as it had been established previously that these sows do not normally exhibit oestrus during lactation (Crighton, unpublished data; Crighton & Lamming, 1969). Data were recorded from twenty-six lactations from the ten control sows in this herd.

Statistical analysis

The t test was used to assess the significance of differences between groups where appropriate.

RESULTS

The oestrous response and pregnancy data from the preliminary experiment are shown in Table 1. None of the control sows exhibited oestrus during lac-

TABLE 2

OESTROUS RESPONSE AND PREGNANCY DATA (EVALUATION OF COMBINED TREATMENT, ESSEX, LARGE WHITE AND LARGE WHITE × LANDRACE SOWS)

Breed	*Treatment*	*No. of sows*	*Litter size on the 21st day of lactation (Mean ± S.E.)*	*No. of sows exhibiting oestrus during lactation*	*Interval between PMSG injection and oestrus in days (Mean ± S.E.)*	*No. of sows carrying pregnancy to term as result of lactational mating*	*No. of young born alive (Mean ± S.E.)*	*Interval between successive parturitions in days (Mean ± S.E.)*
Essex	Control	11	9·8 ± 0·3	0	—	0	10·5 ± 1·1	169·6 ± 1·1
	Separation + PMSG injection	18	9·6 ± 0·3	16	4·2 ± 0·3	11	9·7 ± 0·6*	143·8 ± 1·8* (154·0 ± 3·5)†
Large White and Large White × Landrace	Control	10 (26 lactations)	8·2 ± 0·5	—	—	—	9·7 ± 0·5	168·1 ± 2·0
	Separation + PMSG injection	22	8·4 ± 0·4	17	4·5 ± 0·3	13	9·5 ± 0·8*	143·3 ± 2·7* (154·1 ± 3·1)†

* Mean of sows which carried pregnancy to term as a result of lactational mating.
† Mean of all sows treated.

tation but all returned to oestrus at a mean interval of 3·8 days after weaning. Of the treatments employed, the combination of separation and PMSG injection was clearly the most effective in inducing oestrus during lactation. Only one of the eight sows which exhibited lactational oestrus as a result of any of the treatments failed to carry pregnancy to term after mating during lactation. One sow which mated on the 26th day of lactation returned to oestrus 21 days later and conceived.

The oestrous response and pregnancy data from the evaluation of the combined treatment in the two herds are shown in Table 2. None of the control Essex sows which were tested daily exhibited oestrus during lactation but all returned to oestrus at a mean interval of 4·1 days after weaning. Of a total of forty sows treated overall, thirty-three (83%) exhibited oestrus during lactation. Of these, twenty-four farrowed as a result of mating at the induced oestrus (73% of lactationally oestrous sows; 60% of all sows treated).

The mean number of young born alive in either herd as a result of mating during lactation was not significantly different from the mean number born alive to control sows mated after weaning.

The mean interval between successive parturitions in sows carrying pregnancies established during lactation to term was reduced in both herds by approximately 25 days when compared with controls. When all treated sows were considered, the mean reduction was approximately 15 days. Sixteen sows failed to farrow as a result of treatment (seven Essex, nine Large White/Large White × Landrace). Of these, two were Essex sows which exhibited oestrus during lactation, failed to return to oestrus within 10 days after weaning and were therefore assumed to be pregnant but died subsequently from undetermined causes. No data on the pregnancies of these sows are available. A further seven sows exhibited oestrus during lactation (three Essex, four Large White/Large White × Landrace) and were mated but returned to oestrus at intervals ranging from 5 to 10 days after weaning (mean 6·6 days). Only one of these sows failed to conceive at this oestrus. This Large White animal exhibited oestrus on the 6th day after weaning, was mated but returned on the 31st day after weaning and conceived at this second oestrus. The remaining seven treated sows (two Essex, five Large White/Large White × Landrace) failed to exhibit oestrus during lactation but did so at intervals ranging from 4 to 32 days after weaning (mean 9·3 days).

Continuation of lactation in treated sows

On those days on which separation was imposed, suckling behaviour was resumed immediately on re-uniting sow and litter. Normal suckling behaviour continued throughout the period of lactational oestrus where this occurred and in early pregnancy. It was obvious from the frequency of suckling, the condition of the mammae and the growth of the young that lactation was not seriously disturbed by the treatment or by concurrent early pregnancy.

DISCUSSION

The finding that the Essex sows tested daily with a boar from the 10th day of

lactation onwards failed to exhibit oestrus until about 4 days after weaning confirms numerous previous observations in many breeds (Marshall & Hammond, 1937; Burger, 1952; Heitman & Cole, 1956; Allen *et al.*, 1957; Self & Grummer, 1958; Palmer *et al.*, 1965; Smidt *et al.*, 1965), including observations made previously on Large White sows in the second herd used in the present study (Crighton & Lamming, 1969). While it is known that ovulation without oestrus does not occur during lactation in this strain of Large White sows (Crighton & Lamming, 1969), it was not possible to verify this for the herd of Essex sows.

It has been suggested by Crighton & Lamming (1969) that lactational anoestrus and anovulation in the sow are due to gonadotrophic insufficiency: specifically, failure of release of follicle-stimulating hormone (FSH) and of synthesis of luteinizing hormone (LH). This suggestion was based on information obtained from pituitary FSH and LH bioassays and examinations of the reproductive tract. The latter showed a marked depression of ovarian follicular growth together with uterine atrophy during lactation which were rapidly relieved by weaning when this was effected on the 56th day of lactation, culminating in oestrus and ovulation some 4 to 6 days later.

Weaning at all stages of lactation in the pig results in a return to oestrus and ovulation and this has been the basis of attempts to develop 'early weaning' systems, although there is evidence that the earlier in lactation that weaning is effected, the longer and more variable is the interval between weaning and oestrus (Self & Grummer, 1958). This, and disease problems encountered when young pigs are reared away from the sow, have severely limited the application of 'early weaning' techniques.

'Partial early weaning,' that is the separation of sow and litter for a period each day, was recommended by Marshall & Hammond (1937) as a method of inducing oestrus in the lactating sow. No experimental evidence was offered as a basis for this and Burger (1952) was unable to confirm the efficacy of such a treatment, even when applied for extensive periods as a 12-hr separation overnight. Smith (1961), however, was able to induce oestrus consistently by 12-hr periods of separation during the day beginning on the 21st or 31st to 35th day of lactation, although he observed that sows nursing a first litter required a mean of 14·0 days of separation to elicit oestrus, whereas sows nursing a second litter required a mean of 5·6 days of separation when treatment was begun on the 21st day of lactation. The imposition of 8 hr of separation per day from the 21st day of lactation failed to result in oestrus in any sow until the 8th week of lactation.

In the present preliminary experiment, it was considered that failure to exhibit oestrus within 10 days of the start of separation would render the treatment of little value in practice and so separation was stopped after this period in all sows which had not exhibited oestrus. The results are in agreement with those of Burger (1952), since only one sow exhibited oestrus within the period specified. The variation in the effectiveness of this treatment may be related to the different breeds and strains employed by the investigators and to environmental factors, particularly the effect of proximity of a boar during separation which was excluded in the present study.

The results obtained from the injection of PMSG alone confirmed the results of Cole & Hughes (1946), Heitman & Cole (1956) and Allen *et al.* (1957) in that oestrus was not induced consistently as a result of injections of PMSG on the 21st day of lactation. The finding of Cole & Hughes (1946) and Heitman & Cole (1956) that the response to PMSG improved substantially when injection was carried out after about 40 days of lactation was not examined in the present work.

The finding that the combination of PMSG injection with separation resulted in lactational oestrus in 83% of treated sows when the treatment was begun on the 21st day of lactation demonstrates that this treatment is appreciably more effective than PMSG injection alone. (The reasons for the augmentation of response obtained on combining the treatments may be rather complex.) The ability of PMSG to provoke ovulation in the immature rat has been shown to be due, not to its inherent LH activity, but to the LH-releasing action effected by way of the hypothalamus on the pituitary LH of the rat (McCormack & Meyer, 1962; Zarrow & Quinn, 1963; Szontágh & Uhlarik, 1964; Quinn & Zarrow, 1965). There is evidence suggesting that endogenous FSH is also released by PMSG (Rennels & O'Steen, 1967).

PMSG produces follicle growth and ovulation in the immature pig (Cole & Hughes, 1946). If the mode of action of PMSG is similar in this species, it would presumably be unable to exert its effect under circumstances where insufficient endogenous LH was available. Such circumstances appear to be present during lactation in the sow (Crighton & Lamming, 1969). On this basis, however, it is difficult to explain why, in the work of Cole & Hughes (1946) and Heitman & Cole (1956), the response to PMSG injection was appreciably greater in late lactation than in early lactation, since the results of Crighton & Lamming (1969) suggest that LH synthesis is suppressed up to the 8th week of lactation.

It is clear that, under certain circumstances, separation of sow and litter for a period each day can result in follicle growth, oestrogen secretion and ovulation (Smith, 1961). The combination of circumstances required for this to be achieved is uncertain but one of the main effects is presumably the reduction in suckling intensity which results, negating the inhibition of follicle growth mediated during lactation by the suckling stimulus. The slight evidence available suggests that ovarian insensitivity to circulating gonadotrophin is not a factor in follicular quiescence during lactation in the pig (Kirkpatrick, Lauderdale, First, Hauser & Casida, 1965). Thus, it seems likely that the effect of separation may be to increase synthesis and/or release of endogenous FSH and LH by changes in hypothalamic neuronal activity and the synthesis and release of hypothalamic neurohumoral agents controlling pituitary gonadotrophin secretion. In the present work, while the effects of separation were insufficient to result in oestrus in all but one sow, the initiation of these processes during the period of separation before PMSG injection may have created circumstances in which the gonadotrophin-releasing action of PMSG could be expressed, thus accounting for the greater degree of success achieved compared with PMSG injection alone.

Marshall & Hammond (1937) stated that should a sow be mated during lactation and lactation be continued normally, loss of the pregnancy would

take place due to the competing demands of concurrent lactation on the nutrition available. This did not occur in the present work, confirming the findings of Smith (1961) with a small number of animals in separation experiments. The length of exposure of pregnancy to concurrent lactation in the present study was 2 to 3 weeks and this failed to have any effect on numbers of young born alive. Seven out of thirty-three sows (21·2%) which came into oestrus and were mated during lactation returned to oestrus at intervals ranging from 5 to 10 days after weaning. This might be attributed to loss of pregnancy at about the time of implantation, failure of fertilization or failure to ovulate. Such a percentage failure of conception to mating at the first oestrus after weaning would certainly be considered rather abnormal. It is clear, however, that each of the processes leading to the maintenance of pregnancy was accomplished in the majority of treated sows under circumstances of continued lactation.

It was shown previously that ovariectomy of the sow at 20 to 21 days of lactation had no effect on the subsequent growth of young dependent solely on milk for nutrition (Crighton & Lamming, 1969). The independence of lactation from ovarian and uterine activity is confirmed by the present finding that lactation was not seriously disturbed by the series of ovarian and uterine changes starting from a state of atrophy and leading up to the establishment and maintenance concurrently of the first 2 to 3 weeks of pregnancy.

The results of these investigations suggest that the treatment combining separation and PMSG injection may provide an alternative to 'early weaning' as a means of reducing the interval between successive parturitions in the sow. Further experiments will be required to determine whether the treatment, in its present form, or with modifications, may be employed earlier in lactation than in the present study. While there seems to be no reason why oestrus and ovulation should not be induced earlier, the effects of longer periods than 2 to 3 weeks of concurrent pregnancy and lactation on the maintenance of either are as yet unknown. The value of the treatment in practice will depend on how early in lactation it can be applied without adverse effects on these processes.

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THE INDUCTION OF PREGNANCY DURING LACTATION IN THE SOW: THE EFFECTS OF A TREATMENT IMPOSED AT 21 DAYS OF LACTATION

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SUMMARY

1. Experiments carried out in two herds to examine the effects of a treatment designed to induce oestrus and ovulation during the lactational anoestrus of the sow and to allow the establishment of pregnancy during lactation are described. Two aspects are examined: (a) the effects on the reproductive performance of the sow of repetition of the treatment in successive lactations and (b) the effects of the treatment on the suckled young.
2. The treatment involved the separation of each sow and litter for 12 hr on days 21, 22 and 23 of lactation followed by an injection of 1500 IU pregnant mare serum gonadotrophin (PMSG) at the end of the third period of separation.
3. It is concluded that oestrus and ovulation can be induced with some consistency in the fourth week of lactation, that lactation is not incompatible with the first two to three weeks of pregnancy, and that the establishment of pregnancy during the fourth week of lactation is not detrimental to the growth of the suckled young when supplementary food is provided.

INTRODUCTION

OVULATORY oestrus is inhibited during lactation in the sow (*see* Crighton and Lamming, 1969, for references). In normal husbandry practice, with weaning after six to eight weeks of lactation, lactation and the period after weaning prior to oestrus together represent some 25 to 35% of the interval between successive farrowings.

Three treatments designed to induce oestrus and ovulation during lactation in the sow with a view to reducing the interval between successive farrowings were compared previously and the most successful of these was evaluated in single lactations in two herds (Crighton, 1968, 1970). The present paper describes the application at day 21 of lactation of this treatment, that of following three daily periods of separation of sow and litter with an injection of pregnant mare serum gonadotrophin (PMSG). Two aspects are considered: (1) The effects on the reproductive performance of the sow of repetition of the treatment in successive lactations. (2) The effects of the treatment on the suckled young.

MATERIAL AND METHODS

Animals

Sows from two herds were used. One was the School of Agriculture herd, consisting of Large White and Large White × Landrace sows mated with one of several Landrace boars, and the other was a commercial herd of Essex sows mated with a Large White boar. All sows had reared at least one previous litter before they entered the experiment.

Experimental treatment

The experimental treatment consisted of separation of each sow and litter for 12 hr each day on days 21, 22 and 23 of lactation, followed by an intramuscular injection of the sow with 1500 IU PMSG at the end of the third period of separation. This treatment was imposed during one lactation in the Essex sows and in up to four successive lactations in the Large White and Large White × Landrace sows. In each herd the treated group was compared with a control group of lactating sows.

In the Large White/Large White × Landrace herd, treated sows were tested daily for oestrus with a boar from day ten of lactation until day 10 after weaning. Control sows were not tested daily for oestrus until after weaning since it had been established in previous experiments that the sows in this herd do not exhibit oestrus during lactation (Crighton, unpublished data; Crighton and Lamming, 1969). Data were recorded on 26 lactations from 10 control sows. Weaning was carried out on day 42 of lactation in the Large White/Large White × Landrace herd and on day 49 in the Essex herd.

Growth and supplementary food consumption of young

In the case of the Essex herd, data were obtained from nine treated sows in which pregnancy was induced concurrently with lactation and from 11 untreated controls. The young were numbered individually, males were castrated and a suitable creep feed was offered, all on day 10 of lactation. Litter size was standardized within the range 9 to 12 young on day 14 of lactation. The mean litter size on day 14 was $10{\cdot}0 \pm 0{\cdot}3$ for both control and treated groups and on day 48 was $9{\cdot}9 \pm 0{\cdot}3$ for controls and $9{\cdot}7 \pm 0{\cdot}5$ for treated sows. The young were weighed individually daily from day 14 of lactation to day 42 and on days 45 and 48. The consumption of creep feed, which was offered *ad libitum*, was determined daily from day 14 to day 48. In the Large White/Large White × Landrace herd, while creep feed was offered *ad libitum* from day 10, the young were not weighed and supplementary food consumption was not recorded.

Statistical analysis

The reproductive data were analysed by analysis of variance or chi-square test where appropriate. The growth and supplementary food consumption data were analysed by fitting quadratic regressions on days of lactation and comparing the coefficients obtained for the control and treated groups.

RESULTS

The oestrous response and pregnancy data from the repetitive treatment of Large White/Large White × Landrace sows are shown in Table 1. The

TABLE 1

Oestrous response and pregnancy data

Breed	Treatment	Lactation	No. of sows	Litter size on day 21 of lactation (Mean ± SE)	No. of sows exhibiting oestrus during lactation	Interval between PMSG injection and oestrus (days) (Mean ± SE)	No. of sows carrying pregnancy to term as result of lactational mating	No. of young born alive (Mean ± SE)	Interval between successive parturitions (days) (Mean ± SE)
Large White	Control		10 (26 lactations)	8·2±0·5	—	—	—	9·7±0·5	168·1±2·0
Large White ×Landrace	Separation +PMSG injection	1	22	8·4±0·4	17	4·5±0·3	13	9·5±0·8†	143·3±2·7† (154·1±3·1)†
		2	20	7·8±0·8	16	4·8±0·2	9	9·2±1·0†	144·2±0·5† (157·9±3·2)‡
		3	16	7·9±0·1	12	4·7±0·3	7	8·3±1·2†	144·4±0·9† (156·1±1·3)‡
		4	14	8·4±0·4	12	5·0±0·4	8	7·8±1·5†	144·3±1·1† (157·2±5·3)‡

† Mean of sows carrying pregnancy to term as result of lactational mating.
‡ Mean of all sows treated.

comparable reproductive data from the Essex herd have been reported previously (Crighton, 1968, 1970).

When the treatment was repeated in successive lactations the percentages of treated sows showing oestrus during lactation were 77·3, 80·0, 75·0 and 85·7% in lactations 1, 2, 3 and 4 respectively. These responses were not significantly different from one another.

When the mean intervals between PMSG injection and the onset of oestrus were compared there were no significant differences in the response with repetition of the treatment.

When the numbers of sows carrying pregnancy to term as a result of lactational mating (as a percentage of sows showing oestrus during lactation) were compared in successive lactations the percentages were 76·5, 56·3, 58·3 and 66·7 respectively. There were no significant differences between the responses.

When the mean numbers of young born alive to treated sows were compared there were no significant differences when the treatment was imposed in successive lactations.

The piglet growth and supplementary food consumption data from the Essex herd are shown in Figure 1. The growth of the young of treated sows was faster than that of controls. The growth curves were markedly different both in average live weight ($P<0{\cdot}001$) and in regression coefficients ($P<0{\cdot}001$), the former being the greater effect. The supplementary food consumption curves were also markedly different, most of the difference being due to the higher average level of consumption of the treated group ($P<0{\cdot}001$). The difference in the regression coefficients was, however, also significant ($P<0{\cdot}05$).

The period of separation and injection of the sow and the induced oestrus were not accompanied by an appreciable check in the growth of the young. The establishment of pregnancy was followed by a divergence of the growth curves, the young of treated sows increasing from a mean of 0·2 lb heavier on day 29 to 1·2 lb heavier on day 36. A mean difference of about 1 lb per piglet was maintained until weaning. The supplementary food consumption was significantly higher in the young of treated sows from day 20 to day 48 of lactation. An increase in consumption became apparent during the periods of separation and was again apparent from day 27, the difference relative to controls reaching a maximum of 0·16 lb per piglet per day on days 34 and 37 of lactation.

DISCUSSION

The present work demonstrates the possibility of inducing oestrus in the anoestrous lactating sow with some consistency in successive lactations. Over the whole experiment, out of 72 treatments imposed on day 21 of lactation, there were 57 oestrous responses to the treatment (79·2%) and the results were unaffected by repetition of the treatment. There was also little variation in the time of expression of oestrus after treatment. In 45 of the 57 oestrous responses (78·9%) oestrus began on day 3, 4 or 5 after PMSG injection.

The oestrous response to separation and PMSG injection in the present work represents a considerable improvement on earlier studies in which PMSG injection alone was employed (Cole and Hughes, 1946; Heitman and Cole, 1956; Allen, Lasley and Uren, 1957). The studies of Cole and Hughes (1946)

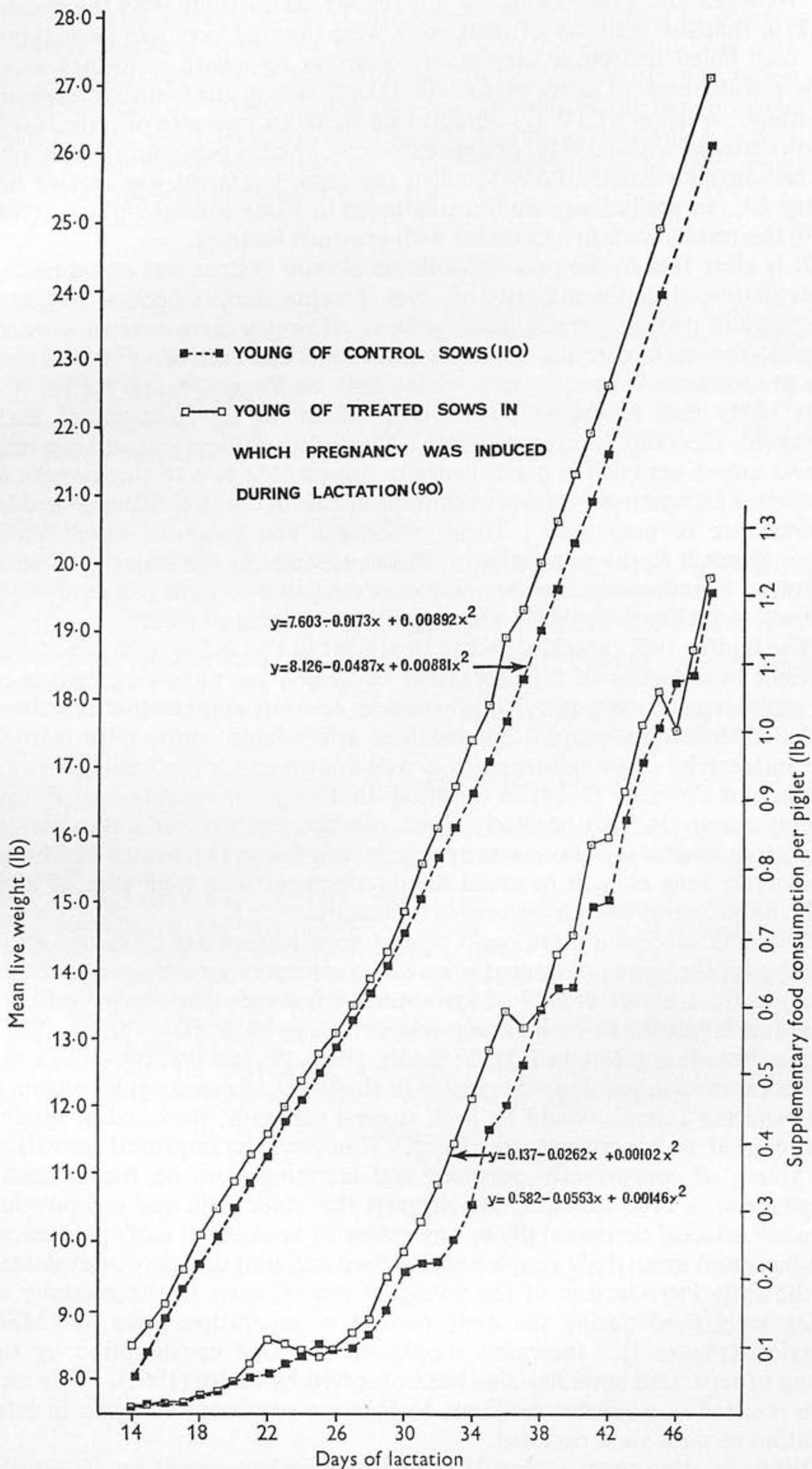

FIG. 1. Piglet growth and supplementary food consumption (Essex herd). Numbers of piglets are in parentheses.

and Heitman and Cole (1956) are not readily comparable with the present work in that the majority of their sows were injected very late in lactation but they failed to obtain satisfactory oestrous responses to PMSG when using a wide range of doses up to 3400 IU per sow in the first four weeks of lactation. Allen *et al.* (1957) obtained an oestrous response of only 16·7% in sows treated with 1000 IU at approximately 20 days post partum although the response increased to 65·4% when the same treatment was carried out at day 40. In preliminary studies conducted in Essex sows (Crighton, 1968, 1970) the results were in agreement with previous findings.

It is clear that in the present work lactational oestrus was accompanied by ovulation, since the majority of sows showing oestrus became pregnant as a result of mating at the induced oestrus. However there were sows which failed to farrow as a result of mating at the induced oestrus. While failure of a proportion of the sows to ovulate might be a contributing factor, it is more likely that failure of fertilization, failure of implantation or early embryonic mortality were responsible. No studies of these factors have been carried out as yet but the present results suggest that two to three weeks of concurrent lactation may create conditions hostile to the establishment and/or maintenance of pregnancy. These conditions are, however, insufficiently severe to result in the prevention of these processes in the majority of sows or indeed to reduce the number of young surviving to term compared with controls in pregnancies which are established and maintained.

The finding that repetition of the treatment in successive lactations failed to result in a decline in the percentage of treated sows showing oestrus or the percentage of sows carrying pregnancies to term suggests that repetition of the PMSG injections did not result in appreciable antibody formation. The antigenicity of gonadotrophins is well known and it has been shown by Garcia and Carrasco (1968) in the sheep that frequent repetition of PMSG therapy results in high antibody titres. In the present work, the interval between successive injections was approximately five to six months which was presumably long enough to avoid the development of a high titre of antibody in the course of four successive treatments.

The data showed a more rapid rate of growth from day 20 to day 48 of lactation of the young of treated sows which were concurrently pregnant and lactating from about day 29 of lactation. Since supplementary food consumption is known to be inversely related to sow milk yield (Smith, 1952; Barber, Braude and Mitchell, 1955; Smith, 1961), the fact that the mean creep feed consumption per day was greater in the litters of concurrently pregnant and lactating animals would by itself suggest that milk yield and/or quality was reduced by concurrent pregnancy. However, the improved growth of the young of concurrently pregnant and lactating sows on the increased supplementary food consumption suggests that milk yield and composition were not affected detrimentally to any extent by concurrent early pregnancy. The increased mean daily supplementary food consumption may be explained by the early introduction of the young of treated sows to the necessity of eating solid food during the daily periods of separation prior to PMSG injection (Figure 1). Increased supplementary food consumption by the young of separated sows has also been observed by Smith (1961). This may have resulted in a greater readiness to increase consumption again in later lactation as milk yield declined.

While treating sows on day 21 of lactation or later might be of value in

producing batch farrowing, the contribution of any treatment in shortening the interval between successive parturitions would depend on how early it could be applied without severely adverse effects on the existing lactation or interference with the establishment and maintenance of the new pregnancy. In the present study, the reduction of the farrowing interval achieved in successful treatments was about 25 days. It remains to be seen whether this can be improved upon in future work.

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PROGESTERONE LEVELS FOLLOWING TREATMENT OF SEASONALLY ANOESTROUS EWES WITH SYNTHETIC LH-RELEASING HORMONE

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Summary. Plasma progesterone determinations were carried out on blood samples collected daily from Clun Forest ewes during the normal oestrous cycle and also after administration of LH-releasing hormone (LH-RH) during seasonal anoestrus.

Levels of plasma progesterone at oestrus ranged from 0·1 to 0·5 ng/ml and luteal phase levels from 3 to 6 ng/ml. Levels found during seasonal anoestrus were within the range of those observed at oestrus. Following treatment with LH-RH, there was an increase in the plasma LH level in all cases and ovulation occurred in twenty-three out of twenty-seven treated ewes. In the animals which ovulated, the plasma progesterone concentration either remained basal (eighteen animals) or rose to a lower level (<2 ng/ml) than that found during the luteal phase of the cycle.

INTRODUCTION

The determination for LH-releasing hormone (LH-RH) of a decapeptide structure (Matsuo, Baba, Nair, Arimura & Schally, 1971) and synthesis of this material (Geiger, König, Wissmann, Geisen & Enzmann, 1971) has provided a valuable tool for the study of reproductive function in the sheep.

The ability of this synthetic decapeptide to induce release of LH and FSH from the ovine pituitary both *in vivo* and *in vitro* is well established (Arimura, Debeljuk, Matsuo & Schally, 1972; Crighton & Foster, 1972; Foster & Crighton 1973; Jonas & co-authors, 1973). Apparent ovulation was induced in most animals when LH-RH was given as a single intravenous injection to seasonally anoestrous ewes (Crighton, Foster, Haresign, Haynes & Lamming, 1973; Foster & Crighton, 1973).

Reports on subsequent luteal function following the induction of ovulation with LH-RH are lacking. The purpose of this communication is to present data on plasma progesterone levels following the treatment of seasonally anoestrous Clun Forest ewes with a single intravenous injection of either 150 μg or 300 μg LH-RH.

A brief preliminary report of this work has already appeared (Crighton *et al.*, 1973).

MATERIALS AND METHODS

Animals

Clun Forest ewes, 4 to 5 years old, were used throughout the experiment. All ewes were housed indoors with a vasectomized ram under natural lighting conditions. All cycling ewes were shown to have had at least two normal oestrous cycles before they were used. All seasonally anoestrous ewes had given birth to lambs during the preceding December and January, and were checked for cyclic activity with a colour-marked vasectomized ram for at least 17 days before treatment.

Collection of blood samples

Blood samples for plasma progesterone determinations were collected daily by jugular venepuncture for 2 days before and 20 days after administration of the decapeptide. Samples for LH assay were collected through an indwelling jugular cannula, inserted 24 hr before treatment, every 10 min for 1 hr before and every 5 min for 3 hr after administration of LH-RH.

The blood was centrifuged immediately after collection at 4°C and 1600 ***g*** for 15 min and the plasma removed and stored at −20°C until assayed.

Experimental design

Experiment 1. Ten seasonally anoestrous ewes, five in each group, were given a single intravenous injection of either 150 μg or 300 μg LH-RH during the period May to June. Four ewes received saline alone or synthetic tripeptide (thyrotrophin-releasing hormone, TSH-RH) to serve a scontrols. Crighton & Foster (1972) showed that this tripeptide did not cause elevation of plasma LH levels. All ewes were subjected to laparotomy 3 to 4 days after treatment for examination of the ovaries.

To provide an estimate of levels of progesterone during the luteal phase for this breed of sheep under similar conditions of surgery to those imposed on the experimental ewes, another group of four ewes was given either saline or TSH-RH on Day 12 of the oestrous cycle. The animals were subjected to laparotomy on Day 14 or 15 of the cycle and again 3 or 4 days after the onset of the next oestrus.

Experiment 2. During May and June 1973 and 1974 a further seventeen seasonally anoestrous ewes were given 150 μg LH-RH by intravenous injection and treated subsequently as described for Exp. 1.

Assay of progesterone

Chemicals and equipment. Petroleum ether (b.p. 30 to 60°C) was Nanograde (Mallenckrodt Chemical Works, St Louis) and was not purified further. The [1,2-^{3}H]progesterone (sp. act. 53 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Dextran–charcoal solution was made by mixing equal volumes of a 0·4% charcoal (Norite A: Sigma Chemical Co.) suspension and a 0·2% dextran (Grade C, British Drug Houses Ltd) solution, both in 0·1 M-phosphate buffer (pH 7·1), and containing 0·1% by weight of gelatin and sodium azide. Liquid scintillation counting was carried out using a Tracerlab coru/matic 200 counter with a scintillator composed of 4·0 g of

2,5-diphenyloxazole and 0·5 g of p-bis-2-(5-phenyloxozolyl)-benzene in 1·0 litre toluene.

Assay procedure. Plasma progesterone was assayed using an antiserum raised in goats. Appropriate duplicate volumes of plasma (0·25 ml for luteal levels, 0·5 ml in other cases) were transferred to test-tubes and extracted twice with 4 vols light petroleum ether. This procedure involved thorough mixing of the plasma and light petroleum on a Vortex mixer, centrifugation at 1500 **g** for 5 min and freezing at −20°C for 30 min. The organic phase was then decanted into a second series of tubes and evaporated to dryness for assay. Ten aliquots from random plasma samples were used for estimating the loss of steroid during the extraction stage in each assay. In these cases, 0·1 ml [1,2-^{3}H]progesterone in benzene (2000 ct/min) were added to each tube and dried down before the addition of the plasma sample. After mixing on a Vortex mixer and incubation at 45°C for 15 min, the samples for recovery estimates were extracted as above and the total extract assessed for radioactivity. A series of standards (0·05, 0·1, 0·2, 0·4, 1·0, 2·0 and 4·0 ng progesterone) were prepared in duplicate. Antiserum at a 1:3000 dilution in phosphate buffer (0·1 ml) was added to both plasma samples and standards and the mixture incubated at room temperature for 30 min. After this time [1,2-^{3}H]progesterone (0·1 ml) in phosphate buffer (10,000 ct/min) was added to all tubes. The contents of the tubes were mixed and incubated at 4°C for at least 8 hr. Two water blanks consisting of 0·5 ml glass-distilled deionized water were processed with the plasma samples in each assay. After incubation, the separation of antibody-bound and free steroid was performed at 4°C. Dextran–charcoal suspension (1·0 ml) was added to each assay tube, the contents were agitated on a Vortex mixer, allowed to stand at 4°C for 10 min and centrifuged at 1500 **g** for a further 10 min. The supernatant solution was decanted into a scintillation vial containing 1·0 ml ethyl alcohol. Scintillation fluid (10 ml) was then added to each vial which was shaken and the radioactivity was counted after allowing time for separation of the two phases.

Reliability criteria for progesterone assay. Specificity studies were carried out on the antiserum and gave results essentially similar to those reported by Furr (1973).

Twenty water blanks measured during the assays contained 26±3 pg progesterone equiv./tube. The average recovery of progesterone (seventeen assays) was 62·7±3·2%. Within assays, the coefficient of variation for recoveries was never greater than ±2·5%. Sensitivity of the assay, defined as twice the S.D. of blank values was 20 pg/tube. Accuracy was assessed by adding known quantities of progesterone to 0·5 ml aliquots of plasma from castrated male sheep and assaying the extracts. The mean concentrations for four samples in each determination were: 0·1 ng added, 0·11±0·01 ng measured; 0·20 ng added, 0·19±0·01 ng measured; 0·40 ng added, 0·40±0·02 ng measured; 1·00 ng added, 0·96±0·03 ng measured; 2·00 ng added, 2·02±0·03 ng measured. The coefficient of variation of duplicate pairs, calculated by the method of Snedecor (1952) and incorporating both intra- and inter-assay variation was 3·30% for values of 0·8 to 4·0 ng/ml (twenty samples) and 9·62% for values <0·8 ng/ml (thirty samples). Duplicate 0·5-ml aliquots of plasma from a seasonally anoestrous ewe and 0·25-ml aliquots from a ewe in the last third of

gestation were measured in each assay. These gave values of $0{\cdot}11 \pm 0{\cdot}01$ ng/ml and $10{\cdot}03 \pm 0{\cdot}66$ ng/ml respectively (fourteen samples).

Assay of LH

A double-antibody radioimmunoassay (Foster & Crighton, 1975) was used to estimate the LH concentrations of plasma samples. The antiserum used was raised in rabbits by intradermal injection of a solution of a partly purified LH preparation in an emulsion with Freund's complete adjuvant at 3-week intervals. The LH preparation used had an activity of half that of NIH-LH-S1 in the ovarian ascorbic acid depletion (OAAD) bioassay. A modified version of the method described by Greenwood, Hunter & Glover (1963) was used for the radioiodination of a highly purified LH preparation with ^{125}I.

The LH activity of this preparation was 1·8 times that of NIH-LH-S1 in the OAAD bioassay.

Assay method. A solution was prepared consisting of normal rabbit serum (NRS) at 1:400 dilution in assay diluent and antiserum at a dilution known to give approximately 50% binding of the labelled hormone (1:20,000); 0·2 ml of this solution was added to reaction tubes containing 0·5 ml of solutions of known concentration of standard LH (NIH-LH-S17, National Institutes of Health, U.S.A.) or known dilution (1:10 or 1:20) of test material. The tubes were incubated at 4°C for 24 hr and 0·2 ml of a solution of labelled LH, diluted to give 10,000 ct/min/0·2 ml, was added. The tubes were counted to determine total counts and were incubated for 18 hr at 4°C before 0·1 ml of a 1:30 dilution of anti-rabbit γ-globulin (MR66, Burroughs Wellcome & Co.) was added. The tubes were incubated for a further 6 hr at 4°C, and after centrifugation, the supernatants were removed and the precipitates counted.

The LH concentrations of plasma samples were calculated by interpolating the % binding obtained on the standard curve. A quantity of 0·5 ng NIH-LH-S17/ml generally gave 10% inhibition of binding. Negligible cross-reactions were observed with ovine FSH, growth hormone and prolactin. A purified preparation of thyroid-stimulating hormone (TSH) was not available. There were no differences, however, between the levels of LH detected by the assay in plasma samples obtained before and after treatment of ewes with 300 μg TSH-RH, suggesting that the cross-reaction with TSH was low.

When a standard curve was prepared using NIH-LH-S17 made up in plasma from hypophysectomized sheep (1:10, v/v) instead of assay diluent, there was a slight ($<2\%$) decrease in % binding at each concentration; an indication that interference from the plasma itself was negligible.

When various preparations containing ovine LH were evaluated by both the OAAD bioassay and radioimmunoassay, the Mean Index of Discrimination $\left(\frac{\text{biological assay}}{\text{radioimmunological assay}}\right)$ was 1·29 (range from 1·00 to 1·97).

The limit of sensitivity of the assay was generally 1 ng NIH-LH-S17 equiv./ml (Foster, 1974).

RESULTS

Experiment 1

Ovarian activity. At laparotomy, four of the five ewes in each of the groups

treated with either 150 μg or 300 μg LH-RH during seasonal anoestrus had apparently normal ovulation points (see Table 1). The other two treated sheep showed considerable follicular development but had no ovulation points. None of the four control ewes that received tripeptide or saline alone showed signs of ovarian activity. The uteri of the treated ewes were still typical of seasonally anoestrous ewes.

Table 1. The effect of treatment with LH-RH on LH and progesterone levels and ovulation in seasonally anoestrous ewes

Ewe	*Treatment*	*Ovulation**	*Plasma LH peak*†	*Luteal function*‡
N	150 μg LH-RH	+ (1)	+	−
O	150 μg LH-RH	+ (2)	+	+
Q	150 μg LH-RH	+ (1)	+	?
R	150 μg LH-RH	− (−)	+	−
T	150 μg LH-RH	+ (1)	+	+
X	300 μg LH-RH	+ (1)	+	+
Y	300 μg LH-RH	+ (1)	+	−
Z	300 μg LH-RH	+ (1)	+	−
A	300 μg LH-RH	− (−)	+	−
C	300 μg LH-RH	+ 1	+	−
S	Saline	− (−)	−	−
B	Saline	− (−)	−	−
U	300 μg TSH-RH	− (−)	−	−
W	300 μg TSH-RH	− (−)	−	−

* The number of ovulation points are indicated in parentheses.
† + indicates elevation; − indicates no rise.
‡ + indicates elevation above basal levels of plasma progesterone; − indicates the plasma progesterone levels remained basal. For sheep Q, see text and Text-fig. 4.

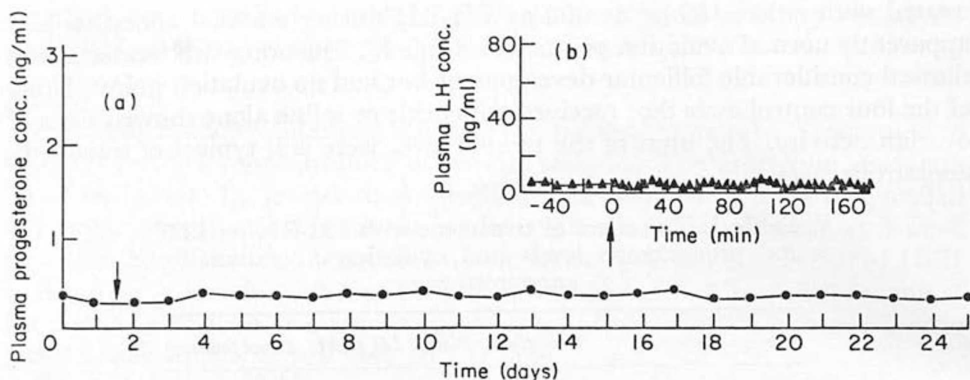
The four ewes receiving saline or TSH-RH on Day 12 of the oestrous cycle (1st day of oestrus = Day 0) had not ovulated by the time of laparotomy on Day 14 or 15, but all these ewes came into oestrus on Days 16 to 18 and had ovulated normally at laparotomy 3 to 4 days later.

Oestrus was not detected in any sheep treated with decapeptide even though most ewes ovulated.

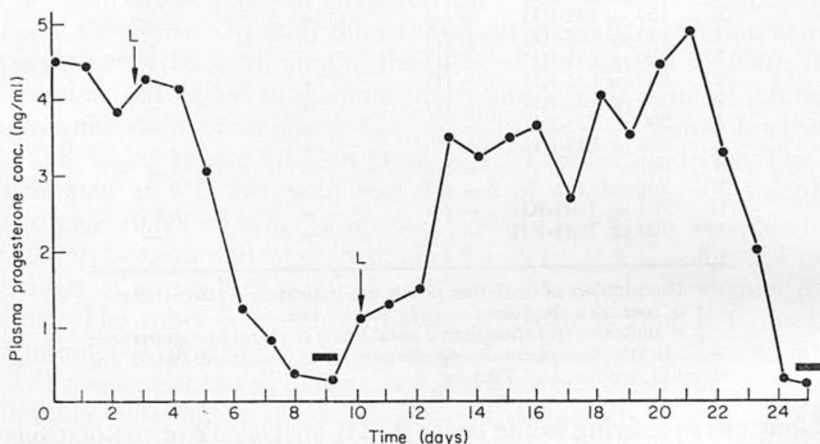
Levels of LH. Treatment with LH-RH resulted in increased plasma LH levels in all ewes (see Text-figs 3, 4 and 5; Table 1). The plasma LH concentration rose within 20 to 40 min and the peak level was reached 90 to 120 min after injection in all ewes. There was no increase in the plasma LH concentration following injection of saline alone or 300 μg TSH-RH (Text-fig. 1; Table 1).

Progesterone levels. Progesterone levels in the four control ewes given saline or TSH-RH during seasonal anoestrus did not rise significantly above pre-injection values (see Text-fig. 1). A typical example of progesterone levels in the four ewes given saline or TSH-RH on Day 12 of the cycle is shown in Text-fig. 2. Mid-cycle levels ranged from 3·6 to 5·4 ng/ml in these ewes.

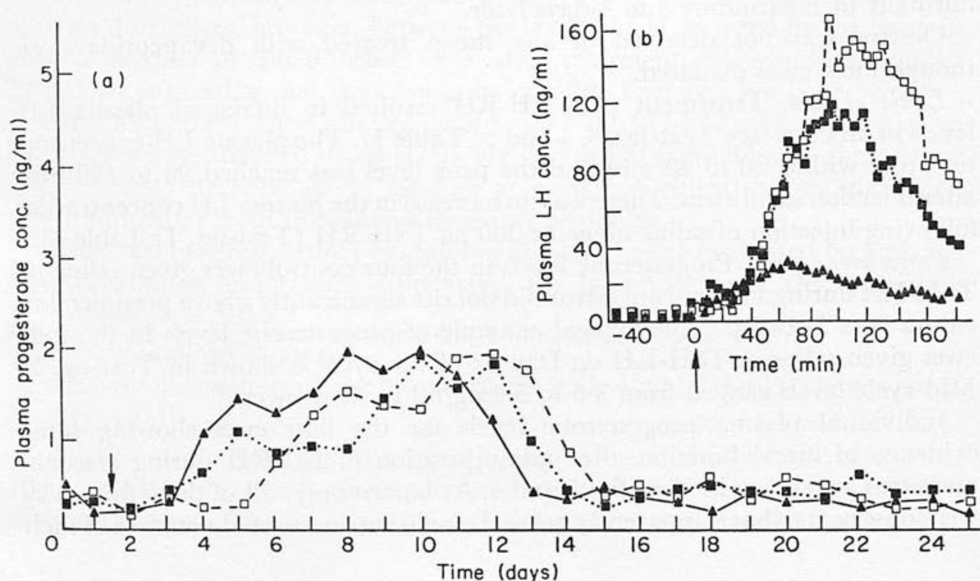
Individual plasma progesterone levels for the four ewes showing some evidence of luteal function after administration of LH-RH during seasonal anoestrus are shown in Text-figs 3 and 4. At laparotomy, all of these four ewes were observed to have apparently normal ovulation points on the surface of their



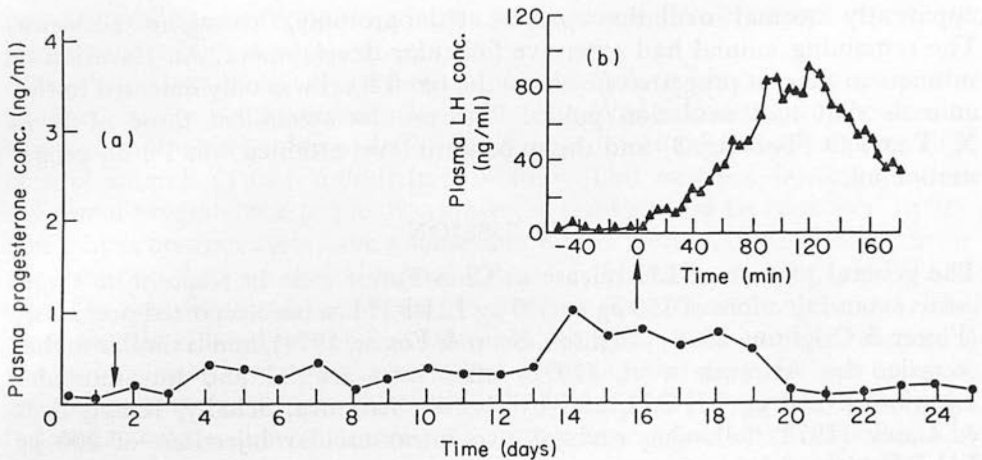
TEXT-FIG. 1. Peripheral plasma progesterone (a) and LH (b) concentrations in an anoestrous ewe given saline (arrows).



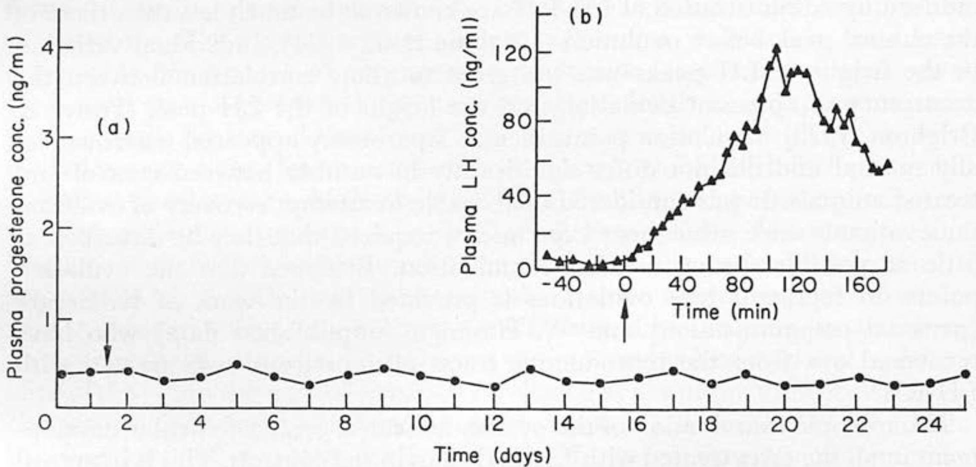
TEXT-FIG. 2. Peripheral plasma progesterone levels in a ewe during the oestrous cycle. The horizontal bars indicate the time of oestrus; L denotes the time of laparotomy.



TEXT-FIG. 3. Peripheral plasma progesterone (a) and LH (b) levels in ewes O (▲), T (□) and X (■). The arrows indicate the time of administration of LH-RH.



TEXT-FIG. 4. Peripheral plasma progesterone (a) and LH (b) in ewe Q. The arrows indicate the time of administration of 150 μg LH-RH.



TEXT-FIG. 5. Peripheral plasma progesterone (a) and LH (b) levels in ewe C. The arrows indicate the time of administration of 300 μg LH-RH.

ovaries. In three of the ewes (Ewes O, T and X; Text-fig. 3), the levels began to rise about 2 days after treatment and remained elevated for 10 to 12 days. The maximum levels attained in these three ewes did not exceed 2·0 ng/ml. The fourth ewe (Ewe Q; Text-fig. 4) was atypical in that a small rise was observed in plasma progesterone between Days 12 to 18 after injection, although the maximum level was only 1·0 ng/ml.

Plasma progesterone levels did not rise significantly (see Text-fig. 5) in the remaining six experimental ewes, four of which had apparently normal ovulation points.

Experiment 2

All seventeen ewes showed an increased plasma LH level and sixteen had

apparently normal ovulation points at laparotomy, averaging 1·25/ewe. The remaining animal had extensive follicular development. An elevation of subsequent plasma progesterone above the basal level was only detected in two animals that had ovulation points. The profiles resembled those of Ewes X, T and O (Text-fig. 3) and the maximum level attained was 1·4 ng progesterone/ml.

DISCUSSION

The general pattern of LH release in Clun Forest ewes in response to single intravenous injections of 150 μg or 300 μg LH-RH has been reported previously (Foster & Crighton, 1973; Crighton, Scott & Foster, 1974), and is similar to that recorded by Arimura *et al.* (1972) after intra-arterial and intramuscular injection of 250 μg LH-RH, and by Reeves, Arimura, Schally, Kragt, Beck & Casey (1972) following each of two intramuscular injections of 200 μg LH-RH given 4 hr apart.

In the present study, considerable variation in LH response was observed with both doses of LH-RH used. The height and duration of the LH peaks induced by administration of LH-RH are known to be much less than those of the natural peak before ovulation (Crighton *et al.*, 1974). Individual variation in the height of LH peaks was too great to allow correlation between the occurrence of apparent ovulation and the height of the LH peak (Foster & Crighton, 1973). Ovulation points seen at laparotomy appeared macroscopically normal and did not differ significantly in number between control and treated animals. It was considered inadvisable to attempt recovery of ova from these animals since subsequent experiments required that they be disturbed as little as possible during ovarian examination. Evidence that the ovulation points do represent true ovulations is provided by the work of K. Seeger (personal communication) and W. Haresign (unpublished data) who have recovered ova from the reproductive tracts of anoestrous ewes treated with LH-RH.

Macroscopic examination of the ovaries indicated greater follicular development in all the ewes treated with LH-RH than in the controls. This is in accord with the results of Crighton & Foster (1972), Crighton (1972) and Jonas *et al.* (1973) which show that LH-RH will induce release of FSH *in vitro* and *in vivo*.

The lack of behavioural oestrus in treated ewes which ovulated was expected, since priming with progesterone is required before oestrus accompanies ovulation in the ewe (Robinson, 1954). The uteri of ewes treated with LH-RH, however, were typical of the anoestrous state. This observation suggests that even though follicular development occurred, this was not accompanied by secretion of a level of oestrogen sufficient to stimulate the uterus to the same degree as that seen at a natural oestrus. The reason for this is not clear.

The pattern of progesterone production obtained from daily samples throughout the oestrous cycle in the four control cyclic ewes was the same as that recorded by other workers (Stabenfeldt, Holt & Ewing, 1969; Thorburn, Bassett & Smith, 1969; Smith & Robinson, 1969; McNatty, Revfeim & Young, 1973), but the mean levels tended to be higher. There is no obvious explanation for this but similar levels were recorded for Clun Forest ewes using a competitive

protein-binding technique for plasma progesterone (W. Haresign and N. B. Haynes, unpublished data).

In all but one of the treated ewes that did show evidence of progesterone production, the pattern was similar to that observed in control cyclic ewes but the maximum levels obtained (2 ng/ml) were lower than those reported for control animals (3 to 6 ng/ml) in this study. This may not, however, reflect abnormal progesterone production since the results could be explained by the fact that anoestrous ewes have a somewhat higher metabolic clearance rate for progesterone than do cyclic animals (Bedford, Harrison & Heap, 1972). In Ewe Q (Text-fig. 4) the progesterone response was definitely abnormal. The levels remained basal for the first 10 to 11 days after treatment, then showed a rise to levels of 1 ng/ml for a period of 5 days. The reason for this atypical response is not clear. Notwithstanding the fact that a proportion of the ewes induced to ovulate show some evidence of progesterone production after LH-RH treatment, the majority do not. It is unlikely that the basal levels of peripheral plasma progesterone recorded throughout in these animals can be explained by changes in the metabolic clearance rate and suggest either a lack of luteal tissue formation, or the formation of luteal tissue which does not produce progesterone. The reasons are not obvious. Laparotomy cannot be considered responsible for the lack of progesterone production, since control cyclic ewes all showed normal peripheral plasma progesterone levels when laparotomy was performed for the second time 3 to 4 days after oestrus, an equivalent time after ovulation to that used for ewes induced to ovulate with LH-RH during seasonal anoestrus. Furthermore, McNatty *et al.* (1973) found that laparotomy increased peripheral plasma progesterone levels. Piper & Foote (1968) demonstrated that when ewes were induced to ovulate on Day 4 of the cycle by injection of oestradiol-17β, the CL was only maintained when oestradiol-17β injections were continued daily, even though the CL formed at the previous ovulation were maintained naturally. This suggests that a stimulus for functional maintenance of the CL in the normal situation is released at about the time of ovulation. Denamur (1968) showed that hypophysectomy soon after ovulation resulted in the formation of a histologically normal CL up to Day 12 of the cycle, but progesterone secretion was abnormal. It has also been suggested that the luteotrophin in sheep may be a combination of prolactin and LH (Denamur, Martinet & Short, 1973). It is possible therefore that the normal luteotrophic stimulus is lacking in most anoestrous ewes induced to ovulate with LH-RH and this could explain the lack of normal luteal function in this study.

The present experiments do not allow a distinction between two possibilities; that the low concentration of plasma progesterone is a result of retarded growth of CL, or development of CL which do not produce progesterone. Further work is in progress to clarify this situation.

Whilst injection of LH-RH will cause LH release and ovulation in anoestrous sheep, subsequent plasma progesterone concentrations usually remain basal. This would seem to preclude the use of LH-RH as a single injection without other therapy for the induction of reproductive activity in the anoestrous ewe.

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EFFECT OF ACTIVE IMMUNISATION OF EWES AGAINST SYNTHETIC LUTEINISING HORMONE RELEASING HORMONE

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ABSTRACT

At the start of the breeding season 13 intact and four ovariectomised ewes were immunised against LH-RH which was rendered immunogenic by conjugation to bovine serum albumin using carbodiimide. The immunogen was emulsified with Freund's complete adjuvant prior to multi-site intradermal injection into a shaved area on the back of each animal. All the ewes were boosted using an identical procedure six and twelve weeks later. LH-RH antibody titres were monitored from weekly blood samples. Oestrous cycles were shown to stop in all but one of the intact ewes after anti-LH-RH titres had developed, but before the seasonal anoestrus. Laparoscopy of the ewes at this time showed that the ovaries and uteri were in various stages of regression. Plasma gonadotrophin levels of ovariectomised ewes fell significantly after immunisation and in intact immunised ewes ovariectomy failed to result in any increase in plasma gonadotrophins. Injection of 150μg synthetic LH-RH or 6μg of an immunologically distinct analogue of LH-RH failed to induce LH or FSH responses approaching those previously demonstrated with identical doses in non-immunised anoestrous ewes. These results suggest that immunisation against LH-RH could provide an alternative to ovariectomy for the suppression of unwanted oestrous symptoms and ovulation but that reversal of the effects of immunisation might be difficult to achieve routinely.

Requests for reprints should be sent to Dr. Crighton.

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INTRODUCTION

The ability of antibodies generated by active immunisation against synthetic luteinising hormone releasing hormone (LH-RH) to prevent oestrous cycles and ovulation has been reported in the rat (1) as has their ability to cause testicular involution and cessation of spermatogenesis (2,3). Such immune responses are of interest because of their potential in controlling reproduction in man and domestic animals. Following encouraging results from preliminary experiments (4,5), the present study was undertaken to examine the effects of active immunisation against synthetic LH-RH on oestrous cycles and ovulation in the ewe.

MATERIALS AND METHODS

Experimental animals: Clun Forest ewes from the School of Agriculture flock were used. Three cyclic ewes were immunised during the 1976-77 breeding season (group A). This was followed by ten cyclic and four ovariectomised animals during the 1977-78 breeding season (group B). At the start of each season all ewes were brought indoors and housed in a single large pen with a colour-marked vasectomised ram in order to detect oestrus. They were all fed twice daily on hay and concentrates, water being available *ad libitum*.

Immunisation: Ewes in both groups A and B were given a course of three identical immunisations against LH-RH starting on the 5 November 1976 for group A and 27 October 1977 for group B, and spaced six weeks apart. On the day before each immunisation the conjugate was prepared using the following procedure: 1 mg LH-RH and 1 mg bovine serum albumin were dissolved in 0.3 ml saline. To this was added 3 mg 1-ethyl-3(3-dimethyl-amino-propyl) -carbodiimide hydrochloride (Sigma Chemical Company) itself dissolved in 0.2 ml saline. The resulting 0.5 ml of reaction mixture was allowed to incubate overnight at room temperature. Immediately before immunisation a further 0.5 ml saline was added and the conjugate was emulsified in 2 ml of Freund's complete adjuvant. Each ewe was immunised after shaving a square area in the middle of the back and swabbing vigorously with a solution of 0.5% chlorhexidine in 70% ethyl alcohol. The immunogen (0.6ml) was injected intra-dermally into 6-8 sites around the edge of the prepared area. This resulted in a total dose of 0.2mg LH-RH per animal.

Releasing hormone administration: Ewes which developed anti-LH-RH titres were injected intravenously with either 150μg synthetic LH-RH (3 animals) or with 6μg of an analogue of LH-RH, $[\text{D-Ser (Bu}^{t})]^{6}$ Des Gly-NH_2^{10} LH-RH ethylamide (3 animals), 110 days after primary immunisation. Blood sampling was carried out at 15-min intervals for one h before and nine h after the injection.

Blood sampling: Weekly blood samples were collected from all ewes throughout the course of the experiment starting immediately prior to the primary immunisation. In addition, the four long-term ovariectomised ewes were fitted with jugular vein cannulae on 19 October 1977 and bled every 15 min for 4 h the next day. This was repeated on the 14/15 February 1978.

Six intact ewes (three group A ewes and three group B ewes) were fitted with jugular vein cannulae on 21 February 1977 and 14 February 1978 respectively. The next day they were sampled at 15-min intervals for one h before and nine h after injection, via the cannula, of either LH-RH or the analogue.

Three intact ewes with high anti-LH-RH titres and one non-immunised cyclic control were fitted with jugular vein cannulae on 14 February 1978 and sampled at 15-min intervals for four h. Two days later they were ovariectomised and then blood sampled, as before, 14 days later. All blood samples were collected into heparinised tubes, centrifuged at 3,000g for 10 min and the plasma removed and stored at -20°C until required for assay.

Surgical procedures: All 13 intact ewes were subjected to laparoscopy after the third immunisation when all but one had failed to show anticipated oestrus. The ewes were anaesthetised with sodium pentobarbital ("Sagatal", May and Baker). Both ovaries were observed for signs of follicle development and corpora lutea, and the state of the uterus was noted. For ovariectomy, anaesthesia was induced with sodium pentobarbital and maintained with halothane ("Fluothane", I.C.I.).

Assays: Plasma samples were assayed for LH and FSH as described previously (6,7). LH-RH antibody titres were initially determined by the ability of a 1:100 dilution of plasma to bind iodinated LH-RH. To determine antibody titre more precisely at the time when the intact ewes stopped cycling,

iodinated LH-RH was added to doubling dilutions of plasma from 1:12.5 to 1:400. For each determination the antibody:antigen complex was precipitated with cold pure ethanol, centrifuged at 3,000g for 15 min at 4°C and the precipitate counted for one min on a gamma spectrometer. Synthetic LH-RH was iodinated using a previously published method (8) with minor modifications. Five micrograms of synthetic LH-RH were added to a tube containing 1mCi^{125}I (Radiochemical Centre, Amersham). ^{125}I-labelled polypeptide was separated by elution from a cellulose CF 11 column with 0.01M phosphate buffer (pH 7.20) to remove the free iodine and damaged polypeptide, followed by 1% egg albumin in 0.01M phosphate buffer to elute the undamaged, labelled polypeptide. This was then diluted and used as labelled antigen in the assay. Using this method, specific activities of approximately 100 µCi/µg were obtained as determined by electrophoresis.

In addition the above procedure was used to test the cross-reactions of the antisera with the LH-RH analogue.

Statistical procedure: The significance of differences between means was determined using Student's 't' test.

RESULTS

LH-RH antibody titre: Mean anti-LH-RH titres for all sheep at a plasma dilution of 1:100 are shown in Figure 1. When antibody titres were determined more precisely at the time when the intact ewes stopped cycling, values ranged from 1:12.5 to 1:300 when dilutions to produce 33% binding of iodinated LH-RH were measured. None of the antisera raised against LH-RH cross-reacted with the analogue at dilutions down to 1:50.

Detection of oestrus: All intact ewes showed regular cycles before the appearance of an antibody titre. One animal from group B continued to show regular oestrous cycles up to the middle of February (the end of the observation period). All the remaining intact ewes from group B had stopped cycling before, or by six days after, the final booster immunisation. The three ewes in group A all stopped cycling by the end of December. In comparison, barren animals from the School of Agriculture flock continued regular oestrous cycles until the end of February in both 1977 and 1978.

Table I. Changes in plasma gonadotrophin concentrations in 4 long-term ovariectomised ewes following immunisation against LH-RH

	Plasma LH concentration (ng NIH-LH-S18/ml)(mean $\pm$ S.E.M.)			Plasma FSH concentration (ng NIH-FSH-S11/ml) (mean $\pm$ S.E.M.)		
Ewe	Before imm.	After imm.	Significance of difference	Before imm.	After imm.	Significance of difference
X	4.9 ± 0.6	1.7 ± 0.2	$P<0.001$	529.2 ± 38.3	67.5 ± 6.0	$P<0.001$
Y	10.5 ± 0.8	8.1 ± 0.6	$P<0.05$	1111.6 ± 53.0	1095.0 ± 41.8	N.S.
G17	21.8 ± 0.6	6.7 ± 0.2	$P<0.001$	265.2 ± 5.3	35.8 ± 8.8	$P<0.001$
R34	19.2 ± 0.5	16.9 ± 0.4	$P<0.01$	229.0 ± 5.1	203.2 ± 7.1	$P<0.01$
Combined	14.1 ± 0.9	8.6 ± 0.7	$P<0.001$	535.6 ± 49.0	346.3 ± 54.0	$P<0.001$

Table II. Changes in plasma FSH concentrations in 3 immunised ewes and one non-immunised control ewe (W) before and after ovariectomy

	Plasma FSH concentration (ng NIH-FSH-S11/ml) (mean $\pm$ S.E.M.)		
Ewe	Before ovariectomy	After ovariectomy	Significance of difference
O	22.1 ± 5.3	25.4 ± 6.3	N.S.
P	24.6 ± 3.2	7.1 ± 1.8	$P<0.001$
T	39.6 ± 3.8	21.9 ± 3.4	$P<0.01$
O,P,T, combined	27.7 ± 2.2	18.0 ± 2.4	$P<0.01$
W	12.0 ± 1.5	330.0 ± 20.0	$P<0.001$

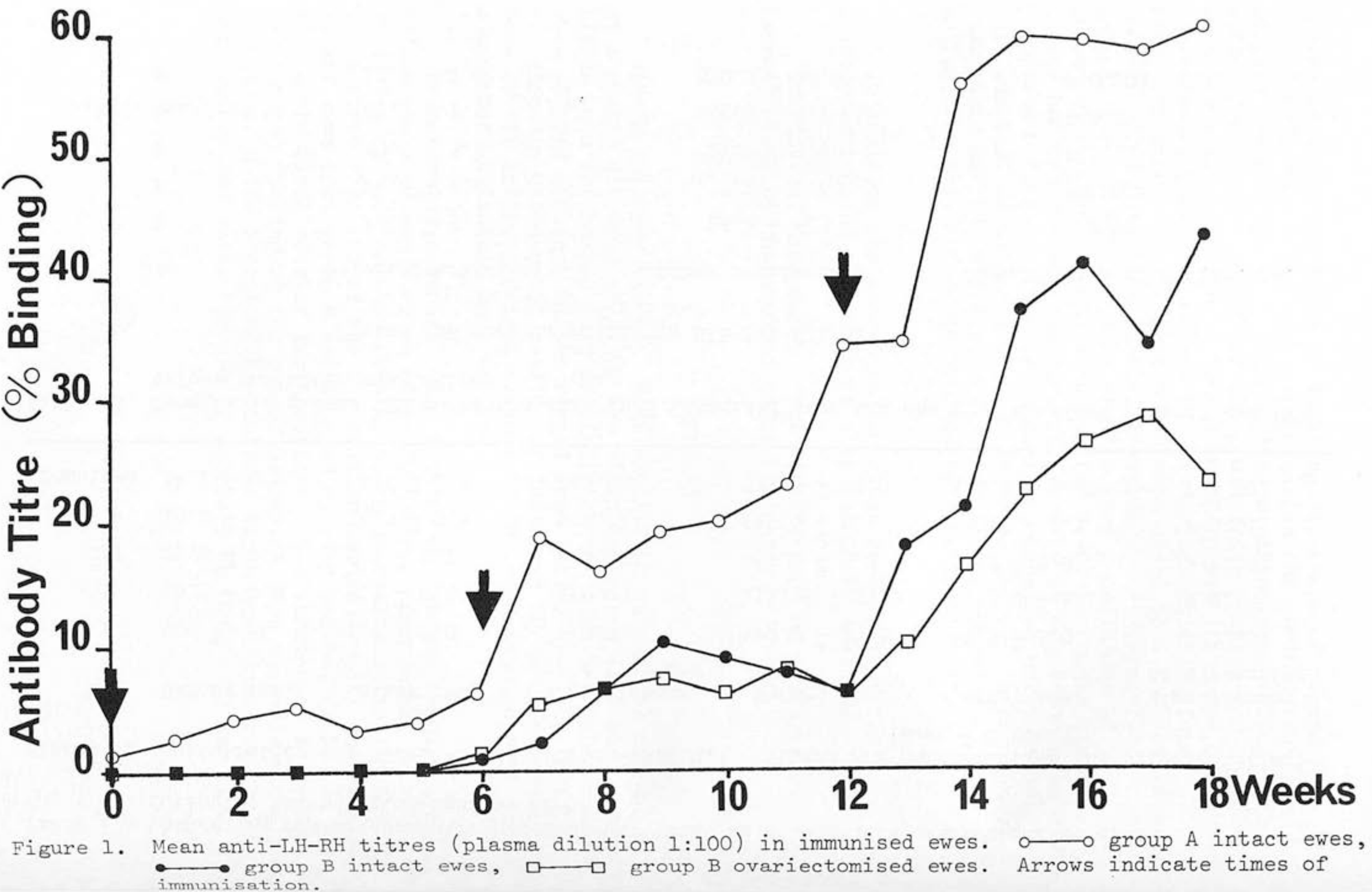

Figure 1. Mean anti-LH-RH titres (plasma dilution 1:100) in immunised ewes. ○——○ group A intact ewes, ●——● group B intact ewes, □——□ group B ovariectomised ewes. Arrows indicate times of immunisation.

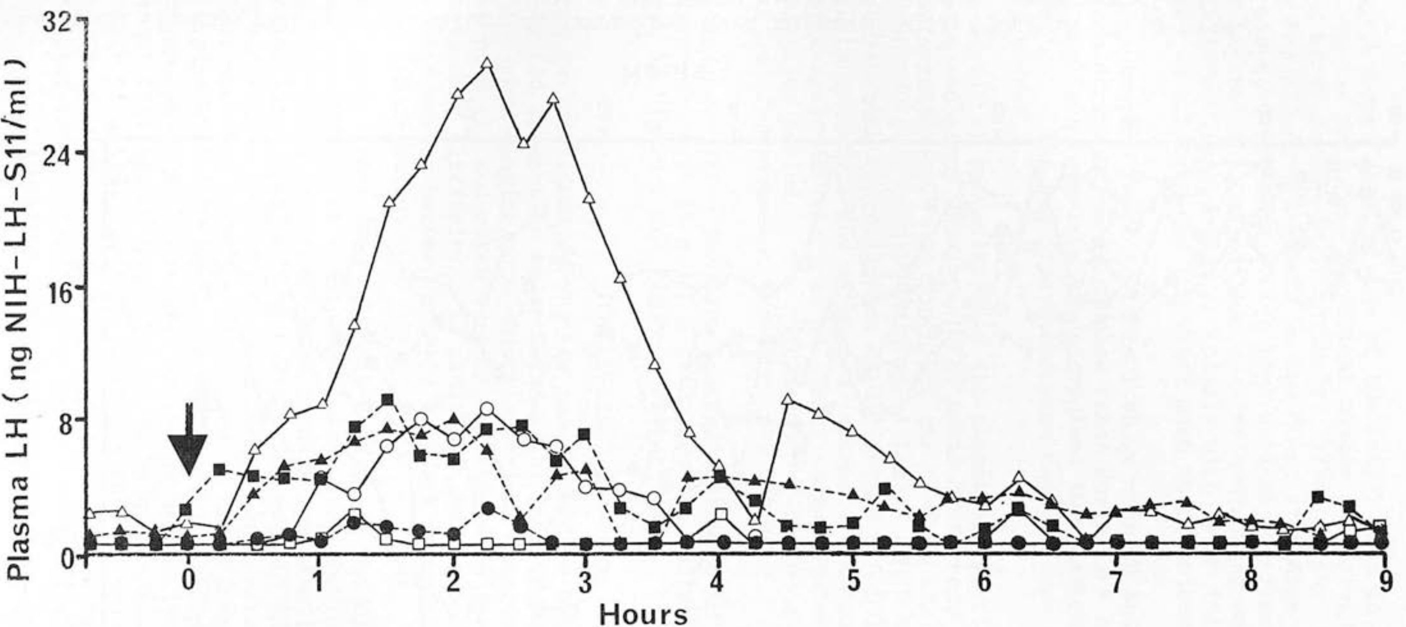

Figure 2. Plasma LH concentrations in immunised ewes injected with 150μg LH-RH (3 ewes, closed symbols, dashed lines) or 6μg LH-RH analogue (3 ewes, open symbols, unbroken lines). Arrow indicates time of injection.

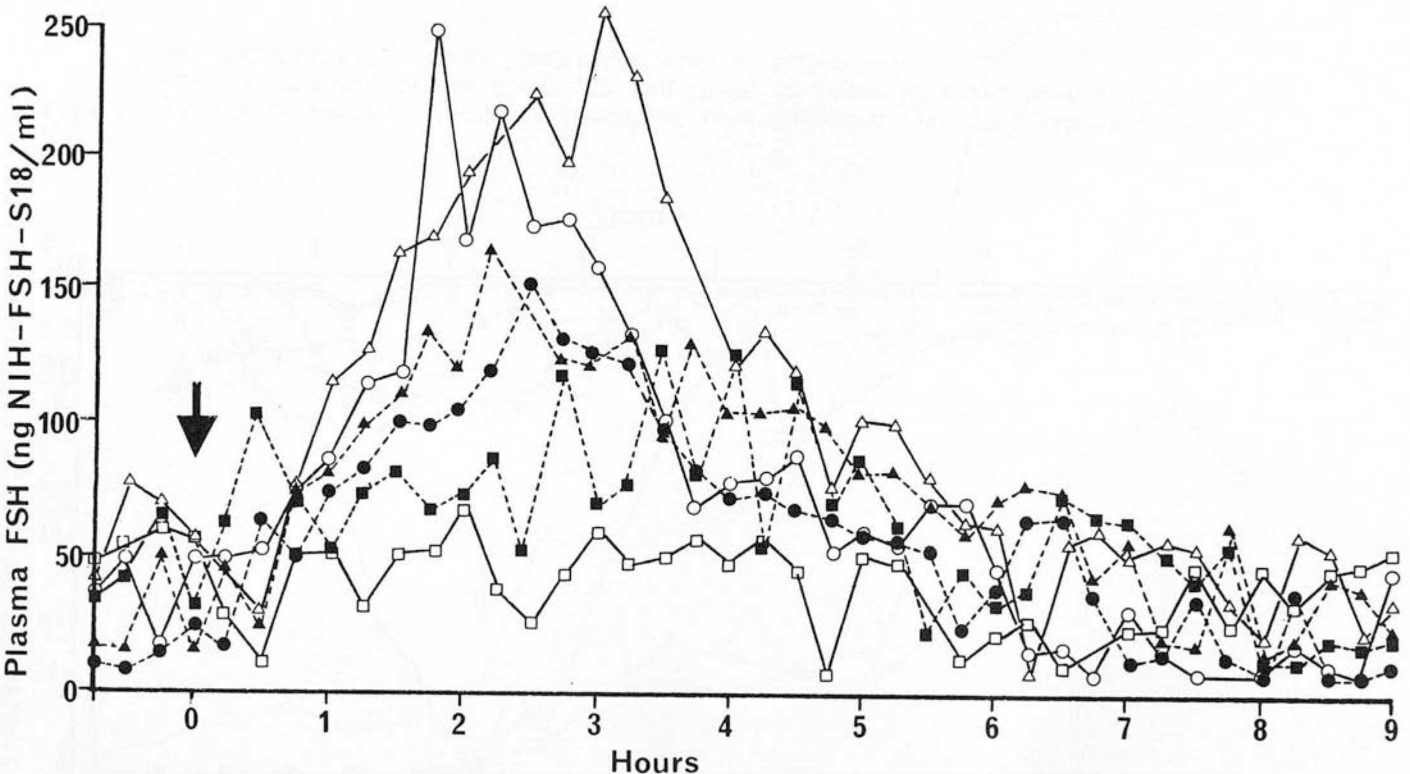

Figure 3. Plasma FSH concentrations in immunised ewes injected with 150μg LH-RH (3 ewes, closed symbols, dashed lines) or 6μg LH-RH analogue (3 ewes, open symbols, unbroken lines). Arrow indicates time of injection.

Laparoscopy: All but one of the intact ewes in 1977 and 1978 showed ovaries and uteri in various stages of regression when examined after detection of antibody. The one ewe that continued cycling had a corpus albicans on one ovary and large follicles on the other. Those ewes with high anti-LH-RH titres at the time of laparoscopy had smaller ovaries containing small follicles and pale infantile uteri. In animals with lower titres the follicles were larger and the uteri more vascularised.

Gonadotrophin levels: Table 1 shows mean LH and FSH data (each mean derived from 17 samples taken during a 4 h blood sampling period) for each long-term ovariectomised ewe, before and after immunisation. The combined means were derived from summated data from all 4 ewes.

LH could not be detected in the plasma of immunised ewes ovariectomised after development of antibody titres, either before or after ovariectomy. In contrast, the control non-immunised ewe had a mean LH level of 0.7 ± 0.3 ng/ml before and 9.1 ± 1.9 ng/ml after ovariectomy (significance of difference $P<0.001$). Mean FSH data taken before and after ovariectomy are shown in Table II. Means were again derived from 17 plasma samples taken during a 4 h blood sampling period. The combined means for ewes O, P and T were derived from summated data from the 3 ewes.

Intact ewes after immunisation and synthetic LH-RH or LH-RH analogue challenge: LH and FSH data from the individual ewes are shown in Figure 2 and Figure 3 respectively.

DISCUSSION

From data obtained during the two successive breeding seasons, a correlation appears to exist between the anti-LH-RH titre and associated physiological and endocrinological changes. This has been reported (1) and makes assembly of mean data for particular treatments very difficult. In addition, gonadotrophin levels varied considerably between animals within a group, both before and after immunisation.

The three intact ewes of Group A followed the classical trend in development of antibodies (9). They showed a very limited transitory response to the primary immunisation, followed by large increases in circulating anti-LH-RH after the two successive booster immunisations. In contrast, the ten intact and four ovariectomised ewes of Group B failed to show a typical primary response after the initial immunisation, the response being delayed until after the second immunisation. Two reasons that can be suggested to explain this failure are either loss of activity of the synthetic LH-RH preparation used in the conjugate or failure of the conjugation reaction. After the final booster immunisation, these 14 ewes attained titres which were

on average similar to those obtained in the previous year in response to the secondary immunisation. In other words it could be postulated that these ewes were about six weeks behind those of Group A with respect to development of antibody titre. This was reflected in data for the cessation of oestrous cycles obtained from the two groups of ewes, in which Group B on average showed their last oestrus 30 days later than had Group A in the previous year.

Immunisation of long-term ovariectomised ewes against LH-RH was effective in reducing circulating levels of LH and FSH, both of which were elevated before immunisation, a characteristic feature of ovariectomised ewes (10,11). The efficiency of immunisation against LH-RH for effecting reduction of circulating gonadotrophin levels appeared to be linked with the anti-LH-RH titre obtained. Thus ewes G17 and X, which both possessed high anti-LH-RH titres, showed the most marked reduction in hormone levels. Ewe R34, which only developed a moderate titre, had a decreased level of gonadotrophin inhibition, while ewe Y, with a low antibody titre showed a small although still significant ($P<0.05$) reduction in LH but not FSH levels.

Immunisation of intact ewes against LH-RH was effective in preventing the post-ovariectomy increase in gonadotrophins which is normally observed within two weeks of surgery in the ewe (10,11), and which was seen in the control non-immunised ewe. Immunisation of rats also prevented the post-ovariectomy increases in gonadotrophins, though LH and FSH levels did rise in animals with lower titres (1). It was also evident that immunisation of intact ewes reduced the basal level of LH from 1-4 ng/ml, normally found during the oestrous cycle (12), to levels below the sensitivity of the assay (0.4 ng/ml LH). At the same time FSH was still detectable in the plasma of these ewes. Maintenance of basal gonadotrophin levels but disruption of the pre-ovulatory peaks of these hormones, has been suggested as the cause of cystic follicles which occur in animals with low circulating antibody titres (1,13). Long-term ovariectomised ewes, however, maintained basal levels of plasma gonadotrophins after immunisation. This implies that either the amount of LH-RH secreted into the hypophyseal portal vessels after ovariectomy fully saturates the neutralising capacity of anti-LH-RH, or that the anterior pituitary after ovariectomy and immunisation is sensitive enough to respond to the very small amounts of LH-RH that might escape neutralisation by the antibody.

Injection of 150µg LH-RH caused a small release of LH and FSH, the effect being smaller in a ewe which had a higher and longer-maintained antibody titre. Synthetic LH-RH has been shown to induce some LH and FSH release in immunised male rats, the response depending on the animal's anti-LH-RH titre and the avidity of its

antibody for LH-RH (3). These results would be expected since the material used in the challenge is the same as the immunogen and would be expected to possess little biological potency in the successfully immunised animal. As a comparison, the same dose of LH-RH (150µg) has been shown to cause LH peaks in the anoestrous ewe of about 110 ng/ml (6).

A single dose of 6µg analogue injected into anoestrous ewes will induce release of LH and FSH to values (177 and 343 ng/ml respectively) within the range of the natural pre-ovulatory surge (7). In addition, incubation of iodinated analogue with plasma from immunised ewes revealed no cross-reaction with anti-LH-RH. Together, these facts suggested that reversal of the effects of immunisation might be achieved with the analogue. However, none of the three ewes tested showed gonadotrophin levels approaching those obtained previously (7) and indeed one animal (□-□ in Figues 2 and 3) failed to show either an LH or FSH response. The animal showing the highest LH and FSH responses to the analogue (△-△ in Figures 2 and 3) had developed a substantial antibody titre (>20% binding at 1:100 dilution) only in the four weeks before injection. The other two animals in the group had maintained substantial titres for 10 weeks. It has been shown in the rabbit (14) and in the rat (2,15) that immunisation against LH-RH reduces the pituitary content of LH and FSH. This is compatible with the view that LH-RH controls both synthesis and release of both gonadotrophins (2,15). Thus, after long-term neutralisation of LH-RH, a depleted pituitary pool of gonadotrophins might explain the absence of a normal surge of these hormones even after injection of the immunologically distinct analogue. In the male rat it has been reported that the same analogue will induce release of LH and FSH even after prolonged active immunity against LH-RH (3) but this discrepancy might be explained by different degrees of neutralisation of LH-RH. Reversal of the effects of immunisation might still be achieved with the analogue but with a revised injection schedule, perhaps employing a series of injections to stimulate gonadotrophin synthesis. A course of five daily injections of analogue into immunised female rats, however, failed to induce ovulation (1).

It would appear from this work that the hormonal events leading up to ovulation are very sensitive to even very low anti-LH-RH titres and hence it is quite simple to block cyclicity. These results suggest that immunisation against LH-RH could provide an alternative to ovariectomy for the suppression of unwanted oestrous symptoms and ovulation. A greater practical application of the technique may arise from its use in the male; this and its use in females of other species are currently under investigation, as are alternative means of reversing the effects of the procedure.

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Effects of active immunization of sheep against synthetic luteinizing hormone releasing hormone. By J. P. Foster, P. D. Webb and D. B. Crighton. *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, LE12 5RD*

Two ovariectomized and four cyclic ewes were immunized against synthetic luteinizing hormone releasing hormone (LH-RH) conjugated to bovine serum albumin. A multiple subcutaneous injection of conjugate in adjuvant was administered initially to each animal and this was followed after 2 months by a booster injection. The anti-LH-RH titre was measured in blood plasma samples obtained at weekly intervals from each animal by determining the binding of ^{125}I-labelled LH-RH to different dilutions of the plasma.

Blood samples were obtained from the ovariectomized ewes every 12 min for a 4 h period before immunization and again after the anti-LH-RH titre had increased. The concentrations of both LH and follicle-stimulating hormone were significantly less in the plasma from samples obtained after the increase in titre.

Two out of the four intact ewes immunized during the breeding season ceased to show cyclic oestrous activity following an increase in anti-LH-RH titre. This occurred at least 6 weeks before the end of the breeding season for the remainder of the School of Agriculture flock. Laparoscopy was performed on these ewes 7 days and 14 days after the expected time of oestrus and the reproductive tracts were examined. No corpora lutea or large follicles were present in the ovaries and the uteri were typical of the anoestrous state.

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Chapter 1

ENDOCRINOLOGY OF MEAT PRODUCTION

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1. INTRODUCTION

In this chapter, the treatment of cattle, sheep and pigs with hormones for meat production purposes is considered. Natural endocrine processes which control reproduction, growth and development, and non-endocrine treatments which bring about changes in the secretion of hormones, are not discussed in any detail. The text is divided into two main sections: reproductive aspects and growth and carcass aspects. In each case, the major classes of hormones used (including synthetic compounds with hormone-like activity) are described and their past, present and likely future contributions to meat production are evaluated.

2. REPRODUCTIVE ASPECTS

Considerable progress has been made in recent years in controlling ovulation in domestic meat-producing species but widespread practical application of many of the techniques developed has not resulted. To be commercially successful, any method of controlling reproduction must be soundly based physiologically, simple and cost-effective. The vital question of cost-effectiveness is complicated by the wide variety of management systems under which breeding stock are kept and by changes in the

popularity and price of different types of meat. The main areas in which hormone administration is important are as follows:

> Increasing the number of young produced at any parturition. Inducing ovulation in animals which fail to ovulate naturally. Hastening or delaying the natural time of ovulation of individuals within a group of animals so that ovulation is synchronised.

2.1. Increasing the Number of Young Produced at any Parturition

This may be achieved in a number of ways, all of which depend on the hormonal induction of superovulation; that is, the production of more ova at one time than is characteristic of the species concerned.

Hormonal techniques for inducing superovulation, without other treatment, are applied to cattle and sheep and generally seek to increase the proportion of twin births while avoiding undesirably larger litter sizes which lead to problems of birth weight and viability. Thus, the level of superovulation desired may be described as 'mild'. Superovulation may, however, be combined with ovum transfer from donors to recipients, in which case the number of ovarian follicles induced to ovulate is much greater. Superovulated individuals (usually cattle) in this case are usually of high genetic merit and the ova, fertilised by semen from a male also of high genetic merit, are recovered from the donor animals and transplanted for gestation into recipient animals which are of lower genetic merit. In the present context, animals of high genetic merit are those carrying genes for such characteristics as rapid growth rate, efficient feed conversion and desirable body conformation. In cattle breed improvement programmes in developed countries, transplantation of fertilised eggs could be used to increase the reproductive rate of females and thus the intensity of selection among them. The availability of large numbers of fertilised eggs from élite cows would increase the average genetic merit of any population within which they were deployed. Were very long-term storage of fertilised ova to become a practical proposition, a supply of genetic controls would become available. Assessment of improvement schemes through concurrent comparison with individuals of the original population would then be possible.[1]

2.2. Inducing Ovulation in Animals Which Fail to Ovulate Naturally

Where the reason for anovulation is pathological, the treatment is applied

to an individual after diagnosis. A good example is cystic ovarian disease in cattle, a common cause of infertility—and hence loss of productivity—in both beef and dairy breeds. Although important, the impact of the successful treatment of ovarian cysts is limited when compared with the production potential of techniques designed to induce reproductive activity during situations of anovulation and anoestrus which occur normally as part of the reproductive pattern of a species. Such situations may place severe obstacles in the path of intensification of production. They include the annual non-breeding season of the sheep and the periods of anovulation and anoestrus post-partum in the cow, sheep and pig.

Under British conditions, lambing generally occurs once a year in the late winter or early spring although the five-months pregnancy of the ewe would theoretically allow for two crops of lambs within one year in the absence of seasonal and post-partum inhibitory effects on reproduction. In the case of mountain sheep, which are maintained under conditions close to those of their wild ancestors, there is no pressure to increase productivity beyond one lamb per ewe per annum. For survival under harsh mountain conditions, where supplementary feeding is not practised except in emergency situations, the single lamb must be born in the spring. However, in the case of lowland sheep, productivity could be greatly increased if it were possible to produce two lamb crops in a year—one in spring and one in autumn.

In the United Kingdom, the beef industry has a very close relationship with the dairy industry. Approximately one-third of all dairy cows in the national herd are inseminated by bulls of beef breeds, either artificially or by natural service. Cross-bred and pure-bred male calves from the dairy herd are reared for beef and, while most pure-bred female calves are used as dairy herd replacements, many of the cross-bred calves are reared as suckler cows. These, together with females from the beef breeds, make up suckler herds in which the cows are inseminated usually by natural service by pure-bred beef bulls, frequently of a different breed. Thus it is necessary to consider both beef and dairy cattle in examining problems which influence the efficiency of beef production. Under modern management conditions, a high proportion of milked dairy cows and suckled beef cows exhibit periods of anovulation and anoestrus post-partum (see Lamming[2] for references). These effects are, at least in part, due to the stimulus of milking or suckling, the latter having the more potent effects on inhibition of reproductive activity;[3] hence the longer periods of anovulation and anoestrus encountered in beef cattle. In order to obtain one calving per year, the recommended interval from parturition to the start of the next pregnancy is

about 60 days. Unfortunately, many herds fall short of this ideal due to a variety of post-partum effects and, in herds where artificial insemination is used, inability of the herdsman to detect the first oestrus post-partum and hence to time insemination accurately.

In the pig, lactation is accompanied by anovulation and anoestrus which, in practice, continue for as long as the young are allowed to suckle. Reproductive activity usually returns within a few days of weaning and 'early weaning' is employed in some herds with a view to increasing annual sow productivity by shortening the interval between successive parturitions. It has been found, however, that the earlier in lactation weaning is carried out, the longer and more variable is the interval from weaning to post-weaning oestrus and ovulation.[4-6] This, together with reduced litter sizes[6] results in some of the reproductive benefits of early weaning being lost. The combination of these effects, together with the knowledge that pregnancy concurrent with lactation is a physiological possibility in the pig (see reference 7 for a review), has led to interest in techniques designed to induce ovulation and oestrus in the lactating sow.

2.3. Hastening or Delaying the Natural Time of Ovulation of Individuals Within a Group of Animals so that Ovulation is Synchronised

Increase in the rate of genetic progress among meat-producing animals demands the widespread use of artificial insemination and the maximal use of semen from highly selected males transmitting the desired characteristics. In the pig and sheep, present limitations to the use of artificial insemination arise in part from problems with the preservation and storage of semen. In the cow this is not the case and inability to detect oestrus is the reason why the proportion of dairy heifers and beef cows which are artificially inseminated is still remarkably small. Any technique which resulted in synchronised ovulation in a group of previously randomly cycling animals at a predictable time after treatment would obviate the necessity for the detection of oestrus, increase the efficiency of the insemination service and considerably reduce the cost of each insemination. It would also result in a widespread upgrading of the quality of progeny produced by the suckler cow herds by allowing the use of sires of superior genetic merit for meat production characteristics.

Thus there is considerable scope for increasing the efficiency of utilisation of breeding stock kept for the production of meat animals, enabling the industry to produce lean meat more economically. The hormone preparations available, and endocrine techniques developed with this in view, will now be considered in detail.

Hormone Preparations

The main classes of hormones which are in use or under investigation at present are:

(a) Gonadotrophins (natural) of other than pituitary origin, which are used for inducing growth of ovarian follicles and ovulation.
(b) Gonadotrophin-releasing hormone (synthetic) and its analogues, which are used for the same purposes as gonadotrophins; but operate by stimulating release of gonadotrophins from the pituitary gland.
(c) Progesterone (synthetic) and its analogues, which are used to delay the occurrence of ovarian follicular growth and ovulation.
(d) Prostaglandin $F_{2\alpha}$ (synthetic) and its analogues, which are used to cause regression of the corpus luteum and hence provoke premature follicular growth and ovulation.

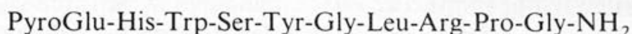
These hormonal agents have been used alone and in combinations and sequences ranging from the simple to the highly complex for various forms of artificial control of reproduction. In addition, brief mention will be made of one synthetic material, methallibure, which is not based on a naturally occurring hormone.

(*a*) *Gonadotrophins.* Two gonadotrophin preparations are widely used for inducing ovarian follicular growth and ovulation: Pregnant Mare Serum Gonadotrophin (PMSG) and Human Chorionic Gonadotrophin (HCG). Neither is of pituitary gland origin; the extraction of pituitary gonadotrophins from abattoir material is a particularly specialised, laborious and expensive process (see reference 8).

(i) *PMSG:* This hormone is present in the blood of the pregnant mare (for details of origin see reference 9). It is a glycoprotein of molecular weight 53 000[10] and is unique in possessing both follicle stimulating hormone (FSH) and luteinising hormone (LH) activities.[11] Various estimates have been made of the ratio of FSH to LH activity in PMSG preparations[12,13] but it is still not clear how variable this ratio may be between unextracted serum samples from different sources or, after extraction or purification procedures, in the preparations produced by different commercial companies. It has, however, been concluded[13] that the wide variation in ovarian response within groups of animals treated with fixed doses of PMSG is unlikely to be due to differences in FSH : LH ratio. The hormone is administered by injection, usually intramuscularly but sometimes subcutaneously.

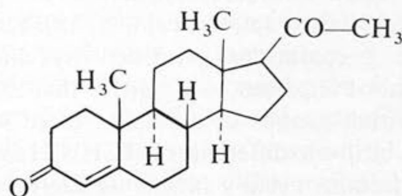
(ii) *HCG:* This hormone is extracted from the urine of pregnant women. It is placental in origin and is a glycoprotein with a molecular weight of about 50 000.[14] The molecule possesses almost entirely LH activity. The hormone is administered by injection, usually intramuscularly but sometimes intravenously.

(*b*) *Gonadotrophin-releasing hormone* (*synthetic*) *and its analogues.* Gonadotrophin-releasing hormone (Gn–RH, also known as luteinising hormone-releasing hormone (LH–RH), gonadorelin, gonadoliberin) is a decapeptide produced by the hypothalamus and made up of the following sequence of amino acids:


PyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

This structure was originally determined for the naturally occurring hormone of the pig.[15] The synthetic hormone releases the gonadotrophins (FSH and LH) from the pituitary glands of a wide variety of species including cattle, sheep and pigs and thus provokes ovarian follicular growth and ovulation indirectly. The hormone is administered by injection, usually intramuscularly but the intravenous and subcutaneous routes are also used. Intravenously injected Gn–RH is cleared rapidly from the bloodstream (half-life less than 10 min in all species studied) and is destroyed rapidly by various tissues.[16] The hormone is also destroyed by enzymes in the digestive tract. Recently, analogues of the Gn–RH molecule have been synthesised which are substantially more potent and longer acting than Gn–RH. Those studied so far have the same plasma half-life as Gn–RH but appear to be destroyed more slowly by certain tissues, notably the pituitary gland.[16]

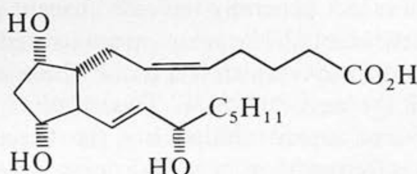
(*c*) *Progesterone* (*synthetic*) *and its analogues* (*progestins*). Progesterone is a steroid hormone produced mainly by the corpus luteum of the ovary during the luteal phase of the oestrous cycle and in pregnancy. One of its actions is to inhibit gonadotrophin release and hence ovarian follicular growth and ovulation. It has the following structure:



Progesterone

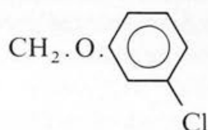
Crystalline progesterone, which has a half-life in blood (cattle) of about 36 min[17] can be administered by injection[18] but can also be given more conveniently for long-term treatment by means of progesterone-impregnated sponges inserted into the vagina (sheep, reference 19), progesterone-impregnated silastic rubber coils inserted into the vagina (cattle, reference 20) or silastic rubber implants placed subcutaneously (cattle, reference 21). Progesterone itself loses activity when administered orally but there are analogues of progesterone which, in addition to being active by the routes mentioned above, are orally active. These include 6-methyl-17-acetoxyprogesterone (MAP) and 6-chloro-Δ^6-17-acetoxy-progesterone (CAP).

(*d*) *Prostaglandin $F_{2\alpha}$ (synthetic) and its analogues.* Prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) is one of a series of naturally occurring unsaturated hydroxy acids of 20 carbon atoms based on a five-membered ring with two side chains. It has the following structure:



Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ is found in the uterine tissue of most species and is luteolytic (i.e. it destroys the progesterone-secreting corpus luteum) and hence permits a new wave of ovarian follicle growth followed by ovulation in a wide variety of species including the cow, sheep and pig. Prostaglandin $F_{2\alpha}$ is very rapidly destroyed when in the systemic circulation (half-life of a few seconds only), producing a variety of breakdown products, some with biological activity. The lung and liver are the major tissues involved in catabolism (see reference 22 for details of the characteristics of the prostaglandin series). There are now available a number of analogues of $PGF_{2\alpha}$ in which the luteolytic property is enhanced at the expense of other activities of $PGF_{2\alpha}$ such as stimulation of smooth muscle contraction. One example is cloprostenol which has the structure shown for prostaglandin $F_{2\alpha}$ except that C_5H_{11} is replaced by



Commercial preparations of $PGF_{2\alpha}$ and its analogues are standardised by weight and they are usually administered by intramuscular, or sometimes subcutaneous, injection.

In addition to these major classes of materials, one other agent, methallibure, should be mentioned. It is a non-steroidal compound (a dithiocarbamoylhydrazine derivative) which blocks ovarian function when administered orally.[23]

The use of these materials in achieving the objectives outlined previously will now be considered in detail.

Increasing the Number of Young Produced at any Parturition

The first step in all endocrine techniques designed for this purpose is to produce superovulation by the administration of gonadotrophin. For practical purposes, the application of superovulation is confined to cattle and sheep. Superovulation can also be produced in pigs[24] but the number of young produced is not generally increased except in individuals with chronically small litter sizes.[25] The gonadotrophin used almost universally in cattle and sheep is PMSG which has usually been administered in the follicular phase of the oestrous cycle. This relatively simple technique undoubtedly produces superovulation but the variability of response among individuals is frequently very wide. For example, in 99 heifers each injected with 2000 iu PMSG, the numbers of ovulations ranged from 3 to 45 and in 60 ewes each given 1000 iu the range was 0 to 23 ovulations.[13]

Attempts to increase the proportion of twin births by producing a 'mild' superovulation without other treatment have met with variable success in both cattle and sheep. Work in cattle up to about 1961 has been reviewed comprehensively.[26] Ovulation results in relation to dosage of PMSG in 416 cattle which showed oestrus shortly after PMSG treatment and were inseminated artificially[26] are shown in Table 1.

Because of heavy losses sustained during pregnancy diagnosis and other experimental procedures in this work,[26] it was not possible to provide complete figures for calving results, but 147 cattle produced single calves and in 44 cattle allowed to have multiple calvings there were 35 sets of twins, eight sets of triplets and one set of quintuplets. The natural incidence of twin calvings was 2·8 %, a figure which seems representative of most populations. The authors concluded, as a result of this detailed study, that the technical problems involved in the commercial use of PMSG for inducing twinning were severe and precluded any practical application at that time.

Subsequent work on superovulation without other treatment has

TABLE 1
OVULATION RATE IN RELATION TO DOSAGE OF PMSG (FROM REFERENCE [26])

Dose of PMSG (iu)	*No. of cows treated*	*No. of cows ovulating*	*Range of no. of ovulations/cow*	*Mean ovulation rate*
800	100	99	1–5	1·43
1 000	145	142	1–15	1·77
1 200	42	42	1–17	2·50
1 600	14	14	1–15	2·71
2 000	115	114	1–25	3·97

involved giving two or more injections of PMSG at various stages of the cycle, the last injection being given during the follicular phase.[27] In this case, the treated cows were also injected with HCG at the time of mating (although the administration of a source of LH is not necessary for ovulation to occur). A high proportion of undesirable triplets and larger litter sizes were again produced. However, using a basically similar technique, but with much lower doses of PMSG, a high proportion of twins was obtained with no litters of more than two calves.[28]

It has been shown that different breeds of cattle show different dose–response curves for PMSG and that, to a certain extent, the response of a given population can be predicted on the basis of results from a representative sample, so that the dose can be modified in the light of this knowledge. In practice, however, one is frequently dealing with populations which are not homogeneous with regard to breed and there is a need for techniques to enable rapid detection of ovarian hyperstimulation and the early termination of undesired multiple pregnancies.[29]

Although reports have appeared of apparently successful attempts to increase the percentage of twin births in cattle by means of the administration of low doses of PMSG together with other hormones,[30] the wide variety of treatment regimes used in this type of work and the range of breeds and types of cattle treated make it impossible to recommend a technique of general practical applicability at present.

'Twinning' in cattle can also be achieved by techniques involving ovum transfer. One procedure which has been adopted[31] is to use superovulated beef heifers as donors of fertilised ova and mated cattle as recipients. Non-surgical transfer of one fertilised ovum per recipient is carried out via the uterine cervix. In order to increase the yield of fertilised ova from each beef heifer, two successive superovulation treatments can be applied in the

month before slaughter for beef. On the first occasion the fertilised eggs are recovered by non-surgical procedures (see reference 31 for details). The donor is slaughtered 3 to 4 days after the second treatment and the fertilised eggs are recovered from the reproductive tract in the laboratory.

Evidence suggests that the use of mated recipients results in a higher rate of survival of transferred ova than when transfer is made into unmated recipients, i.e. the survival rate of 'foreign' embryos is greater when there is also a 'native' embryo in the uterus. The factors affecting embryo survival are, however, complex. They have been reviewed comprehensively,[31,32] together with other aspects of the subject.

The translation of experimental results into the farm situation has already been achieved in a limited way[31] and seems to offer considerable hope for increasing the supply of genetically suitable beef calves from the dairy herd as well as from suckler beef herds. In the dairy herd, however, non-surgical transfer would be useful only where 'native' female progeny were intended for beef production and not as dairy herd replacements, since almost all female cattle born as twins to a male are sterile (freemartinism, see reference 31).

The performance of twin cattle and their dams remains to be assessed under systems of feeding and other aspects of management designed to cope with a high percentage of twins as opposed to systems geared traditionally to the production of one calf per cow. The cost-effectiveness of twinning techniques, when employed under such conditions on a large scale, remains to be established.

The demonstration that the pregnancy rate in cattle, into which fertilised eggs recovered surgically from superovulated donor cows were transferred surgically, was over 90%[33] has led to practical application in situations in which the very high costs of two surgical procedures are justified by the commercial value of the progeny produced. Such circumstances arose between about 1969 (when the paper by Rowson *et al.*[33] was published) and about 1975, due to the finding that certain of the European beef breeds (e.g. Simmental and Limousin) were superior to the traditional British beef breeds in progeny tests in the UK. This led to a great demand for animals of these breeds in North America and Australasia. Stringent quarantine requirements limited severely the number of animals available (for details see reference 34) and very high prices were obtainable. Commercial ovum transfer units were set up with the purpose of multiplying, as rapidly as possible, the small numbers of imported stock. This activity continued on quite a large scale until about the end of 1975 when the prices for these 'exotic' breeds of cattle had fallen to a level at which the techniques were no

longer commercially viable in spite of the fact that by that time commercial pressure had led to the development of techniques and apparatus for non-surgical transfer of ova. In addition to the possibility of a recurrence of this 'artificial' type of situation created by legislation, and the use of superovulation and ovum transfer into mated cows for routinely increasing the percentage of twins,[31] the future of commercial ovum transfer in cattle is likely to be in the area of genetic applications.[1] Here the potential for the improvement of growth rates and body conformation in cattle intended for beef is considerable. These aims would be aided substantially by the development of a simple technique for the long-term storage and long-distance transport of fertilised eggs by deep freezing or other means.[35]

In the case of the sheep, 'twinning', by means of superovulation without other treatment, has been carried out with commercially useful results in some cases.[36] In this work, reported in 1962, the average lambing percentage of 614 treated ewes during 3 years of trials was 178 compared with 116 for control ewes. The technique is still used on a limited scale commercially in Iceland to produce 'twinning' in ewes which have previously given birth to single lambs. Thus, its use is restricted to flocks in which the reproductive history of each ewe is known (H. Pálsson, pers. comm.).

In contrast, in another, earlier, study in Australia no significant increase in the lambing percentage of ewes in PMSG-treated groups was found.[37] More recent work on the sheep appears to be lacking, although there is one report of a significantly increased ovulation rate in ewes injected on day 12·5 of the cycle with the Gn–RH analogue [D–Ser $(Bu^t)^6$] Des Gly NH_2^{10} Gn–RH ethylamide.[38] This finding remains to be verified before any applicability to commercial practice can be considered.

Although ovum transfers have been successfully carried out in sheep it is difficult to see superovulation with ovum transfer ever becoming commercially important in this species in the form described for cattle[31] because the value of the additional lambs is unlikely ever to approach the cost of producing them. It is foreseeable that these techniques could be used for a limited period in a situation such as that which arose in the case of cattle quarantine regulations or for genetic applications.[1]

Inducing Ovulation in Anovulatory Animals

In the situation where one animal becomes anoestrous and anovulatory while others around it are showing normal reproductive behaviour, the problem is one of accurate diagnosis of the underlying causes to allow the application of the correct treatment.[39,40] Of more relevance here are

techniques designed to increase productivity in conditions of anovulation and anoestrus which occur in all females of a species due to seasonal, post-partum or lactation effects.

Of the three meat species considered here, the sheep is the only one to show a pronounced seasonal reproductive pattern. The practice of lambing once a year in the spring in the United Kingdom leads to a characteristic annual pattern of availability of home-produced lamb whereby the market is flooded with fat lambs in the late summer–early autumn with a consequent fall in the price. The inverse situation occurs in late winter–early spring. Thus there is a need for lambs born 'out of season'. If autumn lambing could be achieved in addition to the customary spring lambing, the efficiency of sheep enterprises could be greatly increased. However, the feeding and management of autumn-born lambs presents problems not met with their spring-born counterparts and in the opinion of some the aim of two lamb crops per year is unnecessarily ambitious.[41] Where once-per-year autumn lambing is intended, techniques are now available for inducing reproductive activity during seasonal anoestrus uncomplicated by post-partum effects due to the normal spring lambing. When an increase in the frequency of lambing is required, however, seasonal and post-partum effects combine to create a particularly unfavourable climate for the induction of reproductive activity.[42] In the case of non-lactating ewes which are anoestrous and anovulatory due to the effects of season only, the regime which has been developed is that of treatment with a progestin (originally progesterone, more recently one of its analogues) for a period of about 12 days followed by an injection of PMSG (500–850 iu) at the end of progestin treatment. The history of the development of this regime has been described in detail.[43] It is used quite widely, particularly in France, to obtain three lamb crops in 2 years.[42] This type of treatment is more complex than would be desired ideally. The use of the more recently available synthetic Gn–RH as a single injection or even as multiple injections in anoestrous ewes appears impracticable.[44,45]

When a regime of the progesterone–PMSG type has been attempted with ewes in the early post-partum period the results have been generally very poor. The complex reasons for poor fertility post-partum have recently been discussed in detail.[46] Early attempts to improve fertility of lactating ewes, even when using complex progesterone–PMSG treatments outside the realms of practical application, met with little success.[47,48] More recent attempts to modify the established progesterone–PMSG treatment have also been unsuccessful.[49] In the foreseeable future, hormonal techniques may have their place in the treatment of seasonally anoestrous ewes to

induce autumn–winter lambing for the production of early fat lambs. This system is already in use on a commercial scale in Ireland, the hormone treatment service being run from the cattle artificial insemination stations.[41] For the purpose of increasing the frequency of lambing it may be that more satisfactory results will be obtained by choosing breeds or crosses of sheep with long natural breeding seasons and high fecundity and using manipulation of the diurnal light:dark ratio to induce more frequent periods of reproductive activity. Encouraging results have been achieved with this system when combined with hormonal synchronisation of ovulation and oestrus,[50] but cost-effectiveness remains to be proved.

In domestic cattle, in which there is no marked seasonality of reproductive function, most of the problems are associated with the occurrence of a sufficiently rapid return to oestrus and ovulation after parturition to ensure one calving per cow per annum. One of the reasons for failure to achieve this—ovarian follicular cysts—has already been mentioned. The fact that a wide variety of circumstances associated with the post-partum period in cattle may prevent an early return to reproductive activity makes differential diagnosis essential. While the traditional technique of rectal palpation of the ovaries is still used, the ability to determine the level of progesterone in the milk, which bears a close correlation with that in the blood, has made this task substantially easier.[2]

In the breeding sow, the restriction placed on productivity by lactational anoestrus has led to numerous attempts to induce pregnancy concurrent with lactation by hormonal means (see references 51–54). The results of these treatments, based on the use of PMSG, have been variable and have not led to any widely applicable practical technique. There are undoubtedly substantial differences in the responsiveness of different breeds and strains of pig to PMSG, probably related to the 'depth' of the lactational anoestrus in each case. Non-hormonal techniques, involving the grouping of sows, *ad libitum* feeding, boar presence and other factors, which are successful in practice in some herds,[55] have generally failed to have any effect when subjected to examination in others.[54,56] This is again a reflection of breed and strain variability in the 'depth' of lactational anoestrus. There is thus no widely applicable alternative to early weaning at present and this is an area of research which could reward re-examination since the potential for increased productivity is considerable.

Synchronisation of Ovulation in Groups of Animals

The object of almost all techniques designed to synchronise ovulation is to synchronise the 'end of luteal phase' situation, which precedes the

expression of oestrus and the occurrence of ovulation during the cycl
These are usually not synchronised in any group of cyclic animals. The en
of the luteal phase is characterised by a dramatic decline in the level c
circulating progesterone which is followed by development of ovaria
follicles, oestrus and ovulation. The fact that the interval between the fall i
progesterone and the occurrence of ovulation is consistent in any specie
means that ovulation can be accurately synchronised if the fall ii
progesterone can be accurately synchronised.

Two classes of agent are in use for this purpose—progestins (progesterone and its analogues) and prostaglandins (prostaglandin $F_{2\alpha}$ and its analogues). In this context, progestins are administered so as to maintain a high circulating level after the endogenous progesterone level has fallen. If this is continued for a sufficiently long period in a group of randomly cycling animals, each individual will eventually be in a situation of 'artificial luteal phase' so that stopping administration of the progestin in all animals at the same time will precipitate a synchronised return to oestrus and ovulation. The prostaglandins, on the other hand, are used to destroy the corpora lutea (luteolysis) during the luteal phase of the cycle. Individuals which are in the luteal phase at the time of treatment show a synchronised fall in the circulating level of progesterone and a synchronised return to oestrus and ovulation. Prostaglandins are only effective when a viable (i.e. progesterone-secreting) corpus luteum is present; so that, in a randomly cycling population, a proportion of animals will fail to respond to a single prostaglandin administration.

In cattle, Christian and Casida,[57] using daily injections of progesterone for 14 days, were the first to report the successful synchronisation of ovulation. Since that time a wide variety of progestins has become available and these have been administered by a variety of routes (see reference 58 for review and references). Generally, although satisfactory synchronisation was achieved in many experiments, fertility at the synchronised oestrus was lower than that achieved normally in randomly cycling cattle, normal fertility only returning at the following oestrus.[58] Variation in oestrous cycle length between and within individuals is such that the advantages of synchronisation are, to a large extent, lost at the second oestrus. Much of the subsequent work was aimed at developing a treatment which would result in an ovulation response so precisely timed that artificial insemination of all animals in the group could be carried out at a fixed time after treatment without loss of fertility. One advance in this area was to incorporate treatment with oestradiol (which has luteolytic properties in cattle, possibly through releasing the endogenous luteolytic agent) at the

start of progestin treatment in order to allow shortening of the period of treatment to about 9 days.[59] The reduction achieved in the period of progestin treatment resulted in normal fertility after artificial insemination, not only after oestrus detection as previously reported, but after fixed-time insemination, whether or not the animals were observed on oestrus. The use of prostaglandins in place of oestradiol to shorten the period of progestin administration also produced good synchronisation and normal fertility.[60,61] Progesterone itself can be administered subcutaneously in silastic rubber implants alone or in combination with an injected luteolytic agent.[21] In cattle, progestins may also be administered *per vaginum*. Early attempts to use progestin-impregnated intra-vaginal sponges (as employed with sheep) were unsuccessful due to the high percentage of sponges voided.[58,62] More recently, a progesterone-releasing intra-vaginal device (PRID), which has a high retention rate,[20] has been designed for use in cattle. This is a stainless steel spiral coated with silastic rubber which is impregnated with progesterone. Insertion into the vagina is accompanied by the administration of oestradiol benzoate and progesterone by injection and removal of the device after 12 days is followed by well synchronised oestrus and ovulation with good fertility resulting from artificial insemination.[63]

These two techniques for administering progestins to cattle have been evaluated in extensive field trials and have been found capable of successful application on commercial farms in many parts of the world. Whether or not they are now incorporated into farm practice depends on the demonstration of cost-effectiveness in a variety of circumstances.[64]

The observation[65] that prostaglandin $F_{2\alpha}$, when injected non-surgically into the cow uterus at any time between days 5 and 16 of the cycle, induced regression of the corpus luteum and very exact synchronisation of oestrus, has been followed by many studies on the practical application of prostaglandins to the control of oestrus and ovulation in cattle. Both prostaglandin $F_{2\alpha}$[66] and analogues[67-70] have been employed. Treatment with prostaglandins, whether $PGF_{2\alpha}$ or analogues, has no effect on the bovine corpus luteum when administered during the first 4 to 5 days of the oestrous cycle.[65] The corpus luteum starts to regress spontaneously at about day 17 so that prostaglandins are ineffective from then until about day 5 of the next cycle, leaving a period of about 12 days per cycle in which luteolysis will be provoked. Thus, if a group of regularly but asynchronously cycling cows is given prostaglandin on a particular day, the response in terms of luteal regression will be positive or negative according to the stage of the cycle at treatment. This has resulted in the development

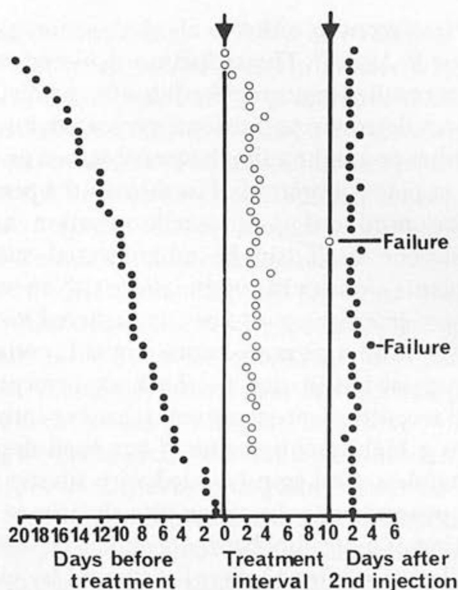


FIG. 1. Synchronisation of oestrus in heifers by means of two injections each of 500 μg cloprostenol given 10 days apart. Closed circles indicate oestrus before and after treatment, open circles indicate oestrus after first injection. Arrows indicate times of injection (from reference 68).

of a treatment regime consisting of two prostaglandin injections separated by 10 to 12 days. At the second injection all cattle in the group are at a stage of the cycle in which they are sensitive to the luteolytic properties of prostaglandin, and luteal regression, oestrus and ovulation are highly synchronised (Fig. 1). Fertility is normal after artificial insemination (see reference 64 for results and discussion).

The prostaglandins are relatively cheap agents and their administration is simple. Their use, particularly that of those analogues in which side effects are virtually absent, has already proved important in commercial practice. Large-scale field trials in beef suckler cows and heifers[71] and in dairy cows[72] have demonstrated the efficacy of the double injection regime.

In the sheep, the same principles have been employed as in the cow for the synchronisation of oestrus and ovulation. Progesterone or its analogues, when administered as 14 daily injections,[18] orally[19] or in intra-vaginal sponges,[19] all resulted in good synchronisation. Poor fertility at the induced oestrus was observed frequently, however, in the field trials and in commercial practice. Improvements in the method of preparation of the

sponges, and the use of PMSG following their removal,[73] has resulted in improved fertility and practical application. In the UK, conception rates of 70 % can be achieved routinely when two artificial inseminations are carried out 50 and 64 h after the withdrawal of sponges impregnated with MAP and when 750 iu PMSG is given at the time of withdrawal of the sponges (see reference 46).

Prostaglandin $F_{2\alpha}$ is an effective luteolytic agent in the sheep when administered as a single intramuscular injection,[74] as is cloprostenol.[75] A similar technique to that employed in cattle, and using two injections of 100 μg cloprostenol 9 days apart (since the oestrous cycle of the sheep is shorter than that of the cow), provides very good synchrony of onset of oestrus after the second injection, with good fertility at the induced oestrus[46,76] (Table 2).

TABLE 2
FERTILITY OF CYCLIC EWES GIVEN TWO INJECTIONS OF 100 μg CLOPROSTENOL 9 DAYS APART (FROM REFERENCE 46)

		Conception rate to first mating (%)	*Prolificacy* (%)
Trial 1	Treated	66·0	133
(n = 50)*	Control	70·0	120
Trial 2	Treated	65·7	161
(n = 35)*	Control	71·4	160

* For both treated and control groups of ewes. There were no significant differences between treated and control ewes in either conception rate or prolificacy. Treated ewes showed oestrus 39·7 $\pm$ 0·7 h (mean $\pm$ SE) after the second cloprostenol injection.

This method of ovulation control shows considerable promise for use in cyclic sheep but it is too early to assess the impact of the technique in practice.

In the synchronisation of ovulation, the pig offers greater problems than either the cow or the sheep. The administration of progestins has frequently (although not always) led to poor fertility and the formation of cystic ovarian follicles (see reference 77 for discussion and references).

It has been found that prostaglandins are ineffective in provoking luteolysis in the pig until about day 11 or 12 of the 21-day cycle. They do

not, therefore, offer a practical means of synchronising ovulation in groups of animals.[78] Techniques designed to render the pig sensitive to prostaglandins by the administration of other hormones are at an early experimental stage and at present seem too complicated for potential practical application.[79]

In contrast to the problems generally associated with the use of progestins and prostaglandins in pigs, a dithiocarbamoylhydrazine derivative, methallibure, administered orally for a period of 20 days, proved to be effective in synchronising ovulation and oestrus.[23] Modification of the technique to include injection of PMSG at the end of the period of methallibure treatment and of HCG 96 h after the PMSG induced a highly synchronised ovulation and a high conception rate in animals inseminated artificially 24 h after the HCG injection without reference to oestrus.[80] This regime could be considered too complex to be ideal for practical use. Nevertheless, the precision of the timing of ovulation, and the ability to inseminate animals artificially without the necessity of detecting oestrus, made the technique of considerable potential importance in the development of controlled breeding programmes and methallibure became available commercially. However, use of the compound was abandoned and regulatory approvals were withdrawn in many countries, after reports of teratogenic effects in pregnant gilts, and in the present-day climate of extreme caution over the side-effects of drugs it seems unlikely that methallibure will again become generally available. Thus, the availability of methods for the synchronisation of ovulation in pigs is very limited and no technique can at present be recommended for practical use.

3. GROWTH AND CARCASS ASPECTS

It has long been recognised that the sex of an animal is a major factor in determining its pattern of growth and development and, in meat animals, the form and composition of the carcass. In the last 20 years or so a large number of experiments have concentrated on differences in rate of growth, feed conversion efficiency and carcass composition and quality between entire and gonadectomised animals of both sexes in cattle, sheep and pigs. Such differences are mediated via the gonadal sex hormones, all of which are steroids, and between which and the hormones of the pituitary gland and hypothalmus there is a complicated interplay, leading to the characteristic reproductive pattern of each species. Withdrawal of these

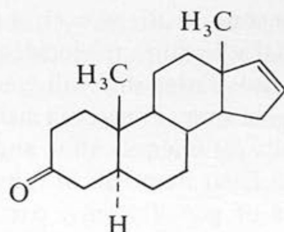
hormones from the circulation due to gonadectomy leads not only to cessation of reproductive capacity but to modification of the secondary sex characters, the abolition or reduction of sex drive and aggressiveness and other behavioural changes. Because of the metabolic effects of the sex steroids, withdrawal also leads to changes in growth rate, in feed conversion efficiency and in the proportions and distribution of muscle, fat and bone in the body.

Gonadectomy, particularly castration but to some extent spaying also, has been used as a production technique through the ages. In the case of castration, the original reason for its use was probably to render the male more easily manageable and to enable males to be grazed along with mature females without indiscriminate breeding. Thus its use was the first step taken to direct genetic changes in populations of farm animals. The practice was probably reinforced by the observation that, under poor grazing conditions, the castrate male had a larger deposition of fat than its entire counterpart. This was particularly important at a time when a large amount of fat was a highly desirable feature of the carcass. Spaying has also been employed to improve the amount of fat deposition, to suppress oestrous behaviour (which leads to disturbance of mixed groups of castrate males and intact females) and to prevent the occurrence of unwanted pregnancy. Because of the greater technical difficulty involved in carrying out the operation, apart from other considerations, such as damage to the hide and the occurrence of abdominal adhesions, the incidence of spaying has been low compared with that of castration and will continue to be so. Where growth performance of spayed animals has been measured it has frequently been poor.[81,82] The necessity for intensification and for the satisfaction of consumer demand for lean meat have led, in recent years, to a detailed examination of the effects of gonadectomy, particularly castration, in cattle, sheep and pigs. Various aspects of this work have been reviewed[83–87] and it is not within the scope of the present chapter to cover this ground again. Suffice it to state that, while conflicting results have been obtained due to different times of castration, different levels of nutrition and to different environmental aspects of the experiments, the overall conclusion is that, regardless of species, intact males make a greater average daily gain in weight (+5 to 20%), have a greater efficiency of feed conversion (+5 to 20%) and deposit less fat in the body. These results alone appear to favour the production of meat from entire animals but against them must be set the following negative factors.

(a) The historical reasons for castration, i.e. improved tractability, reduced aggression and ease of mixing with intact females, are still valid

today under intensive management conditions. Under some management conditions castration may be essential.

(b) In the case of the pig, meat from intact males frequently suffers from a sexual odour or taint on cooking which renders it highly undesirable from the consumer's point of view. In addition, the advantage of boars over castrates in terms of growth rate and feed conversion efficiency is probably the smallest of the three species under consideration. The intact male does, however, have a lesser backfat thickness, an important criterion in judging the quality of pig carcasses. It has been suggested[85] that lower levels of androgens may increase the muscle and reduce the fat in boar carcasses without leading to taint, whereas, when the activity of the androgens reaches a certain level, the sex odour appears. Certainly, not all meat from boar carcasses has a sex odour. In a study on commercial boar carcasses of market weight range[88] it was found that 28 % had a strong odour, 36 % had a slight odour and 36 % were free from odour. In boars slaughtered below about 70 kg liveweight the incidence of odour is very low[89] and, in older animals, the administration of diethylstilboestrol by implantation or in the feed has been used as a means of suppressing it.[90] The odour is caused by a steroid, 5α-androst-16-en-3-one, which was originally isolated from boar fat.[91] This compound is very similar to the male gonadal steroid hormones. Its structure is shown below:



5α-androst-16-en-3-one

The steroid does not have androgenic activity, however. Its physiological function is that of a pheromone, imparting a characteristic odour to the breath and saliva of the boar which intensifies the sexual response of the oestrous sow. The development of a radioimmunoassay for 5α-androst-16-en-3-one[92] has enabled its detection in biopsy samples from the fatty tissues of boars and in much smaller amounts in the fat of gilts and castrate males.

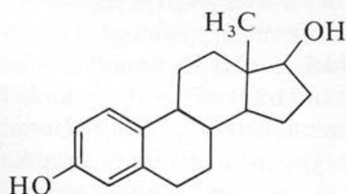
These aspects make it certain that castration will continue to be practised widely in spite of its proven disadvantages in terms of growth rate and feed conversion efficiency. A further disadvantage of surgical castration is that it is, of course, irreversible so that the genetic characteristics of individuals

which prove to have outstanding performance in the growing period and highly desirable body conformation are lost to the breeding industry. Surgical castration results in complete absence from the circulation of testicular androgen and oestrogen, no grading of effects is possible and the benefits of the metabolic properties of androgen and oestrogen are exchanged for the benefits of sterility, tractability, relative ease of management and, in the pig, absence of sex odour of the meat associated with the castrate animal. An attempt to obtain all these benefits together has been embodied in the development and use, since the late 1940's, of anabolic agents. The effectiveness of such agents was first demonstrated in 1949 in castrate lambs when it was found that subcutaneous implantation of the synthetic oestrogen stilboestrol or of testosterone increased weight gain and improved feed conversion efficiency.[93] Anabolic agents are now applied not only to castrates but also to entire males and females although the nature of the agent which is ideal in each of the sex types is different, androgen and oestrogen both being necessary for the maximum realisation of growth potential.[94]

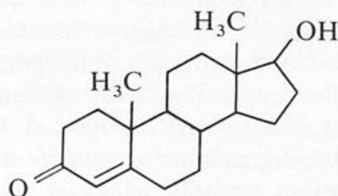
Anabolic agents may be defined as compounds or mixtures of compounds which stimulate accretion of body protein. This is usually accomplished by an increase in nitrogen retention. Almost without exception, anabolic agents possess hormonal activity as well as anabolic activity, those which are currently employed having hormonal activity similar to that of the gonadal steroids. The compounds used can be classified, depending on their chemical structure and occurrence, into:

(a) Steroid hormones which are normally present in the animal body.
(b) Steroids which are not normally present in the animal body.
(c) Compounds other than steroids which are not normally present in the animal body.

Commercially produced anabolic agents frequently consist of mixtures of these compounds. Within group (a) the major hormones used are oestradiol-17β and testosterone. Their structures are shown below:

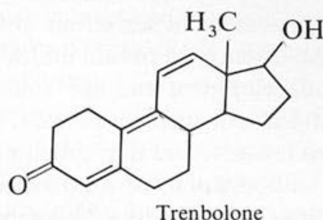


Oestradiol-17β



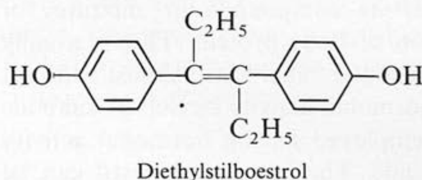
Testosterone

Commercially, these steroids are frequently produced as simple esters, e.g. oestradiol benzoate and testosterone propionate, in which form they are not exactly identical in structure to the native hormone and are considered properly under (b). Also under (b) should be grouped synthetic steroids such as trenbolone, frequently used as trenbolone acetate.

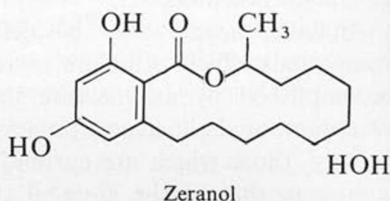


Trenbolone

Group (c) includes the stilbene derivative diethylstilboestrol and a substituted resorcylic acid lactone, zeranol.



Diethylstilboestrol



Zeranol

These compounds have been administered in a variety of ways—orally, by previous incorporation in the feed or as a separate additive at feeding, as subcutaneous or other implants and by injection. The natural steroid hormones lose much of their biological activity on oral administration, being broken down into less active metabolites which are, in turn, rapidly degraded, and are usually applied now as implants. Diethylstilboestrol,[95,96] trenbolone acetate[97] and zeranol[98] are also frequently applied in this way. Oral administration has been used frequently for diethylstilboestrol.[99] Injection is rarely used because of the necessity for repetition and implants with slow release characteristics are much preferred. The use of removable implants is a development which has highly desirable features since the supply of anabolic compound can be cut off suddenly at will, as with oral administration, instead of a gradual decrease taking place in an uncontrolled manner as with the earlier types of implants or injection.

The first anabolic agent to be used on any scale commercially was diethylstilboestrol, a highly potent oestrogen, with a structural formula

different from those of the naturally occurring oestrogens. The linking of this material with the occurrence of cancer in certain circumstances[100] led to prohibition or restriction of the use of synthetic oestrogens as anabolic agents in certain countries, although in others they are still used quite freely. Some features of the legislation and the closely related public health aspects have been dealt with recently in specialised review articles.[101–104] The only problem in these areas which will be considered here is that of determining and assessing the implications of levels of anabolic compounds in animals treated with them as a basis for methods designed to control their use and misuse.

There is no doubt that, when applied correctly, compounds of the types listed earlier have anabolic effects on farm livestock. Literally hundreds of investigations have shown various degrees of improvement in liveweight gain and feed conversion efficiency from their use. For details of these investigations the reader is referred to excellent recent reviews on the subject.[94,105,106] As far as growth and feed conversion efficiency are concerned, the use of anabolic agents in castrate animals is effective in compensating for the deleterious effects of castration. Abnormalities of behaviour in treated animals are only occasionally referred to in these papers although in one of the earliest reports[107] treatment with diethylstilboestrol resulted in nymphomania and treatment with testosterone caused restlessness and nervousness in heifers. These problems have recently been referred to again[94] and it has been pointed out that new formulations and longer periods of administration may lead to further difficulties.

The mode of action of anabolic agents is at present a subject of great interest and of practical importance in the search for new compounds. The work has been aided by the development of sensitive and specific radioimmunoassays for hormones and the realisation of the dynamic nature of secretion of many hormones which has followed has led recently to much better-designed experiments on the hormonal effects of anabolic agents.

Although the question is by no means answered fully, much recent evidence suggests that at least some anabolic agents act by causing an increase in the secretion of growth hormone from the pituitary gland of the treated animal. A number of early studies, using various parameters of growth hormone activity, had suggested that there were increases in the secretion of this hormone following treatment with oestrogens (for references see Trenkle[105] and Davis and Borger[108]). Plasma insulin levels were also higher in treated animals (for references see Trenkle[105]), possibly

due to the increased growth hormone release. Recent well-designed studies, using radioimmunoassay for plasma growth hormone, confirm the suggestions from earlier work. In a study comparing entire rams with castrates, it was found that entire rams showed growth hormone secretory episodes of greater amplitude than the castrates, and their mean baseline and mean overall plasma growth hormone concentrations were higher. Treatment of the castrates with either testosterone propionate or diethylstilboestrol by repeated subcutaneous injection resulted in increased baseline and overall growth hormone concentrations.[109] In another recent study[110] plasma growth hormone and insulin levels were increased in castrate lambs treated with oestradiol-17β injected subcutaneously twice weekly. Both hormones returned to baseline levels within one week of cessation of the oestradiol treatment.

A number of the effects of oestrogen treatment of sheep, such as increased plasma insulin levels, decreased plasma urea nitrogen and amino acids and decreased urea nitrogen excretion, are also observed after administration of purified growth hormone to sheep (for references see Trenkle[105]). These data support the view that, in sheep at least (since some recent evidence in cattle seems to implicate increases in thyroid hormones[111]), the anabolic action of the oestrogens and testosterone is due to their effects on growth hormone secretion. Whether the testosterone effect is direct or occurs after aromatisation is not clear.[109]

The anabolic nature of oestrogens of various types appears to be linked to their oestrogenicity although the choice of assay animal used to assess oestrogenic activity may be very important in this context. Certain anabolic agents, including zeranol, which had no oestrogenicity in the mouse, showed signs of oestrogenicity in the sheep, in which species they were anabolic.[110] In view of the link between certain oestrogenic compounds and carcinogenesis, this is an area which requires further exploration and standardisation of procedures.

There is no doubt that the aspect of the use of anabolic agents which leads to the most debate is that of possible tissue residue formation and danger to the consumer arising from their ingestion. The presence or absence of detectable residues in the carcass of an animal treated with an anabolic compound depends on a variety of factors, including the means of administration, the withdrawal time before slaughter (if any), the metabolic clearance rate of the compound, and last, but by no means least, the sensitivity of the assay method used to test for its presence.

The qualitative and quantitative evaluation of tissue residues in animals treated with anabolic agents is fraught with difficulty, not only in the

development of the tools for the job in the form of specific and sensitive assays for the compounds concerned but in assessing the validity of the assay results from the point of view of legislation and consumer protection. Some of the problems arising from the prohibition of the use of all anabolic agents in some countries and the partial prohibition in others have recently been discussed.[112] The authors concluded in 1975 that methods of assessment for anabolic compounds were not sufficiently developed to allow efficient control where their use is permitted. Since that time further

TABLE 3

TESTOSTERONE IN FAT OF TREATED AND UNTREATED FEMALE CALVES 77 DAYS AFTER IMPLANTATION WITH 20 mg OESTRADIOL-17β + 200 mg TESTOSTERONE AND IN THE FAT OF UNTREATED MATURE BULLS (FROM REFERENCE 115)

Group	*No. of animals*	*Testosterone (ng/g) (mean ± SD)*
Control calves	5	0·18 ± 0·12
Treated calves	5	0·34 ± 0·26
Untreated mature bulls	5	11·05 ± 8·56

advances in methodology have been made, including the development of radioimmunoassays for the quantitation of the non-natural steroid, trenbolone acetate, and its major metabolites in bovine tissues and plasma.[113] In the case of the naturally occurring steroid hormones, information on levels in the blood and tissues of untreated animals and after administration as anabolic agents is accumulating,[114] as are data on catabolism.[110] It has become obvious in recent years that measurable—and, in some cases, quite high—levels of hormones are present in various products from untreated animals (e.g. testosterone in meat and fat,[115,116] oestrogens in muscle, fat, kidney and liver[117] and progesterone and testosterone in milk and dairy products.[116,118] Comparison of treated and untreated animals[115] has shown, with regard to testosterone, that the levels found in treated animals, although greater than those in comparable controls, may be substantially lower than those present in untreated animals of a different age and sex (see Table 3).

These and other similar results make it extremely difficult to judge, in the case of treatment with naturally occurring steroids, what are acceptable levels of hormone in the carcasses of treated animals. As has been pointed out,[115] such findings clearly make nonsense of the terms 'free of hormone'

or 'free of residues' where the endogenous steroids are concerned. This point has also been taken up in a recent report on the use of removeable silicone rubber implants containing oestradiol-17β.[110] Under the conditions of the experiments described, not only did extremely low doses of oestradiol-17β produce an anabolic response in cattle, but, when implants were removed, values for both oestradiol-17β, and for a metabolite oestrone, in kidney fat (the slowest tissue to clear these hormones) were

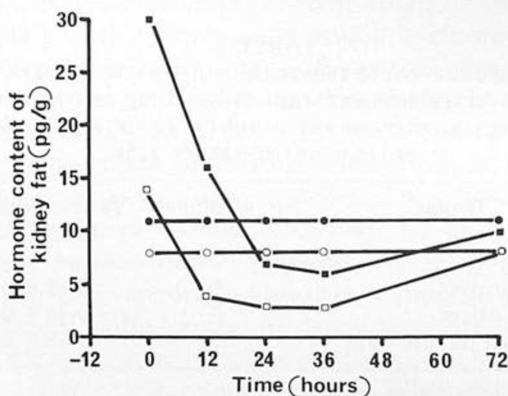


FIG. 2. Content of oestradiol-17β (open squares) and oestrone (closed squares) in kidney fat at intervals after removal of oestradiol-17β implants from cattle. Baseline values for untreated animals are indicated for oestradiol-17β (open circles) and oestrone (closed circles) (from reference 110).

below the established baseline, for the class of cattle concerned, during the period between 24 and 36 h after implant removal (see Fig. 2). It was suggested, therefore, that slaughter of oestradiol-treated animals within the period 24–36 h after implant removal could result in lower levels of oestrogen in edible tissues than would be encountered in those from untreated animals.

Much more work will be required on this subject before the complicated problems are solved. At least in the case of the administration of naturally occurring steroid hormones with anabolic activity, these are destroyed rapidly on ingestion so that levels of residue in treated animals, which are within the range recorded in untreated animals, are unlikely to represent an increased risk to the consumer. Some compounds with anabolic activity which do not occur naturally in the body, such as diethylstilboestrol, are resistant to enzymic degradation and are orally effective. Thus the presence

of residues of these materials in tissues presents a considerable problem. Questions of suitable forms of application, such as removable implants (which allow withdrawal periods to be timed from the cessation of treatment and not from its initiation) and the rigorous observation of statutory withdrawal periods, become very important. Whether the benefits arising from the increased efficiency of animals treated with anabolic agents outweight the potential problems which may arise from their use and misuse is a question on which research workers and public health authorities in different countries are far from unanimous at present.

Surgical gonadectomy is irreversible and has drastic effects on the levels of androgen and oestrogen to which the animal is exposed, leading to slower growth and poorer feed conversion efficiency. Gonadectomised animals often suffer quite a severe growth check immediately after the operation and infection of wounds is a common occurrence. If it were possible to suppress gonadal function by other means, gradation of effect might be achieved so that fertility, sexual aggression, other aspects of behaviour and, in pigs, the presence of sexual odour, could be inhibited whilst retaining sufficient circulating androgen and oestrogen to produce anabolic effects. Reversal of these effects might be possible in animals proving to be of particular merit during the growth period so that they could then be used for breeding.

Immunological approaches to these problems show promise and deserve consideration here for their novelty although no technique applicable in practice has yet emerged. Antibodies to hormones have played an increasingly important role in endocrinology in recent years, both in the detection and measurement of hormones in tissues and fluids and in the diagnosis and treatment of endocrine disorders. In the former case, radioimmunoassay is important in the present context because of its use in the detection of hormone residues. In the latter case, radioimmunoassays are now being used routinely in diagnosis and, in the treatment of endocrine disorders both in man and animals, the fact that antibodies may be developed against the hormones administered, and may partially or completely neutralise their effects, is a well-recognised problem. Immunological neutralisation of a particular hormone can also be used in the study of its rôle in the endocrine system.[119] The rather surprising fact is now well-documented that man and animals can produce antibodies against administered hormones identical to those which are normally present in the body even when these are administered without adjuvants[120] and indeed that, occasionally, untreated individuals spontaneously develop antibodies against their own hormones.[121] Against this background, it is important to consider whether hormone antibodies could have applications to meat

animal production and, in the present context, particularly to the development of a form of 'immunogonadectomy'.

With regard to the specific problem of taint in meat from boars, the possibility of producing active immunisation against the steroid concerned has been investigated.[122] A 3-carboxymethyloxime of the steroid was synthesised, bound to bovine serum albumin (BSA) and administered to boars with Freund's complete adjuvant (FCA). The object was to suppress 5α-androst-16-en-3-one selectively, leaving the testicular anabolic hormones unaffected; hence the cross-reaction of the antiserum obtained was important. Antibody titres were obtained with specificity characteristics of the antiserum which were favourable for this purpose, although difficulties were encountered in obtaining sufficiently high antibody titres to neutralise all of the 5α-androst-16-en-3-one produced. Repetitive immunisation was necessary in order to achieve satisfactory results and, in this form, the technique has proved too costly for practical application (R. Claus, pers. comm.).

In more general terms, for the production of castration-like effects, one could consider disrupting the endocrine control of the testis at testis level, at the level of the pituitary gland or at the level of the hypothalmus. The production of antibodies against the testicular sex steroid hormones, as with the boar taint steroid, is quite possible, and indeed forms the basis for the radioimmunoassay of these hormones. For the production of antibody the steroid (hapten) is usually linked to a carrier protein and, in this conjugated form, when administered with an adjuvant, is capable of consistently producing an immunological response. Immunisation of male rabbits with testosterone is followed by certain changes similar to those seen after castration, i.e. loss of libido, atrophy of the accessory structures of the reproductive tract and increased gonadotrophin release. Paradoxically, however, these effects are accompanied by hypertrophy and hyperfunction of the testis with serum testosterone levels 30 to 100 times greater than those of the controls. However, the biologically active free testosterone fraction is thought to be reduced.[123,124] A problem in raising antibodies to steroids is that steroids other than the one used as antigen may cross-react with the antibodies produced. Where this occurs with the adrenal hormones, e.g. corticosterone, undesirable effects on metabolism may occur.

Antibodies to the gonadotrophins are easily produced because of their high molecular weight and glycoprotein nature. Conjugation to a carrier protein is not necessary but an adjuvant is usually used to intensify the immunological response. Loss of libido,[125] and testicular atrophy with accompanying decline in the level of testosterone,[126] occur in animals

immunised with gonadotrophin. Thus, immunisation with gonadotrophin produces castration-like effects. The problems which are foreseeable in a possible practical application to meat-producing animals are several. First, the structures of the gonadotrophins of cattle, sheep and pigs are different, although there are various degrees of cross-reaction between them. Secondly, the gonadotrophins are not available in synthetic form due to the complex natures of their molecules, and preparations have to be extracted from pituitary gland collections. This is a highly specialised, laborious and expensive procedure from which there is a low yield of gonadotrophin.[8] The cost of producing purified pituitary gonadotrophin would be far too high for its use in such a practical application.

The structure of Gn–RH was elucidated in 1971[15] and the synthesis of its decapeptide molecule followed rapidly.[127] When used as a hapten linked to a carrier protein such as BSA, Gn–RH elicits the production of antibodies in both males and females of laboratory species.[128–131] In the male, the development of an antibody titre results in testicular atrophy with involution of the seminiferous tubules, suppression of testosterone secretion and atrophy of the accessory reproductive structures. In the female, reproductive cycles cease and luteal tissue disappears from the ovaries. Follicle development may continue in some animals, depending upon the degree of inhibition of Gn–RH.[131]

The results of these studies are encouraging. Long-term potential applications include non-surgical castration and suppression of unwanted oestrus and ovulation in domestic pets and bloodstock and the meat production applications described earlier. Early results in the meat-producing species confirm the findings in laboratory animals. In the sheep, Gn–RH conjugated to BSA and administered with FCA, suppressed oestrus and ovulation in cyclic ewes.[132,133] It would appear from this work that the complex of hormonal events leading to oestrus and ovulation is sensitive to even quite low titres of Gn–RH antibody and hence it is very simple to block cyclicity. More specifically, it was found that the degree of suppression of gonadotrophin levels was related to the antibody titre obtained, offering hope that graded effects might be possible. The finding[134] that the secretion of ovarian steroids was continued in similar circumstances reinforces this view. The results obtained to date suggest that immunisation against Gn–RH could, with some refinement of technique, provide an alternative to spaying for the suppression of unwanted oestrus and ovulation.[133]

In adult rams, and in ram lambs eight months of age at the start of treatment, immunisation with synthetic Gn–RH leads to the development

of antibody titres similar to those seen in ewes, and gradual testicular regression is produced. Libido is suppressed, fighting within groups is reduced and testosterone levels are lowered after the development of Gn–RH antibody titres (I. A. Jeffcoate and D. B. Crighton, unpublished observations). It remains to be seen whether the suppressive effects of immunisation on sexual activity and aggression can be obtained while retaining some of the anabolic effects of testicular steroids. One of the major obstacles to achieving this consistently may arise from the fact that the immune response system is genetically controlled, thereby leading to variability of response among individuals. So far, different antibody titres have been achieved by chance and not by design.

The question of reversibility of the effects of immunisation has also been examined in a preliminary manner in the ewe.[133] An analogue of Gn–RH, [D–Ser(Bu^t)^6] Des Gly NH_2^{10} Gn–RH ethylamide, was shown not to cross-react with Gn–RH antisera from any of the ewes. This analogue has potent gonadotrophin-releasing properties in the sheep.[16,135] When administered as a single low dose injection to ewes which were acyclic as a result of immunisation, however, the gonadotrophin release obtained was small. It is probable that more complex therapy would be necessary for reversal of the effects of immunisation if this was required while titres of antibody were high. Since reversal would be an occasional, rather than a routine, procedure in practice, such a complex technique might be acceptable in this case. It is possible that reversal may take place naturally in time with a fall in antibody titre in the absence of 'boosting' injections and this is under investigation (I. A. Jeffcoate and D. B. Crighton, unpublished work).

One other aspect of this work deserves mention. The adjuvant used almost universally is FCA. While extremely effective, this adjuvant always causes tissue destruction since complement-fixing antibodies of the IgM and IgG classes are stimulated. These antibodies are not required for the neutralisation of hormone activity and only lead to the adverse side-effects seen.[136] The development of severe tissue reactions which may lead to the formation of abcesses under farm conditions is highly undesirable and a search for other adjuvants which are effective when used with Gn–RH, but do not produce these effects, should be a priority.

4. SUMMARY AND CONCLUSIONS

While many endocrine techniques for controlling reproduction in the meat-producing species have been devised or are in the course of development,

relatively few have found a place on the farm so far. Where background knowledge of the particular area of reproductive endocrinology concerned has been good and a simple and reasonably cheap hormonal treatment has resulted from research, it has been taken up enthusiastically by the industry. An example is the use of prostaglandins for inducing luteal regression and synchronising ovulation in cattle, a technique which, applied by the veterinary surgeon on the farm, has widened the use of artificial insemination and made a significant contribution to meat production in the short time since its development.

Several techniques not yet in widespread use have considerable potential. Ovum transfer after hormonal superovulation in cattle has already been used in meat production in transporting valuable genetic material from one part of the world to another. The perfection of non-surgical collection and transfer techniques and methods for the long-term storage of fertilised ova will probably lead to a much wider application in the industry in future. Organisation of specialist centres, similar to those used by artificial insemination services, rather than local veterinary application, seems likely in this case. Extension to the sheep of ovulation synchronisation by means of prostaglandins is likely, although the development of effective semen storage techniques will be necessary before the full potential of artificial insemination can be realised in this species.

There are certain physiological states, such as the lactational anoestrus of the sow and the combination of seasonal and post-partum anoestrus in the ewe, in which the potential for intervention with hormones is great but knowledge of the endocrine background is still relatively poor. In such cases, continued basic research is essential if sound practical techniques for improving the efficiency of reproduction of the meat-producing species are to be developed eventually.

The recognition that the sex of an animal is a major factor in determining its pattern of growth and development, and, in meat-producing animals, the form and composition of the carcass, has led to detailed investigation of the effects on these aspects of gonadectomy, a production technique which was employed originally for reasons of ease of management of gonadectomised animals, particularly castrates. The findings that intact males grow faster, have a greater efficiency of feed conversion and deposit less fat in the body than castrates led to attempts to mimic these characteristics of intact males without losing the management advantages by treating castrates with anabolic agents. The use of these agents was rapidly extended to intact males and females.

Those anabolic agents which are used in practice at present are generally

similar in activity to the gonadal steroid hormones, indeed these hormones themselves are often employed. Their anabolic effects in meat-producing species have been demonstrated in hundreds of experiments but their detailed mode of action is only now coming under investigation. It is suggested that some at least act by causing an increase in the secretion of growth hormone from the pituitary gland of the treated animal. The aspect of the use of anabolic agents which has led to the most debate is that of possible tissue residue formation and danger to the consumer from their ingestion. The linking of certain anabolic agents with carcinogenesis in particular circumstances has led to complete prohibition of their use in some countries. Techniques for the detection and accurate measurement of tissue residues in animals treated with anabolic agents are now being developed and knowledge is accumulating on the levels of endogenous hormones present in the blood and edible tissues of untreated animals—a necessary requirement for the drafting of legislation designed to control the use of naturally occurring anabolics.

Immunological alternatives to surgical gonadectomy for meat-producing animals are under investigation. Active immunisation of animals against synthetic gonadotrophin-releasing hormone leads to gonadectomy-like effects which appear graded, depending on the antibody titre developed, and are potentially reversible. While the practical applicability of such techniques remains to be demonstrated, they could have a considerable impact on meat production in the future, both from the point of view of the genetics and reproduction of breeding stock and from that of the growth and development of meat-producing animals.

5. REFERENCES

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CHAPTER 4. METHODOLOGY

Development and application of biological and radioimmunological assays for hormones.

(See also papers 7, 17 and 21)

Papers in scientific journals (refereed)

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BREEDING MICE FOR THE BIOLOGICAL ASSAY OF FOLLICLE-STIMULATING HORMONE

D. B. Crighton

BREEDING MICE FOR THE BIOLOGICAL ASSAY OF FOLLICLE-STIMULATING HORMONE

D. B. Crighton

In the female mammal, the follicle-stimulating hormone (FSH) secreted by the anterior pituitary gland is responsible for the growth of ovarian follicles and their preparation for ovulation. Measurements of the levels of FSH in the anterior pituitary glands of experimental animals can provide valuable information about reproduction. Such measurements are being made in some of our current experiments.

It is possible to measure hormone levels in glands and body fluids by three types of procedure:— biological assay, chemical assay and immunological assay. Up to the present, techniques have not advanced sufficiently to enable the assay of FSH accurately by any but the first type of procedure. This is largely because the structure and properties of the FSH molecule have not yet been elucidated.

In a biological assay, animals are used as test objects in which some kind of reaction, known as the response, is observed. The response must be a specific effect of the hormone concerned. In the case of FSH, a number of biological assay procedures were available when this work began in 1962, but most possessed disadvantages in the form of complicated techniques or lack of specificity or sensitivity. One procedure which did not seem to possess such disadvantages was that described by Steelman and Pohley[1] and later modified by Brown[2]. The assay depends on the increase in weight of the ovaries caused in immature rats (Steelman and Pohley) or immature mice (Brown) by the injection of FSH when the animals are concurrently given a fixed augmenting dose of human chorionic gonadotrophin (HCG). Separate groups of test animals are injected with suitable doses of the 'unknown' material or of a standard 'pure' FSH preparation, each augmented with the chosen fixed dose of HCG. One group, injected with HCG alone, acts as the control. The usual number of animals employed per group is five to nine. The immature test animals are placed on the assay at 21 days of age and are selected within a narrow weight range (in the case of mice, 8.0 to 10.0 g.). The doses are given in five subcutaneous injections over three days and the test animals are autopsied 24 hours after the last injection. The ovaries are then removed, cleaned and weighed in pairs and the increases in mean total ovarian weight produced by each dose when compared with the control are taken as the responses. These are then subjected to a statistical analysis, from which a potency for the 'unknown' in terms of the standard can be calculated.

It was decided to set up a colony of mice and to carry out preliminary work to find a suitable type of animal with which this assay procedure could be carried out under local conditions.

The requirements of a strain or cross of mice suitable for producing assay animals are as follows: the strain or cross must be productive, i.e. fertile with a long reproductive life; the young should be healthy and should have a rapid and uniform growth rate. The two factors of most importance in determining the precision of the assay procedure itself are the steepness of the slope of the dose-response line and the variation in response of the animals. The greater the former and the smaller the latter, the better will be the precision of the assay.

A considerable and well-established disadvantage of inbred lines is the decline in vigour often associated with inbreeding. This is manifested as a decline in fertility, fecundity and health. It has been known for many years that F_1 hybrids between inbred lines are characterised by high vigour. It seemed certain, therefore, that the requirements with regard to productivity mentioned above would be met by the use of F_1 hybrids.

As far as the precision of assay procedures is concerned, there is a certain amount of information in the literature comparing F_1 hybrids with inbred lines. It was found[3] that when two oestrogens were administered by two routes, the dose-response curves of F_1 hybrids of the C57 and CBA strains had steeper slopes than those of the inbreds in each case. With regard to variation in response, more evidence has accumulated. The action of oestrone on the CBA strain of mice was studied[4] and it was found, surprisingly, that individuals of this strain were significantly more variable in response than those of the randomly-bred colonies normally used at the time. This was the first pointer to the possible inadvisability of using inbred strains of mice for biological assay purposes. Grüneberg[5] concluded that there is evidence that pure lines of mice are much less homogeneous genetically than is widely believed. Grüneberg's work dealt with morphological variation, but McLaren and Michie[6] held that the same genetic rules should apply also to the "physiological" characters measured in biological assay. These workers obtained information to show that, for the response they measured (duration of narcosis in response to sodium pentobarbital) the variability of inbreds of the C57BL strain was 3.5 times greater than that of F_1 hybrids produced by crossing the C57BL strain with the C3H strain. This finding was confirmed[3] in C57 and CBA mice using the vaginal response to oestrone and oestradiol-17β. In this work, the hybrids of these two inbred lines had 25% of the variability of the inbreds.

For the reasons referred to above, it seemed likely that crossbred mice would be more suitable than inbred mice for FSH biological assay work. Accordingly, three samples of inbred strains were obtained for crossing purposes. These were the albino strain A and the wild-type strains C3HF and CBA. The following crosses were made and the progeny were used for assay purposes, while suitable stocks of the inbred strains were kept for breeding replacements:

A x C3HF — cross 1
C3HF x A — cross 2
A x CBA — cross 3
CBA x A — cross 4

In each case, the female is quoted first.

Records were kept on the breeding performance of the inbred strains within and between strains over a representative number of litters and on the performance of the crosses as assay animals over a representative number of assays.

BREEDING RESULTS

Inbred Strains

To provide replacement inbred animals for the production of crossbreds, small numbers of males and females from each inbred strain were placed on a *post-partum* mating system, two females caged with one male (later one female to one male). From the records, the following general conclusions can be drawn:—

The females of the A strain proved to be very fertile, although in the period immediately after the establishment of the colony, they tended to become sterile rather early in life. The males at this time also tended to become sterile early but improved as the strain settled down. The average number of young born per litter was 6.3.

In the C3HF strain, both the males and females were very fertile with as long a breeding life as those of the A strain. The average number of young born per litter was 5.8.

In the CBA strain, both males and females were often sterile. The average number of young born per litter was 6.5.

To produce the crossbred animals for assay, the males and females were placed on a different mating system to that previously described. The ratio of females to males was again 2: 1 but in this case the males were left with the females for seven days only. This regime ensured the birth of the young over a short period and provided successive groups of assay animals of a reasonably constant age.

The single factor considered in comparing the crosses as producers of assay animals was the number of potential assay animals (i.e. females raised to 21 days old) produced per female mated. This criterion embraced fertility of both males and females, fecundity, rearing ability of females and viability of young. Information on this criterion was kept over three litters from each strain, i.e. over a period of approximately 18 weeks, and using from 50 to 100 breeding females from each strain. The following results were obtained:—

Cross 1 — 2.16 females per female mated
Cross 2 — 1.31 females per female mated
Cross 3 — 1.57 females per female mated
Cross 4 — 0.97 females per female mated

ASSAY RESULTS

It was necessary for reasons of continuity within the experiments of individual workers to allocate an individual cross to each of several research projects during the period of investigation, and hence the results quoted below were not all obtained under identical circumstances as far as the persons carrying out the procedures are concerned. All animals were, however, subjected to the injection and autopsy regimes described above and the measurements taken should have provided good indices of their suitability for assay purposes.

The variability of the ovarian weights produced in the young of each cross were examined in response to:—

(a) a standard dose of 40 i.u. of HCG;

(b) doses of standard 'pure' preparations of FSH (NIH-FSH-S1; NIH-FSH-S2) augmented with the standard dose of 40 i.u. of HCG. The doses of FSH used were 25μg. and 75μg. (except where otherwise stated). These procedures were carried out over a series of three assays using each cross. The results are shown in Table 1.

It was necessary, in deciding on the most suitable cross for assay purposes, to take into account both performance in breeding and in assays. It was, however, obvious at the outset of the breeding programme that because of the large number of workers requiring assay animals, the productivity of the two inbred strains mated to produce any cross would be the factor of overriding importance, subject to the cross producing reasonable assay results. In breeding, the CBA females gave markedly the worst results over the period observed and regardless of the assay results obtained, these had to be discarded on grounds of poor productivity. Similarly, the males of this strain showed premature sterility

TABLE I

DOSE—RESPONSE RESULTS FROM FOUR F_1 HYBRIDS

Cross No.	NIH-FSH Prep. used	Ovarian weights (mg.)		
		Control Mean±S.E.	25 μg. NIH-FSH Mean±S.E.	75 μg. NIH-FSH Mean±S.E.
1	S_2	4.6 ± 0.2	5.5 ± 0.3	7.9 ± 0.6
	S_2	5.6 ± 0.3	6.4 ± 0.3	8.4 ± 0.3
	S_2	4.8 ± 0.2	5.8 ± 0.4	8.2 ± 0.5
2	S_1	4.4 ± 0.2	5.5 ± 0.1	8.7 ± 0.6
	S_1	5.2 ± 0.5	7.3 ± 0.5	9.4 ± 1.1
	S_1	4.8 ± 0.5	6.1 ± 0.7	8.9 ± 0.3
3	—	4.9 ± 0.5	not tested	not tested
	—	4.5 ± 0.4	not tested	not tested
	S_2	4.4 ± 0.4	5.7 ± 0.4*	9.6 ± 0.7**
4	S_2	5.3 ± 0.3	6.4 ± 0.5	7.3 ± 0.7
	S_2	5.1 ± 0.3	6.2 ± 0.5	7.8 ± 0.5
	S_2	5.2 ± 0.3	6.4 ± 0.3	8.1 ± 0.5

*=20 μg. NIH-FSH injected

**=80 μg. NIH-FSH injected

and were undesirable for crossing. Of the others, the C3HF females had as long a breeding life as those of the A strain but on the basis of crossing results, the A females showed to best advantage. The most productive cross was that between A females and C3HF males, more assay females being reared to three weeks of age by each female mated. This cross also produced assay results better in terms of variability than any of the other crosses (Table 1). This is emphasised when the root-mean-square standard error is expressed as a percentage of the mean ovarian weight for each cross: i.e.

Cross 1 — 1.9%

Cross 2 — 2.9%

Cross 3 — 3.8%

Cross 4 — 2.4%

It has been decided on the basis of the above evidence that the cross between A females and C3HF males (cross 1) provides, under our conditions a suitable type of animal for the assay of FSH by the augmentation method. The present breeding policy is to maintain stocks of these parent strains and to breed the above cross on a large scale using a *post-partum* mating system. This provides a constant supply of young female mice from which groups of assay animals can be selected when required for any of the various research projects. Assays can thus be carried out immediately pituitary material is collected which obviates storage for variable periods prior to assay.

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DEPLETION OF RAT OVARIAN ASCORBIC ACID BY A FACTOR OTHER THAN LUTEINIZING HORMONE PRESENT IN THE BLOOD OF THE PIG

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The ovarian ascorbic acid depletion (OAAD) assay of Parlow (1958) is an established method for the measurement of luteinizing hormone (LH). Tests using purified preparations of other anterior pituitary hormones have shown the bio-assay to be specific for LH (McCann & Taleisnik, 1960; Schmidt-Elmendorff & Loraine, 1962) but vasopressin possesses appreciable activity (McCann & Taleisnik, 1960). Pelletier (1964) has reported that hypophysectomized ewe plasma caused a significant response when assayed by this method. Evidence has been produced suggesting that the plasma acted by releasing LH from the pituitary glands of the assay rats (Pelletier, 1965).

Recently, determinations of the pituitary content of LH in the sow at various reproductive stages and after ovariectomy have been carried out in this laboratory. A preliminary report of this work has already been presented (Crighton, 1967). In the course of this work, serum was obtained from the jugular venous effluent at the slaughter of each sow.

After storage for variable periods at $-20°$ C the samples of serum were tested individually and concurrently for LH activity using the OAAD method. The procedure used was a modification of that described by Schmidt-Elmendorff & Loraine (1962). Each rat was anaesthetized with an intraperitoneal injection of sodium pentobarbitone (Veterinary Nembutal Solution, Abbott Laboratories Ltd). One ovary was then removed using the dorsal approach. The test sample was immediately injected into the femoral vein. Each rat was killed 1 hr$\pm$3 min after injection and the second ovary was removed. Each sample of serum was injected into six rats, each rat receiving a volume of 2·0 ml. In addition, ten rats were each injected with 2·0 ml of 0·9% NaCl. The ascorbic acid concentrations of the two ovaries were then determined and the results were expressed as the percentage OAAD produced in the treated ovary (removed at autopsy) compared with the control ovary (removed before injection). The mean percentage OAAD for each sample was calculated. The significance of the depletion produced by each sample of serum was tested using the '*t*'-test and the signifi-

cance of the differences between the groups in their ability to produce OAAD was tested by analysis of variance. The groups of sows and the group mean results are shown in Table 1.

Table 1

OAAD PRODUCED BY SERUM OF EXPERIMENTAL SOWS

Group	*No. of sows*	*Reproductive status at slaughter*	*Mean OAAD produced by 2·0 ml serum (% ± S.E.)*
1	3	Lactating 45 to 46 days, anoestrous	44·8 ± 3·6
2	3	Lactating 45 to 46 days, ovariectomized 25 days previously	48·9 ± 10·4
3	4	Cycling, Day 5 of oestrous cycle	48·1 ± 2·0
4	4	Ovariectomized 25 days previously on Day 5 of oestrous cycle	50·8 ± 5·2

The injection of isotonic saline produced a non-significant increase in ovarian ascorbic acid of 1·1%. All samples of serum produced significant OAAD ($P<0·001$ in each case). There were no significant differences between the groups in the mean depletions produced.

It seemed obvious that the depletions did not represent the levels of LH present in the blood of the sows in life. In order to determine whether the observed depletions were due to LH or to some other factor, two experiments were designed.

Table 2

OAAD PRODUCED BY PURIFIED LH AND SOW SERUM INCUBATED IN THE PRESENCE AND ABSENCE OF 6 M-UREA

Sample	*Mean OAAD produced by 1·0 ml solution (% ± S.E.)*
NIH-LH	29·0 ± 3·3
NIH-LH + urea	13·0 ± 3·6
Serum	29·4 ± 3·6
Serum + urea	30·0 ± 2·2

In the first experiment, a sample of sow serum previously demonstrated to produce OAAD was incubated under suitable conditions with 6 M-urea. Such incubation has been shown to destroy the LH activity of purified preparations of LH alone and when present as a component in a mixture of other gonadotrophic hormones and the LH activity of such preparations as Pergonal, PMSG and HCG (Ellis, 1961; Schmidt-Elmendorff, Loraine & Bell, 1962). The effect of 6 M-urea on sow serum was compared with its effects on a purified preparation of LH (NIH-LH-S9; National Institutes of Health, U.S.A.).

Before this experiment, preliminary investigations were carried out in order to determine doses of serum and NIH-LH required to produce comparable depletions. The purified preparation of LH was dissolved in 0·9% NaCl to provide a concentration of 10·0 μg/ml. Two 25·0 ml aliquots of this solution were taken and urea added to one to provide a concentration of 6 M. Similarly, two 25·0-ml aliquots of serum were taken and urea was added to one to provide a concentration of 6 M. All four samples were incubated in 100 ml conical flasks in a water bath at $40 \pm 1°$ C for 24 hr. After incubation the samples were dialysed against 0·9% NaCl at 3° C for 24 hr. They were then assayed using the method as before, 1·0 ml of solution being injected into each rat and ten rats being used for each sample. The results are shown in Table 2. The incubation of purified LH with 6 M-urea resulted in a significant decrease ($P < 0·01$) in the OAAD produced, whereas similar treatment of sow serum did not affect its activity.

For the second of these experiments, a sample of hypophysectomized gilt plasma was obtained. The plasma was transported from the U.S.A. in the frozen state and was stored at −20° C until required for assay. The assay method was as described previously. The dosage per rat of the gilt plasma was 1·0 ml. Two replicates of the experiment were carried out, using ten rats in each. In both replicates of the experiment, the plasma produced a significant OAAD ($P < 0·01$ in both cases). The mean percentage depletions with S.E. produced were $25·1 \pm 3·6$ and $24·1 \pm 5·0$ in the first and second replicates respectively.

The results of these experiments provide evidence that the OAAD produced by the serum of the experimental sows was not due to LH or to any other hormone of pituitary origin. They demonstrate that the OAAD method is unsuitable for the assay of the unextracted serum or plasma of the pig and hence agree with the results of Pelletier (1964) in the sheep.

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A STUDY OF THE ANTIHORMONAL ACTIVITY OF AN ANTISERUM TO OVINE PROLACTIN USING THE LOCAL LACTOGENIC RESPONSE IN THE RABBIT

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SUMMARY

A rabbit antiserum to a purified preparation of ovine prolactin was prepared. The capacity of the antiserum to counter the biological effect of the preparation of ovine prolactin was determined. When injected intraductally before the injection of prolactin into the same duct the antiserum inhibited the lactogenic effect of prolactin on the rabbit mammary gland. The weight, nitrogen content and reducing sugar content of the mammary glands were used as criteria to judge the effect of the antiserum. The results of specificity tests on the antiserum, using the techniques of double diffusion and immunoelectrophoresis, are also reported.

INTRODUCTION

The capacity of prolactin to elicit antibody formation has been demonstrated by Kabak & Stulova (1937), Kabak (1938), Young (1938), Strangeways (1938), Rowlands & Young (1939), Bischoff & Lyons (1939), Levy & Sampliner (1961, 1962) and Emmart, Bates, Condliffe & Turner (1963). Hayashida (1962) used the pigeon-crop sac assay of Riddle, Bates & Dykshorn (1933) and showed that an antiserum to ovine prolactin, when injected concurrently with prolactin caused inhibition of the effects of the latter.

Lyons (1942) observed that the rabbit responds with lactogenesis in the injected sector of the mammary gland after the intraductal injection of preparations rich in prolactin. This observation was confirmed by Meites & Turner (1947) and by Bradley & Clarke (1956). The latter workers adapted the technique for the quantitative estimation of prolactin, using the total reducing sugar content of the mammary gland as the criterion of response. Chadwick (1962, 1963) investigated the factors which affect the local lactogenic response of the rabbit to prolactin and found that there were distinct breed differences. He also devised a method for the separate estimation of the lactose content of the mammary gland in preference to the estimation of total reducing sugar.

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In investigations concerned with the development of an immunological assay procedure for prolactin, it became necessary to demonstrate the antihormonal activity of an antiserum to prolactin. To date, we have seen no studies in which the antihormonal activity of an antiserum to prolactin has been evaluated by means of a mammalian lactogenic test. For these reasons, a procedure was designed to use the local lactogenic response in the rabbit to study the ability of an antiserum to ovine prolactin to inhibit the biological activity of a purified preparation of ovine prolactin.

MATERIALS AND METHODS

Preparation of antiserum

Animals. The antiserum to ovine prolactin was prepared in adult albino rabbits of mixed New Zealand–Dutch stock weighing approximately 3–4 kg.

Antigen. Three preparations were employed as antigen. These were ovine hypophysial preparations (NIH-P-S 4, NIH-P-S 5 and NIH-P-S 6) containing respectively 21·0, 17·0 and 24·8 i.u. prolactin/mg. as measured by the pigeon-crop sac assay.

Adjuvant. The quantity of ovine prolactin required for injection was adsorbed on to an equal amount (w/w) of bentonite (British Drug Houses) in 1·0 ml. of 0·9% NaCl solution. The material was administered by the intradermal, subcutaneous and intravenous routes in various combinations (Table 1).

Table 1. *Raising of antiserum samples and resultant titres*

Rabbit no.	Doses of prolactin (mg.)	Routes of administration	Schedule of injections and bleeding	Haem-agglutination titre
1	0·5 1·0 (booster)	Intradermal (foot pad) Intravenous (ear vein)	Once per week for 3 weeks. Booster dose in 4th week. Schedule repeated 4 times. Bleeding 10 days after last booster	1:10,000
2	0·5	Intravenous (ear vein)	Once per week for 14 weeks. Bleeding 10 days after last injection	1:620
3	0·5 0·5 0·5 (booster)	Intradermal (foot pad) Subcutaneous (chest wall) Intravenous	Once per week for 4 weeks, followed by s.c. injection once per week for 5 weeks. Booster dose 7 days after last s.c. injection. Bleeding 10 days after booster. Schedule repeated from 1st s.c. injection onwards as blood required	1:20,000

Antibody titres. The antibody titres of the antiserum samples were measured by the haemagglutination technique of Boyden (1951), using the modification of Butt, Crooke & Cunningham (1961) in which sheep erythrocytes stabilized with pyruvic aldehyde after the method of Ling (1960) are employed. Antiserum samples with high antibody titres were pooled and stored in 1·0 ml. glass ampoules at −15° until required.

Specificity tests. The double diffusion technique of Ouchterlony (1953) and the immunoelectrophoresis test of Scheidegger (1955) were employed to determine the specificity of the antiserum and the homogeneity of the antigen.

Preparation of rabbits for local lactogenic response assay

Animals. The rabbits used were albino virgin females, 9–12 months old, of mixed New Zealand–Dutch stock, bred in a closed colony. They were maintained in a controlled environment, at $65 \pm 5°$ F and a schedule of 12 hr. light and 12 hr. darkness. Pseudopregnancy was induced by a single intravenous injection of 50 i.u. of human chorionic gonadotrophin (Lutormone, Burroughs Wellcome and Co.) dissolved in 2·0 ml. 0·9 % NaCl solution.

Intraductal injections. The intraductal injections of prolactin and the antiserum were made on the 11th day of pseudopregnancy and the rabbits were killed by dislocation of the neck on the 7th day after the injections. The methods of anaesthesia and preparation of the rabbits were those of Chadwick (1963). In the rabbit there are four pairs of mammary glands which cover almost the whole of the thorax and abdomen. The thoracic pair were employed as uninjected controls throughout. Of the remaining three pairs, the glands on the left were each injected with 0·15 ml. undiluted and untreated antiserum, while those on the right were injected similarly with the same amount of normal rabbit serum (Plate). In each case only one sector of each gland was injected and the injected ducts were marked with a permanent dye to facilitate identification for the subsequent injections of prolactin. After 45–60 min., the ovine prolactin was injected dissolved in 0·15 ml. saline. The injections were made into the ducts which had been injected previously with antiserum or normal rabbit serum, the same dose of prolactin being administered to both glands of any one pair (Plate). Each gland was massaged gently after injection with the duct occluded in order to distribute the material and prevent leakage.

Doses of prolactin providing clear lactogenic responses were selected after preliminary experiments. Doses of 25, 50 and 75 μg. were found to be suitable. Since the inguinal mammary glands in the rabbit are always smaller than the others, the experimental design was such that in each repetition of the experiment the paired doses of prolactin were administered to different pairs of mammary glands. Three replicates were carried out of each combination, thus a total of nine rabbits was used for the experiment.

Removal of mammary glands and chemical measurements

The mammary glands were exposed by cutting the skin on the mid-line and dissecting away the two sides. After visual examination, the mammary glands were removed together and photographed. Each mammary gland was then dissected out carefully, so as not to damage the sectors filled with milk. The whole glands were weighed individually and stored at $-15°$ for 1–2 hr., before being divided into small pieces and homogenized in 5·0 ml. of de-ionized water in a top-drive blender (Folley & Watson, 1948). An 0·5 ml. sample of the homogenate was de-proteinized with 5 % zinc sulphate and 0·3 N-barium hydroxide (Nelson, 1944). From the supernatant, an aliquot of 2·0 ml. was taken for the estimation of the total reducing sugar against a glucose standard, using the method of Somogyi (1945). A further 1·0 ml. sample of the original homogenate was used for the estimation of the total nitrogen content by a micro-Kjeldahl technique (Sreenivasan & Sadasivan, 1939; Redemann, 1939; Bradstreet, 1954).

RESULTS

Antibody titres

All rabbits used for raising the antiserum lacked demonstrable antibodies to ovine prolactin before immunization. Quantitative haemagglutination assays showed that two rabbits had developed particularly high titres of the antibodies against ovine prolactin. Those antiserum samples which gave complete agglutination at dilutions of 1:10,000 to 1:20,000 were used.

Specificity tests

The immuno-diffusion results obtained using the double diffusion technique and immuno-electrophoresis demonstrated that the rabbit antiserum to ovine prolactin (NIH-P-S4) cross-reacted with both the ovine prolactin preparations used (NIH-P-S4; NIH-P-S5) forming broad single precipitin lines. These lines were confluent, showing the immunological identity of the two preparations. A single precipitin line was also observed as a result of reaction between the antiserum to ovine prolactin and a sheep pituitary extract obtained by dissolving the powdered gland in saline. This precipitin line was confluent with that of the purified prolactin, thus establishing the immunological identity of the two antigens. No precipitin lines were observed between the antiserum to ovine prolactin and ovine follicle-stimulating hormone (NIH-FSH-S2) or ovine luteinizing hormone (NIH-LH-S7).

The antiserum to ovine prolactin also cross-reacted with sheep serum, forming a faint precipitin line at dilutions of 1:32 and 1:64 only. This precipitin line was not confluent with the precipitin line of the purified prolactin, thus demonstrating lack of immunological identity with the purified prolactin. The faint precipitin line demonstrated a low titre of antibodies, probably against sheep serum protein.

Effect of the antiserum on the lactogenic response to prolactin

Occasionally, a small quantity of milk was observed in the ducts of mammary glands treated with the antiserum. When this occurred, traces of milk were also found in the uninjected control thoracic glands, and this effect was therefore assumed to be due to endogenous prolactin. Visible milk secretion in response to ovine prolactin was completely inhibited in mammary glands treated with the antiserum. In contrast, lactogenesis was clearly apparent in those mammary glands which were treated with normal rabbit serum and prolactin (Plate).

In demonstrating the antihormonal effect of the antiserum to ovine prolactin, it was thought adequate to use the criteria of weight, nitrogen content and total reducing sugar content of the mammary glands. The technique of Chadwick (1962) for determining the lactose content of the mammary glands is rather time-consuming and is more suitable for use in quantitative assays.

The results of the analysis of variance of the above criteria are summarized in Table 2. The antiserum produced a highly significant depressant effect ($P < 0{\cdot}01$) on the response to prolactin when judged by any of the three criteria used. Increasing the dose level of prolactin over the range employed produced significant increases in response ($P < 0{\cdot}05$) as judged from changes of weight and nitrogen content and a

Table 2. *Mean effects of antiserum on assay criteria*

	Controls* (means ± S.E.)	Prolactin dose levels (μg.)† With antiserum			Without antiserum				With antiserum v. without antiserum	Effect of increasing dose
		25	50	75	25	50	75	± S.E.		
Mammary gland weight (g.)	1·57 ± 0·11	2·62	2·46	3·23	3·20	3·87	5·18	± 0·25	$P < 0{\cdot}01$	$P < 0{\cdot}05$
Total nitrogen content (mg.)	9·64 ± 0·51	13·87	15·06	17·02	20·18	25·06	29·80	± 1·48	$P < 0{\cdot}01$	$P < 0{\cdot}05$
Total reducing sugar content (mg.)	1·90 ± 0·18	3·09	3·59	4·63	5·02	9·31	12·83	± 0·63	$P < 0{\cdot}01$	$P < 0{\cdot}01$

* Each figure is the mean of 16 observations (two values missing).

† Each figure is the mean of three observations. The S.E. is calculated for all observations on injected animals.

highly significant increase ($P < 0{\cdot}01$) in the case of total reducing sugar content. The variation between rabbits was less in the case of measurements of total reducing sugar than with the other two criteria.

DISCUSSION

The present study demonstrates that the rabbit antiserum prepared against ovine prolactin inhibits one biological effect of purified ovine prolactin, namely lactogenesis. The results show that 0·15 ml. antiserum neutralized 50 μg. or more of ovine prolactin (NIH-P-S5). Although visible milk secretion in response to ovine prolactin was inhibited at all three dose levels of prolactin, the weight, nitrogen content and total reducing sugar content of mammary glands treated with the antiserum were higher after 75 μg. prolactin than at lower doses. This may indicate a lactogenic effect of non-neutralized prolactin.

In spite of the careful selection of rabbits uniform with regard to age and body weight, considerable variation was encountered between rabbits in their response to prolactin. This variation was more marked when the criteria of weight and nitrogen content were considered than in the case of estimations of total reducing sugars.

The exact mechanism of the antigen-antibody reaction in the injected sectors of the mammary glands is not clear. Presumably, the conditions within the ducts and alveoli were favourable for the reaction. The lactogenic response to prolactin was so marked on the right side of the rabbits (i.e. the side without the antiserum) that there could have been no appreciable diffusion of the antiserum across the epithelium of the alveoli to the adjacent tissues, nor could significant amounts have been present in the general circulation.

The results obtained show that the antiserum to purified ovine prolactin (NIH-P-S4) was effective in inhibiting the biological effect of prolactin, as judged by the local lactogenic response in the rabbit.

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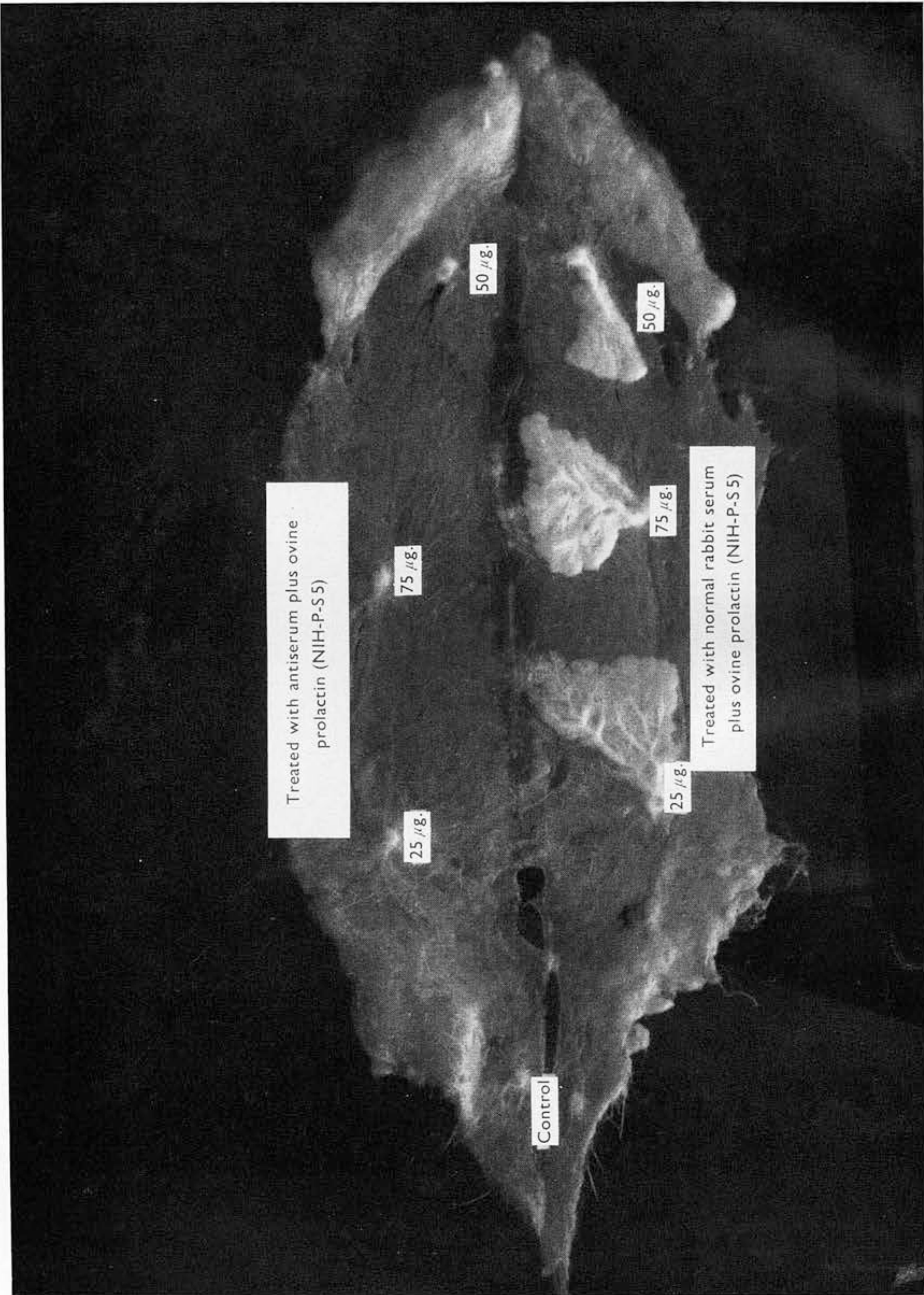
50 μg.
50 μg.
Treated with antiserum plus ovine prolactin (NIH-P-S 5)
75 μg.
75 μg.
Treated with normal rabbit serum plus ovine prolactin (NIH-P-S 5)
25 μg.
25 μg.
Control

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DESCRIPTION OF PLATE

Mammary gland area of a rabbit after dissection, showing uninjected control glands (thoracic pair), the lactogenic response to graded doses of prolactin (three abdominal glands) and the inhibition of this response by prolactin antiserum (contralateral abdominal glands).

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THE SPECIFICITY OF AN OVINE PITUITARY INCUBATION SYSTEM LINKED WITH GONADOTROPHIN ASSAYS FOR DETECTING GONADOTROPHIN RELEASING ACTIVITY

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SUMMARY

A sheep pituitary incubation system was developed which may be used to study the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as assayed biologically. Specificity was examined by adding at high concentration various materials present in the hypothalamus, a crude acid extract of sheep cerebral cortex or crude or partly purified extracts of ovine hypothalamic tissue.

Of the materials tested for LH and FSH releasing activity, oxytocin, adrenaline, synthetic lysine vasopressin and cerebral cortex extract failed to influence LH or FSH release. Natural vasopressin containing unknown proportions of the lysine and arginine forms did not affect FSH release but produced a significant increase in the LH content of the medium, the reason for which was not clear. Noradrenaline failed to influence FSH release but produced an apparent depression in the LH content of the medium due to an action on the LH released or upon the ovarian ascorbic acid depletion assay since the addition of noradrenaline to standard ovine LH reduced the potency in the assay to an almost identical degree.

Extracts of ovine hypothalamic tissue consistently increased the LH and FSH content of the medium.

The method described may be applicable to the detection and estimation of LH and FSH releasing activity in ovine hypothalamic tissue extracts and to detecting changes in the responsiveness to hypothalamic stimulation of sheep pituitary tissue.

INTRODUCTION

In the sheep, information is available concerning pituitary gland luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations (Robertson & Hutchinson, 1962; Robertson & Rakha, 1966) and serum LH concentrations (Geschwind & Dewey, 1968; Niswender, Roche, Foster & Midgley, 1968; Wheatley & Radford, 1969) in various reproductive states. However, little work has been carried out on the role of the hypothalamus in controlling changes in gonadotrophin syn-

thesis and release. Dománski & Kochman (1968) found seasonal differences in the response of ewes to intrapituitary infusion of hypothalamic extracts and suggested that pituitary sensitivity to neurohumoral agents may fluctuate during the year. Reeves, Arimura & Schally (1970) suggested, similarly, that pituitary responsiveness to releasing factors may change during the oestrous cycle, possibly due to changes in the levels of the gonadal hormones.

The present study has used a sheep pituitary incubation system, coupled with biological assays specific for LH and FSH, to detect LH- and FSH-releasing activity in hypothalamic extracts. Such a system was used by Pelletier & Ortavant (1968) but the specificity of this technique was not adequately established. In the present work, the following materials were tested for their ability to influence LH and FSH release: oxytocin, vasopressin (natural and synthetic), adrenaline, noradrenaline, a crude acid extract of sheep cerebral cortex, a crude acid extract and partly purified extracts of ovine hypothalamic tissue. Where appropriate, materials were also tested in the gonadotrophin assays after 'blank' incubations identical except for the absence of pituitary tissue.

MATERIALS AND METHODS

Incubation procedure

The method was based on that used by Crighton, Schneider & McCann (1970) for rat pituitary tissue. Since Samli & Geschwind (1967) and Wakabayashi & McCann (1970) have reported that in the rat, the basal release of LH and the response to added hypothalamic extract were markedly enhanced by castration, castrated male sheep were used as pituitary donors. Each sheep was stunned with a captive bolt pistol and then exsanguinated between 14.00 and 15.00 h on the day of incubation. The pituitary gland was removed within a few minutes, the anterior lobe separated, placed dorsal surface down on a Stadie–Riggs microtome and sectioned horizontally. It was assumed that gonadotrophs are distributed more or less equilaterally (Mikami & Daimon, 1968; Nakane, 1970). In early experiments each slice (approximately 0·5 mm thick) was halved and each half slice was placed in 2·0 ml of Medium 199 in a chilled Erlenmeyer flask. Each flask was paired with another containing tissue from the opposite half of the gland. Two glands were used in each experiment and four slices were taken from each gland. Each of eight flasks contained a half slice from each gland, i.e. two half slices per flask. In all but the first few experiments each half slice was further cut into four pieces and these were distributed as shown in Fig. 1, so that although each flask contained tissue equivalent to two half slices, any differences in gonadotroph distribution dorsoventrally were avoided. All flasks were incubated at 37 °C in a shaking reaction incubator at 60 cycles/min in 95 % oxygen: 5 % carbon dioxide. After pre-incubation for 30 min, the medium was discarded and replaced with 2·0 ml medium plus 0·5 ml experimental solution or solvent, the pH of this mixture having been adjusted previously to 7·6. Incubation was continued for 6 h and the medium was then decanted and stored at −15 °C until required for assay. The pieces of pituitary tissue from each flask were blotted and weighed to the nearest 0·1 mg. Each flask usually contained approximately 3·0 mg pituitary tissue.

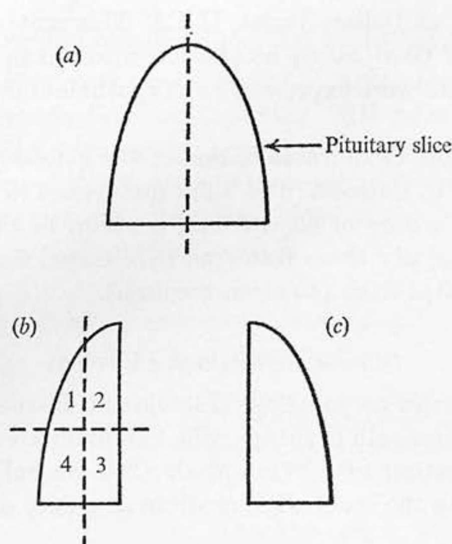

Fig. 1. Cutting procedure for sheep pituitary tissue and distribution of pieces of pituitary between different flasks. (*a*) Initial cut; (*b*) Each half-slice was cut into four pieces as shown and distributed among the 'treated' flasks as shown below. (*c*) Each half-slice was cut as shown in (*b*) and distributed among the 'control' flasks.

	Flask 1	Flask 2	Flask 3	Flask 4
First half-slice	1	2	3	4
Second half-slice	4	1	2	3
Third half-slice	3	4	1	2
Fourth half-slice	2	3	4	1

Assay methods

The medium was assayed for LH by the ovarian ascorbic acid depletion (OAAD) method (Parlow, 1958) using an inbred strain of rat of Wistar origin and for FSH by the augmentation method (Steelman & Pohley, 1953) using an inbred strain of rat of Sprague–Dawley origin. Both assays had minor modifications. The design in each case was that of a three-point assay with two doses of standard (0·8 and 4·0 μg NIH-LH-S16 or 50 and 150 μg NIH-FSH-S7; National Institutes of Health, U.S.A.) as appropriate and one dose of medium designed to produce a response between the responses to the two doses of standard. The results were calculated as described by Gaddum (1953) and the hormone content of the medium was expressed as μg NIH-LH or NIH-FSH equivalents/mg anterior pituitary tissue incubated as appropriate.

Hypothalamic and cerebral cortex extracts

A crude acid extract of the hypothalami of 100 miscellaneous sheep was prepared. The combined fragments were homogenized in 100 ml 0·1 M-HCl, centrifuged, and the supernatant was partially lyophilized and stored at -15 °C. A crude extract of sheep cerebral cortex was also prepared, using the same ratio of tissue weight to acid volume. A concentrated ultrafiltrate of a glacial acetic acid extract of sheep pituitary stalk medium eminence (SME) tissue with a concentration of approximately 200 SME/ml was kindly donated by Dr C. P. Fawcett, University of Texas South-

western Medical School at Dallas, Texas, U.S.A. This material was known to elicit LH release at a dose of 60 μl/2·0 ml incubation medium in a rat pituitary in-vitro system. Doses of extracts were expressed as 'hypothalamus equivalents' or 'cortex equivalents'.

Aliquots from Sephadex G-25 fractionation of the glacial acetic acid extract were also donated by Dr C. P. Fawcett. The tube numbers 125 to 144 were known to provoke LH release at a dose of 50 μl/2·0 ml medium in the rat pituitary in-vitro system. In the present study these fractions were tested for LH and FSH releasing activity at a dose of 100 μl/flask (40 μl/ml medium).

Other materials tested in vitro

Oxytocin and vasopressin preparations (Pitocin and Pitressin, Parke, Davis & Co.) and synthetic lysine vasopressin (Syntopressin, Sandoz) were added to the incubation medium at a concentration of 500 mu./flask (200 mu./ml). Adrenaline and noradrenaline were added to the incubation medium at a dose of 25 μg/flask (10 μg/ml).

RESULTS

Basal release of LH and FSH in vitro

Anterior pituitary tissue from castrated male sheep released readily measurable quantities of LH and FSH into the incubation medium. Because of the low sensitivity of the FSH assay, the medium from four flasks was pooled for assay whereas in the

Table 1. *Basal release of luteinizing hormone (LH) in sheep* in vitro

Replicate no.	LH content of medium (μg NIH-LH equiv./mg anterior pituitary tissue incubated)	λ
1	1·28 (0·67–3·10)	0·23
	1·01 (0·55–2·05)	0·22
2	1·11 (0·61–2·57)	0·22
	0·91 (0·49–1·73)	0·22
3	1·09 (0·64–2·08)	0·19
	1·90 (0·95–6·65)	0·25
4	1·88 (1·04–5·04)	0·21
	1·73 (0·94–4·74)	0·21

The two values for each replicate represent paired untreated flasks. Figures in parentheses are fiducial limits of error at $P = 0·95$. There were no significant differences between the values for each replicate.

case of LH, medium from each individual flask could be assayed. Thus in an experiment involving FSH release only one comparison was possible whereas in the case of LH up to four comparisons could be made concurrently. It was necessary to show that basal hormone release from members of a pair of flasks was not significantly different. This proved to be the case justifying the use of one flask in each pair as a 'control' and the other as a 'treated' flask in subsequent experiments. The results for LH are shown in Table 1. In the case of FSH the results of a single experiment were 4·85 (3·08–8·12) and 4·57 (2·84–7·35) (relative potency with fiducial limits of error at $P = 0·95$) in terms of μg NIH-FSH equivalents/mg tissue incubated.

Effects of oxytocin and vasopressin on LH and FSH release in vitro

Oxytocin failed to influence the release of LH (two experiments) or FSH (two experiments) and vasopressin failed to influence FSH release (three experiments). In the case of Pitressin, the natural vasopressin used at first, there was a significant increase in the LH content of the medium in three out of four experiments. When two experiments were carried out with the same dose of Syntopressin, the synthetic material failed to influence LH release (Table 2).

Table 2. *Effects of vasopressin on luteinizing hormone (LH) release in sheep* in vitro

Replicate no.	Treatment*	LH content of medium (µg NIH-LH equiv./mg anterior pituitary tissue incubated)†	λ	Significance of difference‡
1	Vasopressin (Pitressin)	1·40 (0·84–2·24)	0·17	NS
	Control	1·00 (0·51–1·66)	0·21	
2	Vasopressin (Pitressin)	2·30 (1·53–3·47)	0·17	S
	Control	1·23 (0·63–1·97)	0·19	
3	Vasopressin (Pitressin)	2·45 (0·71–3·51)	0·14	S
	Control	1·23 (0·63–1·97)	0·11	
4	Vasopressin (Pitressin)	1·93 (1·45–2·58)	0·12	S
	Control	1·29 (0·90–1·86)	0·14	
1	Vasopressin (Syntopressin)	1·76 (1·29–2·40)	0·13	NS
	Control	2·21 (1·61–3·03)	0·13	
2	Vasopressin (Syntopressin)	1·29 (0·89–1·86)	0·14	NS
	Control	1·80 (1·31–2·47)	0·13	

* The quantity of vasopressin was 500 mu.
† Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.
‡ NS = not significant; S = significant.

Effects of adrenaline and noradrenaline on LH and FSH release in vitro

The effects of adrenaline and noradrenaline on LH release are shown in Table 3. Adrenaline failed to influence LH. Noradrenaline apparently decreased the output of LH in two experiments out of three. This effect was further examined in two ways: 'blank' incubations of Medium 199 plus noradrenaline at a dose of 25 µg/flask were carried out. The medium, diluted to the same degree as after the tissue incubations, failed to influence the ovarian ascorbic acid level of LH assay rats but when noradrenaline was added at the usual dilution to the standard doses (0·8 and 4·0 µg) of NIH-LH used routinely in the LH assay and this mixture was assayed in a four-point design against the same doses of standard NIH-LH alone, the addition of noradrenaline diminished the potency of the standard by 35%. Both adrenaline (two experiments) and noradrenaline (two experiments) failed to influence FSH release.

Effects of cerebral cortex extract on LH and FSH release in vitro

Cerebral cortex extract failed to influence LH release (five experiments) or FSH release (one experiment). In one experiment, the highest dose used (1·6 equivalents) produced a marginally significant increase in the LH content of the medium. In a

Table 3. *Effects of adrenaline and noradrenaline on luteinizing hormone (LH) release in sheep* in vitro

Replicate no.	Treatment*	LH content of medium (μg NIH-LH equiv./mg anterior pituitary tissue incubated)†	λ	Significance of difference‡
1	Adrenaline	2·21 (1·64–2·98)	0·12	NS
	Control	2·33 (1·63–3·32)	0·14	
2	Adrenaline	1·00 (0·75–1·33)	0·11	NS
	Control	1·35 (0·98–1·87)	0·13	
1	Noradrenaline	1·07 (0·73–1·56)	0·13	S
	Control	1·67 (1·23–2·27)	0·11	
2	Noradrenaline	1·44 (1·11–1·87)	0·10	S
	Control	2·40 (1·93–2·98)	0·09	
3	Noradrenaline	1·32 (1·01–1·73)	0·10	NS
	Control	1·72 (1·25–2·37)	0·13	

* The quantity of adrenaline or noradrenaline was 25 μg.
† Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.
‡ NS = not significant; S = significant.

Table 4. *Effects of a crude acid extract of sheep hypothalamus on luteinizing hormone (LH) release in sheep* in vitro

Replicate no.	Treatment (quantities expressed as hypothalamus or cortex equivalents)	LH content of medium (μg NIH-LH equiv./mg anterior pituitary tissue incubated)*	λ	Significance of difference†
1	Hypothalamus (0·00136)	1·02 (0·74–1·39)	0·12	NS
	Cortex (0·00136)	0·96 (0·69–1·33)	0·13	
	Hypothalamus (0·00625)	0·83 (0·59–1·13)	0·14	NS
	Cortex (0·00625)	0·67 (0·43–1·03)	0·18	
	Hypothalamus (0·025)	0·65 (0·46–0·93)	0·15	NS
	Cortex (0·025)	0·68 (0·50–0·93)	0·13	
	Hypothalamus (0·1)	1·39 (0·89–2·19)	0·15	S
	Cortex (0·1)	0·67 (0·38–1·19)	0·16	
	Hypothalamus (0·4)	1·37 (0·96–1·96)	0·12	S
	Cortex (0·4)	0·83 (0·61–1·12)	0·12	
	Hypothalamus (1·6)	1·85 (1·33–2·57)	0·11	S
	Cortex (1·6)	1·22 (0·84–1·77)	0·14	
2	Hypothalamus (0·00136)	0·99 (0·67–1·46)	0·14	NS
	Cortex (0·00136)	1·02 (0·70–1·49)	0·14	
	Hypothalamus (0·00625)	0·51 (0·33–0·78)	0·14	NS
	Cortex (0·00625)	0·51 (0·33–0·78)	0·14	
	Hypothalamus (0·025)	0·56 (0·36–0·87)	0·15	NS
	Cortex (0·025)	0·42 (0·19–0·71)	0·18	
	Hypothalamus (0·1)	1·19 (0·78–1·81)	0·15	S
	Cortex (0·1)	0·46 (0·26–0·74)	0·17	
	Hypothalamus (0·4)	1·67 (1·07–3·36)	0·14	S
	Cortex (0·4)	0·60 (0·41–0·88)	0·14	
	Hypothalamus (1·6)	1·35 (0·90–2·02)	0·14	S
	Cortex (1·6)	0·64 (0·42–0·96)	0·15	

* Figures in parentheses are fiducial limits of error at $P = 0{\cdot}95$.
† NS = not significant; S = significant.

duplicate experiment, this dose failed to influence the LH content of the medium. The same dose was ineffective with FSH. After 'blank' incubations of Medium 199 plus cerebral cortex extract (1·6 equivalents), the medium, diluted to the same degree as after the tissue incubations, failed to influence the ovarian ascorbic acid level of LH assay rats.

Effects of hypothalamic extracts on LH and FSH release in vitro

Crude acid extract

The effects on LH release of a crude extract of sheep hypothalami are shown in Table 4. In these experiments the LH content of media to which graded doses of

Table 5. *Effects of a glacial acetic acid extract of sheep hypothalamus after ultrafiltration on luteinizing hormone (LH) release in sheep* in vitro

Replicate no.	Treatment (quantities expressed as hypothalamus equivalents)	LH content of medium (μg NIH-LH equiv./mg anterior pituitary tissue incubated)*	λ	Significance of difference†
1	Hypothalamus (0·1)	0·64 (0·31–1·19)	0·24	NS
	Control	1·03 (0·43–2·80)	0·32	
	Hypothalamus (0·4)	0·63 (0·18–1·50)	0·33	NS
	Control	0·70 (0·18–1·81)	0·35	
	Hypothalamus (1·6)	0·67 (0·12–1·46)	0·29	NS
	Control	0·42 (0·06–0·95)	0·28	
	Hypothalamus (6·4)	1·38 (0·54–3·01)	0·28	S
	Control	0·48 (0·07–1·08)	0·29	
1	Hypothalamus (3·2)	5·13 (3·54–7·43)	0·14	S
	Control	2·31 (1·57–3·41)	0·16	
	Hypothalamus (6·4)	4·14 (3·03–5·64)	0·12	S
	Control	1·89 (1·34–2·68)	0·14	
	Hypothalamus (12·8)	5·33 (3·70–7·67)	0·14	S
	Control	2·18 (1·56–3·04)	0·14	
	Hypothalamus (25·6)	4·91 (3·55–6·78)	0·12	S
	Control	1·26 (0·87–1·81)	0·13	
1	Hypothalamus (1·6)	1·21 (0·93–1·58)	0·10	S
	Control	0·77 (0·59–1·02)	0·09	
	Hypothalamus (2·26)	1·95 (1·58–2·42)	0·08	S
	Control	0·77 (0·59–1·01)	0·09	
	Hypothalamus (3·2)	2·78 (2·24–3·45)	0·08	S
	Control	1·13 (0·92–1·40)	0·07	
	Hypothalamus (4·53)	3·05 (2·50–3·73)	0·07	S
	Control	0·78 (0·58–1·04)	0·09	
2	Hypothalamus (1·6)	2·63 (1·93–3·58)	0·12	NS
	Control	2·59 (1·83–3·67)	0·14	
	Hypothalamus (2·26)	3·36 (2·46–4·59)	0·12	S
	Control	2·38 (1·62–3·50)	0·16	
	Hypothalamus (3·2)	4·18 (3·03–5·76)	0·12	S
	Control	1·67 (1·25–2·23)	0·12	
	Hypothalamus (4·53)	4·81 (3·42–6·77)	0·12	S
	Control	2·32 (1·76–3·05)	0·11	

* Figures in parentheses are fiducial limits of error at $P = 0·95$.
† NS = not significant; S = significant.

hypothalamic extract had been added were compared with those of media containing equivalent graded doses of cerebral cortex extract. A log-dose interval of log 4 was employed. The hypothalamus extract increased the LH content of the medium significantly at the higher doses used. In both replicates of the dose–response experiment, the minimal effective dose of hypothalamus extract was 0·1 equivalents but there was no indication of a graded response to increasing doses above this level. After 'blank' incubations of Medium 199 plus hypothalamus extract (1·6 equivalents), the medium, diluted to the same degree as after tissue incubations, failed to influence the ovarian ascorbic acid level of LH assay rats.

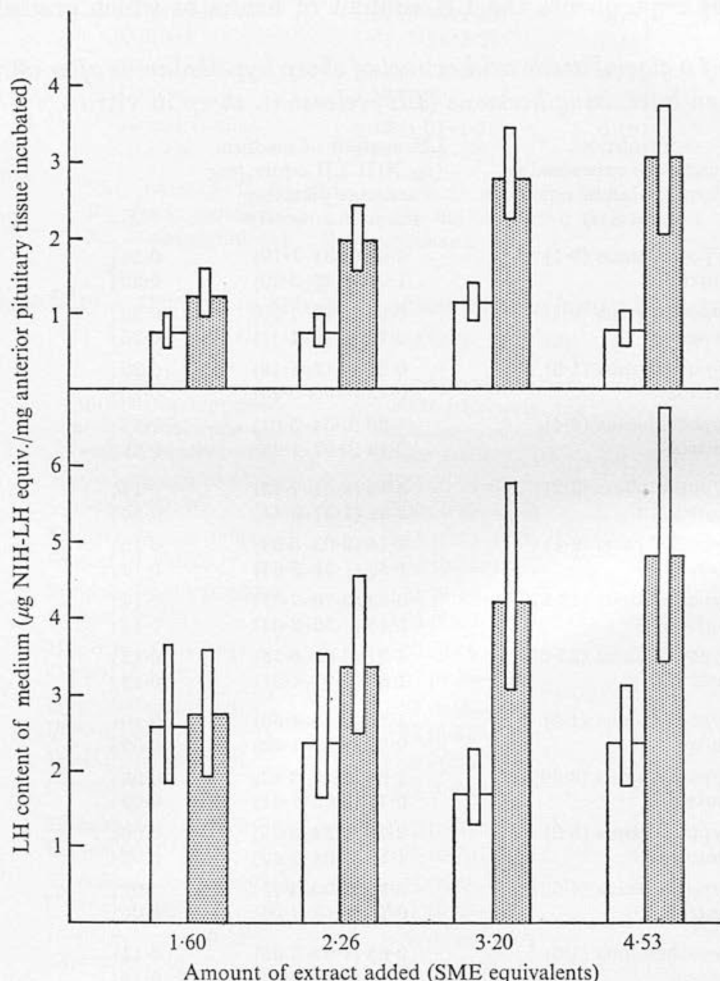

Fig. 2. Results of replicate experiments showing the effects of a glacial acetic acid extract of sheep hypothalamus on luteinizing hormone (LH) release from sheep pituitary tissue *in vitro*. Open bars = LH levels in control media; shaded bars = LH levels in experimental media. Fiducial limits of error at $P = 0{\cdot}95$ are shown. SME = stalk-median eminence.

Partly purified extracts

Glacial acetic acid extract after ultrafiltration. The effects on LH release of a partly purified extract of sheep SME tissue are shown in Table 5. In these experiments the LH content of media to which graded doses of extract had been added were compared with those of media to which equivalent graded doses of cerebral cortex had been added. A log-dose interval of log 4 was employed initially. This was subsequently narrowed to log 2 and later to log 1·42. At the wider log-dose intervals there was no indication of a graded response but at the narrowest log-dose interval a graded response was apparent in both replicates of the experiment (Fig. 2).

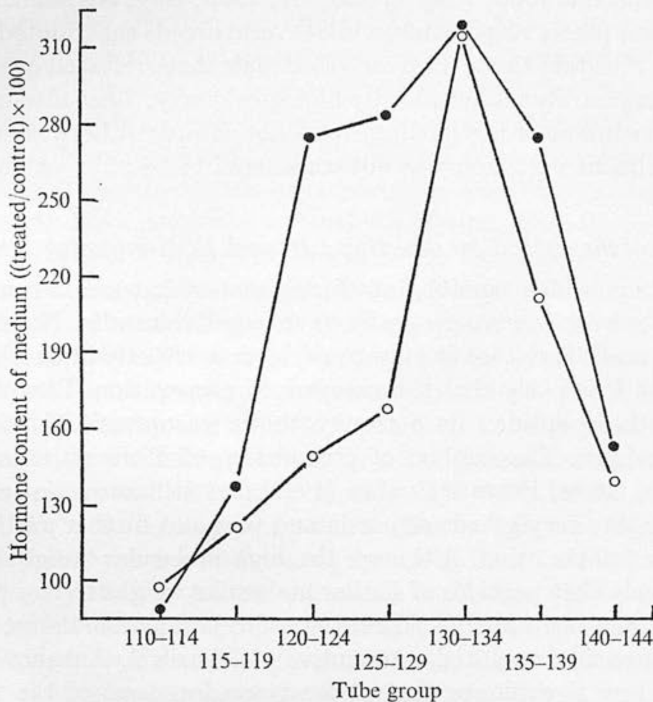

Fig. 3. Effects on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release in sheep pituitary tissue *in vitro* of fractions obtained from a glacial acetic acid extract of sheep hypothalamus subjected to gel filtration on Sephadex. ●, LH releasing activity; ○, FSH releasing activity.

Fractions obtained from a glacial acetic acid extract by gel filtration on Sephadex. The effects on LH and FSH release of various fractions of a glacial acetic acid extract of hypothalamus separated by gel filtration on Sephadex G-25 are summarized in Fig. 3. Luteinizing hormone releasing activity was located over the range of tubes 120–139. Follicle-stimulating hormone releasing activity was found over the same range although the lower precision of the FSH assay and the fewer experiments carried out made the result less reliable than in the case of LH. In spite of some variation in response, peak values of both LH and FSH releasing activity were found in tube group 130–134. No separation of LH releasing activity from FSH releasing activity was apparent.

DISCUSSION

Basal release of LH and FSH in vitro

Our results show that there were no differences in basal LH and FSH levels within pairs of flasks. However, variation in basal release of both hormones between pairs within an experiment and between experiments was encountered. Other workers have noted such variations in basal release of gonadotrophins *in vitro* (Mittler & Meites, 1966; Watanabe & McCann, 1968). The between-experiment variation is partly attributable to inherent differences between the sheep used as donors of pituitary tissue for incubation. Instability in the pituitary gonadotrophin concentration of gonadectomized animals (Gay & Midgley, 1969; Gay, Niswender & Midgley, 1970) may have been partly responsible, while several breeds contributed to the crossbred animals used. Pituitary tissue from castrated male sheep released quantities of LH and FSH which were readily measurable by biological assay. Since absolute values for the gonadotrophin content of the medium were not compared between experiments, the between-experiment variation was not considered to be of great importance.

Specificity of the method for detecting LH- and FSH-releasing activity

The experiments in which possible interfering materials were assessed for effects on gonadotrophin release *in vitro* generally gave negative results. No effect of vasopressin (Pitressin) on FSH release *in vitro* could be demonstrated but LH release was increased (Table 2). It is likely that the vasopressin preparation (Pitressin) was contaminated with other peptides since the synthetic vasopressin Syntopressin had no effect on LH release. The method of preparation of Pitressin is based on that of Kamm, Aldrich, Grote, Rowe & Bugbee (1928) and utilizes acetic acid extraction of mixed posterior pituitary glands of cattle and pigs and further purification using acetone and ether fractionation. Although the high molecular weight proteins are removed it is unlikely that peptides of similar molecular weight to vasopressin would be excluded. During gel filtration of acid extracts of hypothalamic tissue, LH releasing activity and vasopressin are eluted consecutively (Dhariwal, Antunes-Rodrigues & McCann, 1965). There is evidence that at least one fragment of the LH releasing factor molecule may possess some LH- but not FSH-releasing activity (Bowers, Chang, Sievertsson, Bogentoft, Currie & Folkers, 1971). The reasons for the present observation are not clear. The apparent depressant effect of noradrenaline on LH release (Table 3) but not FSH release seems to be explicable in terms of a direct effect on the hormone or on the assay animals rather than on the release of LH. Noradrenaline diminished the potency of a standard LH preparation by 35%. Correction for this effect in the assay results for the incubation media led to almost identical values for 'control' and 'treated' flasks. Van Loon & Kragt (1970) found that dopamine denatured FSH in an in-vitro system, and Kamberi, Schneider & McCann (1970) recorded a slight decrease in FSH release at the highest dose of noradrenaline used in specificity studies with rat pituitary tissue *in vitro*. The dose used was approximately equivalent to half the dose used in the present experiments. No further investigations were carried out in the present study so that it is not clear whether noradrenaline denatured ovine LH or affected the assay animals. The levels

of noradrenaline in hypothalamic tissue are low enough to make any influence on the specificity of the system very unlikely.

When crude hypothalamus extract was tested in the system, doses above 0·1 equivalents consistently produced increases in LH release (Table 4). This finding is similar to that of Schally & Bowers (1964) with rat pituitary tissue *in vitro*. In their experiments, 0·1–0·2 equivalents of a sheep hypothalamus were necessary to stimulate LH release. In the present work no graded response to crude hypothalamic extract above the minimal effective dose was observed. It is clear from the results of other experiments (Table 5; D. B. Crighton, unpublished data) that this was due to the wide log-dose interval employed.

When the ultrafiltered glacial acetic acid extract of sheep hypothalamus was tested it was seen that some activity had apparently been lost in processing or storage of this extract since the minimal effective dose was approximately two equivalents (1·6 equivalents effective in one out of three experiments, 2·26 equivalents in two out of two experiments). Graded responses were observed when a narrow log-dose interval was employed (Table 5, Fig. 2). Graded dose–response relationships have been established previously for the gonadotrophin releasing activity of hypothalamic extracts in rat pituitary incubations (Mittler & Meites, 1966; Jutisz, Bérault, Novella & Ribot, 1967; Serra & Midgley, 1970) and in the rat *in vivo* (McCann, 1962). Jutisz *et al*. (1967) found a linear relationship between the LH released and the log dose of LH releasing factor added to rat pituitary tissue over a limited range with a maximal response. Doses above this produced a diminished response.

When the fractions of hypothalamic extract resulting from gel filtration on Sephadex were tested, LH releasing activity was found to be present in tubes 120–139. This compared with the finding of C. P. Fawcett (personal communication) in a rat pituitary in-vitro system of activity in tubes 125–144. In the present work FSH releasing activity was found in the same region as LH releasing activity (Fig. 3). These findings do not agree with the early results of Dhariwal, Nallar, Batt & McCann (1965) and Schally, Saito, Arimura, Sawano & Bowers (1967) who reported that LH releasing and FSH releasing activities were separated by Sephadex fractionation. Subsequent studies by Schally, Arimura, Kastin, Reeves, Bowers, Baba & White (1970) indicated that such separation could not be obtained and the homogeneous purified ovine and porcine LH releasing factor obtained by Amoss, Burgus, Blackwell, Vale, Fellows & Guillemin (1971) and Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk & White (1971) respectively, had FSH releasing activity which was apparently intrinsic to the LH releasing factor. Failure to separate the LH from the FSH releasing activity in the Sephadex fractions tested in the present work agrees with the more recently published studies.

Although the quantitative nature of the responses to added hypothalamic extracts could be demonstrated clearly only over a very narrow dose range and when assays were of very high precision, it was clear that the method could be used readily for the establishment of minimal effective doses of active material. The LH and FSH releasing activity of ovine hypothalamic extracts and changes in the responsiveness to hypothalamic stimulation of sheep pituitary tissue could thus be assessed on this basis.

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COMPARISON OF THE REDOX BIOASSAY WITH OTHER ASSAYS FOR LUTEINIZING HORMONE

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SUMMARY

The cytochemical (redox) bioassay for LH has been compared with established LH assays. Measurements made by redox bioassays were considerably lower than those made by radioimmunoassay in human female plasma samples obtained at mid-cycle. There was no apparent relationship between measurements on incubation media from cultures of sheep pituitary glands made by redox bioassay and the ovarian ascorbic acid depletion (OAAD) assay. After polyacrylamide gel electrophoresis of a crude extract of a human pituitary gland, redox LH measurements were lower than those of the OAAD assay and radioimmunoassay in the cathodal segments of the gel. By contrast, there was reasonable agreement between LH measurements made by radioimmunoassay and redox assay in cathodal fractions from gel electrophoresis of a purified pituitary LH preparation. Follicle-stimulating hormone, and the α- and β-subunits of LH depressed the response of intact LH in the redox assay; this might explain the relatively low levels of LH measured by redox assay in some of the experiments described. Which type of assay best reflects the biological activity of LH in man remains to be determined.

INTRODUCTION

A highly sensitive cytochemical bioassay for the measurement of luteinizing hormone (LH), the 'redox LH bioassay', has been described (Rees, Holdaway, Kramer, McNeilly & Chard, 1973). The specificity of this assay technique was established by testing a variety of other materials in the system, and of these, only human chorionic gonadotrophin induced a reaction similar to that of LH. However, when estimates were made on plasma samples and compared with those obtained by radioimmunoassay, significant discrepancies were observed. In particular, determinations carried out at mid-cycle showed high levels in the days preceding and following the peak of immunoreactive LH, and low levels on the day of the immunoreactive peak (Holdaway, Kramer, McNeilly, Rees & Chard, 1974). There are two possible explanations for these findings. First, that the immunologically active LH is not biologically active under varying physiological conditions. Secondly, that there is one form of LH, but the redox activity is influenced by the presence of other hormones or

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hormone subunits in biological samples. The present experiments were designed to evaluate these possibilities by comparison of redox LH levels with those obtained by radioimmunoassay and by the classic ovarian ascorbic acid depletion (OAAD) bioassay.

MATERIALS AND METHODS

Samples for assay

Plasma samples

Samples of heparinized plasma were obtained during normal menstrual cycles in female volunteers aged between 19 and 29 years. Samples were also taken from normal men, post-menopausal women and women taking oestrogens. These samples were assayed by radioimmunoassay and redox bioassay.

Media from maintenance cultures of sheep pituitary tissue

Pituitary tissue (approximately 25 mg in each of eight flasks) from castrated sheep was incubated for 6 h in Medium 199 (Hartley, Crighton & Lamming, 1973). Tissue in four flasks was treated with gonadotrophin releasing hormone while the remaining four flasks acted as controls. These samples were assayed by OAAD and redox bioassays.

Human pituitary extract

A crude tissue extract was prepared from a single pituitary gland obtained at post-mortem from a post-menopausal female subject with neoplastic disease. The tissue was homogenized in 0·18 M-Tris–HCl buffer, pH 8·3, containing 10^{-3} di-isopropylphosphofluoridate, at 4 °C for 30 s and centrifuged at 3000 *g* for 25 min at 4 °C. The supernatant was then kept at 4 °C for 20 h until submitted to electrophoresis. After electrophoresis, the eluates were assayed in radioimmunoassay, OAAD and redox systems.

Electrophoresis in polyacrylamide gels

Gel electrophoresis was carried out on 100 μl aliquots of crude pituitary extract and on pituitary LH (MRC LH 68/40) made up to 10 i.u./ml in 0·05 M-phosphate buffer, pH 7·6, containing 2·5 mg human serum albumin/ml. Gels contained 10 % polyacrylamide (4·8 % bis) prepared in 0·18 M-Tris–HCl buffer, pH 8·3, and were run at 4 mA/gel at 4 °C. The cathodal buffer was 0·04 M-Tris 0·04 M-glycine buffer, pH 9·1, and the anodal buffer was 0·04 M-Tris 0·34 M-glycine buffer, pH 8·5. The migration front was identified using bromophenol blue. After electrophoresis the gels were divided into 0·5 cm sections and eluted into assay diluent at 4 °C for 12 h. The eluates were divided into three aliquots and stored at −70 °C until assayed by each of the three methods.

Assay procedures

Radioimmunoassay of LH was performed by a double antibody method using a rabbit antiserum, as described by McNeilly & Hagen (1974). Redox bioassay of LH was carried out using segments of luteinized rat ovary maintained *in vitro* as described by Rees *et al.* (1973) and using technical modifications as outlined by Kramer, Holdaway, Rees, McNeilly & Chard (1974). Ovarian ascorbic acid depletion bioassay of LH was based on the method of Parlow (1958) as modified by Crighton, Hartley & Lamming (1973).

Standard hormone preparations

The following materials were used as LH standards: MRC LH 68/40 assuming a content of 40 i.u./ampoule; NIH-LH-S17, assuming a potency of 2117 i.u./mg; and NIH-LH-S18, assuming a potency of 1545 i.u./mg. Other hormone preparations used were FSH CPDS 6

(kindly supplied by Dr W. R. Butt), MRC LH α-subunit, and LH β-subunit (kindly supplied by Dr R. Lequin). Plasma LH was assayed against MRC LH 68/40 (radioimmunoassay and redox bioassay), and media from cultures of sheep pituitary gland were assayed against NIH-LH S17 (OAAD) and NIH-LH-S18 (redox bioassay). Fractions from gel electrophoresis were assayed against MRC LH 68/40 (radioimmunoassay and redox bioassay) and NIH-LH-S17 (OAAD).

Studies with follicle-stimulating hormone and the subunits of LH

The possibility of interference from follicle-stimulating hormone (FSH) and the α- and β-subunits of LH in the redox bioassay was studied in several animals. Tissue segments from one animal were exposed to four dilutions of FSH alone and four dilutions of LH standard plus 10 ng FSH/ml as well as four dilutions of LH standard. Tissue segments from a second animal were exposed to dilutions of standard LH plus 1 ng FSH/ml, or 0·1 ng FSH/ml. Segments from a third animal were exposed to dilutions of LH standard plus 10 ng LH α-subunit/ml, or 10 ng LH β-subunit/ml. Standard curves were obtained from each animal.

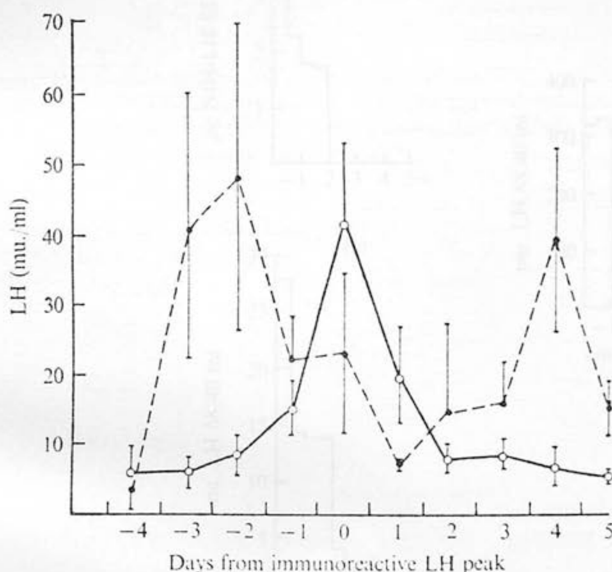

Fig. 1. Pattern of LH release during 6 normal menstrual cycles (means ± S.E.M.) as measured by radioimmunoassay (○) and redox bioassay (●).

RESULTS

There was no apparent relationship between the LH concentrations determined by radioimmunoassay and redox bioassay in human mid-cycle plasma. In five out of six subjects studied, the levels recorded by redox bioassay on the day of the mid-cycle peak of immunoreactive LH were considerably lower than values of immunoreactive hormone. In addition, the values measured by redox assay at mid-cycle were considerably lower than values recorded by the same assay on the days preceding and following the immunoreactive LH peak (Fig. 1). There was no apparent relationship between the LH values detected by the OAAD assay and the redox bioassay in culture media from sheep pituitaries incubated *in vitro* (Table 1). In six out of eight samples the redox bioassay again gave values considerably lower than the OAAD assay.

Electrophoresis of a human pituitary extract showed a slow migrating fraction (up to 1 cm

from the origin) active in both radioimmunoassay and the OAAD bioassay, but showing little activity in the redox bioassay (Fig. 2*a*, *b*, *c*). The bulk of the redox activity was found 1–2 cm from the origin. Faster migrating fractions showed a small amount of activity in both radioimmunoassay and redox bioassay but none in the OAAD bioassay. By contrast, electrophoresis of purified human LH (MRC 68/40) showed a slow migrating fraction

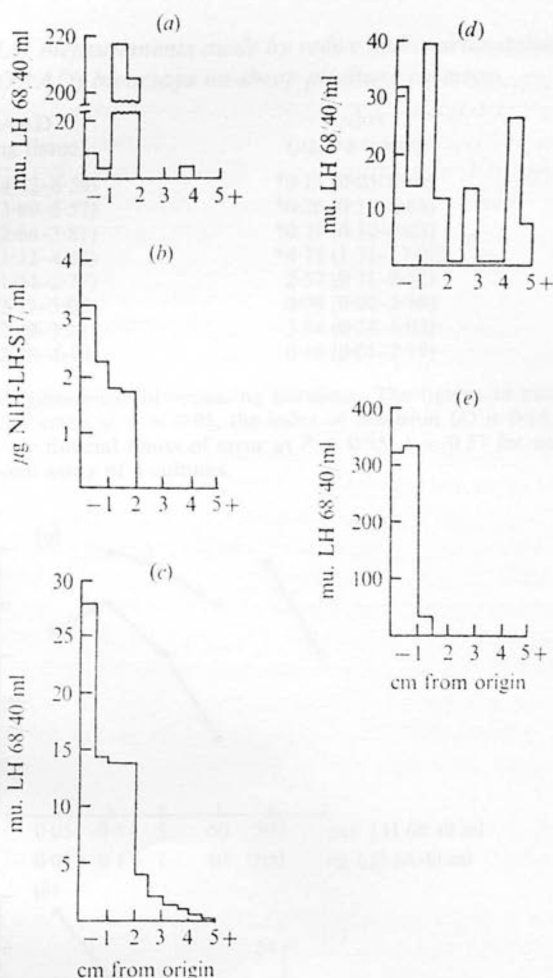

Fig. 2. Polyacrylamide gel electrophoresis of a crude pituitary extract with LH measurements made by (*a*) redox bioassay with MRC LH 68/40 as standard, (*b*) ovarian ascorbic acid depletion bioassay with NIH-LH-S17 as standard, (*c*) radioimmunoassay with MRC LH 68/40 as standard, and of a purified LH preparation (MRC LH 68/40) with LH measurements made by (*d*) redox bioassay and (*e*) radioimmunoassay, both using MRC LH 68/40 as standard. Where a zero level is recorded, no activity was detectable in the assay used.

active in both the redox bioassay and the radioimmunoassay (Fig. 2*d* and *e*). Correcting for recovery after electrophoresis, 2 % of the bioactive material appeared in the first 0·5 cm of gel of the crude extract, while 20 % appeared in the first 0·5 cm of the gel of the pure LH preparation. Fifty-five per cent of the LH immunoreactive material was present in the same 0·5 cm eluate of the crude extract and 46 % in the pure preparation. In addition, fast migrating components were present in the pure preparation which were biologically but not

immunologically active. Analysis by OAAD bioassay was not possible due to the insensitivity of the assay and the limited amount of material available for electrophoresis.

Follicle-stimulating hormone and the α- and β-subunits of LH at levels of 10 ng/ml considerably reduced the activity of LH in the redox bioassay (Fig. 3*a*, *c*). FSH alone cross-reacted only slightly in the assay and concentrations of 0·1 and 1 ng/ml had no effect on the LH response in the assay (Fig. 3*b*).

Table 1. *Comparison of LH measurements made by redox and ovarian ascorbic acid depletion (OAAD) bioassays on sheep pituitary cultures*

OAAD (μg/mg tissue)	Redox (μg/mg tissue)
*5·37 (4·42–6·59)	*0·17 (0·05–0·65)
*4·66 (3·89–5·57)	*0·26 (0·11–0·64)
*3·19 (2·66–3·81)	*0·31 (0·12–0·83)
*3·98 (3·32–4·82)	*4·73 (1·31–17·09)
2·32 (1·94–2·77)	2·57 (0·71–9·22)
2·54 (2·11–3·05)	0·08 (0·02–3·98)
2·74 (2·29–3·27)	3·44 (0·24–6·03)
3·57 (2·95–4·40)	0·16 (0·01–2·19)

* Pituitary tissue stimulated with gonadotrophin-releasing hormone. The figures in parentheses in the OAAD column are fiducial limits of error at $P = 0·95$, the index of precision (λ) is 0·18. The figures in parentheses in the Redox column are fiducial limits of error at $P = 0·95$, $\lambda = 0·37$ for one of the assays of 4 cultures, $\lambda = 0·23$ for the second assay of 4 cultures.

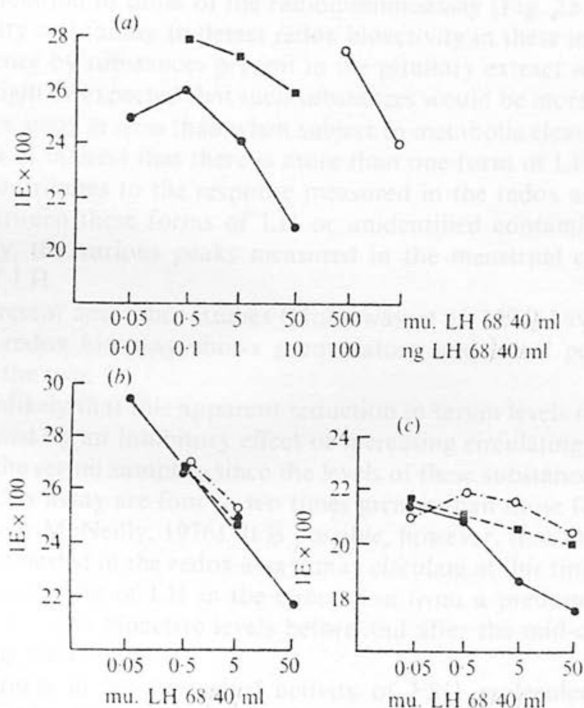

Fig. 3. Response of LH in the redox bioassay in the presence of FSH and LH α- and β-subunits: LH standard curves (●). (*a*) LH + 10 ng FSH/ml (■); FSH 10 ng/ml and 100 ng/ml (○). (*b*) LH + 1 ng FSH/ml (■); LH + 0·1 ng FSH/ml (○). (*c*) LH + 10 ng LHα/ml (■); LH + 10 ng LHβ/ml (○).

DISCUSSION

Comparison with existing techniques is an essential part of the validation of a new assay. In the case of the redox bioassay for LH, earlier studies showed this to be a sensitive and specific procedure when applied to purified materials (Rees *et al.* 1973; Kramer *et al.* 1974). It might, therefore, be expected that measurements made using the redox bioassay would be similar to those of other methods. However, under many conditions major discrepancies are observed, in particular, in plasma samples collected from women at mid-cycle.

This discrepancy might be explained by the presence of some agent interfering in the redox system. Specific materials which might interfere include FSH, and the subunits of LH. The present studies show that both FSH and the subunits of LH can interfere with the redox activity of intact LH and might, therefore, be responsible for the relatively low activity of samples collected at the time of the mid-cycle peak of immunoactive LH, and in the pituitary incubation media. Further evidence for interfering factors is provided by the studies on the crude pituitary extract: the initial fractions containing the bulk of the material active in the radioimmunoassay showed little activity in the redox bioassay. By contrast, electrophoresis of a purified LH preparation showed both redox bioactivity and immunoreactivity in these fractions. This suggests that slow migrating material was present in the crude preparations which decreased activity in the redox bioassay but not in the radioimmunoassay, and that this material was removed by purification. In contrast to the redox assay results, the OAAD assay measured LH activity in the slowest migrating fractions and results showed a reasonable correlation to those of the radioimmunoassay (Fig. 2*b* and *c*). The presence of OAAD bioactivity and failure to detect redox bioactivity in these initial fractions may be related to interference by substances present in the pituitary extract which remain at the origin of the gel. It might be expected that such substances would be more effective during static culture in the redox assay *in vitro* than when subject to metabolic clearance in the OAAD assay *in vivo*. It is also of interest that there is more than one form of LH present in the MRC LH 68/40 which contributes to the response measured in the redox assay. The assay does not distinguish between these forms of LH or unidentified contaminants of the preparation. Consequently, the various peaks measured in the menstrual cycle may reflect these different forms of LH.

The present and other studies (Holdaway *et al.* 1974) have shown that material active in the LH redox bioassay shows preovulatory and luteal peaks, with an apparent trough between the two.

It is unlikely that this apparent reduction in serum levels of bioactive LH at the mid-cycle is explained by an inhibitory effect of increasing circulating levels of FSH, LHβ- or α-subunits in the serum samples, since the levels of these substances required to affect LH activity in the redox assay are four to ten times greater than those found in the circulation (Hagen, McNatty & McNeilly, 1976). It is possible, however, that other substances, as yet unidentified and untested in the redox assay, may circulate at this time and may modify the apparent biological activity of LH in the circulation from a predominantly steroidogenic role, emphasized by high bioactive levels before and after the mid-cycle peak, to an ovulatory and luteinizing bioactivity.

Alterations in the biological activity of FSH molecules extracted from pituitaries of animals under different steroid environments have been shown in the rat (Diebel, Yamamoto & Bogdanove, 1973; Bogdanove, Campbell, Blair, Mula, Miller & Grossman, 1974) and monkey (Peckham, Yamaji, Dierschke & Knobil, 1973). Thus two alternative explanations for our results may be suggested. First, the bioactivity of the LH molecules may vary throughout the cycle in a similar way to that suggested for FSH and may be increased before and after, or decreased at the time of the mid-cycle immunoreactive peak of LH. If this was

the case immunoreactive levels of LH would not reflect this change in bioactivity. Secondly, an alteration in the biological activity of FSH may occur which while not reflected by an increase in immunoreactivity, may be sufficient to inhibit LH action in the redox bioassay. Whichever explanation is correct, it is interesting to note that peaks of LH activity demonstrated by redox bioassay correspond well to the pre-midcycle rise in oestrogen levels, and the post-midcycle rise in oestrogens and progesterone. Since the redox assay is likely to reflect the steroidogenic activity of LH, it may be that the biological activity of LH is more closely related to steroid production than is usually considered to be the case.

Earlier observations have also suggested that the peak of biologically active plasma LH (Watson, 1972) and urinary LH (Fukushima, Stevens, Gantt & Vorys, 1964) is broader than that of immunoreactive material. Multiple peaks of bioactive LH have been reported in human urine (Brown, Wells & Cunningham, 1964), and in bovine serum collected during complete cycles, two or three peaks of bioactive and/or immunoreactive LH have been detected (Schams & Karg, 1969; Snook, Saatman & Hansel, 1971).

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An *in vitro* technique for the study of hypothalamic control of gonadotrophin secretion in the sheep

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The work of Domanski & Kochman (1968) in the sheep suggests that changes occur with season in the sensitivity of the pituitary gland to hypothalamic stimulation, while, in the rat, fluctuations in the hypothalamic content of gonadotrophin-releasing factor have been observed (Chowers & McCann, 1965; Ramirez & Sawyer, 1965).

We have developed an *in vitro* sheep pituitary incubation system which may be used to elucidate the relative importance of these factors in controlling reproduction in the sheep. The results presented here refer only to the release of luteinizing hormone (LH) *in vitro* and to the specificity of the method for detecting hypothalamic LH-releasing factor (LH-RF) activity when the incubation is coupled with a biological assay specific for LH. Specificity was examined by testing, at high levels of inclusion, various materials which are known to be present in the hypothalamus, and also a crude acid extract of sheep cerebral cortex. The effects of these preparations were compared with those of a crude acid extract and a partly purified extract of ovine hypothalamic tissue.

Castrate male sheep were used as pituitary donors. Two pituitary glands were used for each experiment and each flask contained two pituitary half-slices. Flasks containing corresponding half-slices were paired (control and treated) during incubation. Incubation in medium 199 (Morgan, Morton & Parker, 1950) with test substances lasted for 6 hr. The LH content of the medium was determined using the ovarian ascorbic acid depletion (OAAD) assay (Parlow, 1958) and the results were expressed as μg ovine LH standard per mg pituitary wet weight incubated.

In preliminary experiments, the LH contents in the media in paired flasks were not significantly different. Of the materials tested for LH-releasing activity, oxytocin, adrenaline, synthetic vasopressin and cerebral cortex extract failed to influence LH release. Natural vasopressin produced a significant increase in the LH content of the medium. In the absence of activity from synthetic vasopressin this was almost certainly due to contamination of the preparation with hypothalamic LH-RF. Noradrenaline produced an apparent significant depression in the LH content of the medium, but this effect proved to be one upon the LH released or upon the OAAD assay since noradrenaline reduced the potency of standard ovine LH in the assay to an almost identical degree.

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Extracts of ovine hypothalamic tissue of an adequate dose consistently caused significant elevations of the LH content of the medium. When the dose interval employed was wide the response appeared to be all-or-none but when the interval was narrowed a graded dose–response curve was apparent.

We suggest that the method described may be used for the detection and estimation of LH-RF in ovine hypothalamic tissue extracts and for detecting changes in the responsiveness to LH-RF of sheep pituitary tissue.

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CHAPTER 5. MISCELLANEOUS

Possible oestrogenic effect of kale. Effects of the male on the oestrous cycle of the underfed female rat. Steroid hormones and nitrogen retention in the pig.

Papers in scientific journals (refereed)

Br. vet. J. (1967), **123**, 64

AN INVESTIGATION INTO THE POSSIBLE OESTROGENIC EFFECT OF KALE

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SUMMARY

The possible oestrogenic effect of kale was investigated by the biological assay in rats of kale extracts. Neither oral administration nor subcutaneous injection of acetone extracts demonstrated the presence of oestrogenic activity in the kale samples used. There was no difference in response to an acetone extract and an extract obtained by alkaline alcoholic distillation when both were given by subcutaneous injection.

INTRODUCTION

It has been suspected for some time that the practice of feeding high levels of kale can be the cause of reduced fertility in dairy cattle (Reed, 1961; Melrose & Brown, 1962). Reproductive abnormalities have also been observed in sheep fed high levels of kale (Williams, Hill & Alderman, 1965). Chury (1960) has suggested that reproductive disturbances in cattle fed kale might be due to the presence of oestrogenic material. Using a biological assay technique, Chury (1960) found that the oestrogen content of kale was equivalent to 24 μg. of oestradiol benzoate/kg. kale. The present series of experiments was designed to investigate whether oestrogenic activity could be demonstrated in samples of kale grown under British conditions, in an attempt to provide an explanation for certain effects of high levels of kale feeding on reproductive performance.

EXPERIMENT I: MATERIALS AND METHODS

Kale

The kale was of the marrow stem type, grown on the farm of the University of Nottingham School of Agriculture, Sutton Bonington.

Extraction procedure

The extraction procedure employed was that recommended by Bickoff, Booth, Livingston, Hendrickson & Lyman (1959) with certain modifications. The kale leaves were washed in water to remove soil and aphides and were shaken dry. They were then chopped into pieces about one-quarter of an inch square. Three samples, each of 10·0 g., were taken for moisture determination and 2·0 kg. were macerated in a blender for one minute using 7·0 litres of acetone. The plant debris was filtered off under vacuum and the acetone extract was evaporated under vacuum at 50 to 60°c to a volume of about 150 ml. This

aqueous solution was extracted three times with 300 ml. portions of ether. The ether extracts were combined and taken to dryness under vacuum.

Assay method

The assay method was based on that of Wiberg & Stephenson (1957). Forty-nine intact, immature female Wistar rats, 21 to 23 days old and weighing 38 to 55 g. were caged individually and treatments were allocated at random. The treatments were: 0·15, 0·30 and 0·60 μg. hexoestrol/day; kale extract equivalent to 5·0, 10·0 and 20·0 g. fresh kale/day; and control. The standard hexoestrol and unknown kale extracts were added to the normal rat ration by solution in acetone/ether followed by evaporation of the solvent. The control group received the normal rat ration without addition. The ration was fed at the rate of 6·0 g./rat/day for eight days, being offered in the form of a stiff paste with water to avoid wastage. Twenty-four hours after the last feed the rats were killed in ether, the uteri were removed, cleaned, blotted dry and weighed on a torsion balance.

RESULTS

TABLE I

UTERINE WEIGHT RESPONSE TO FEEDING WITH HEXOESTROL OR KALE EXTRACT

Treatment	*Mean body weight (g.)*	*Mean uterine weight (mg.)*	*Mean uterine weight (mg./100 g. body weight)*
Control (normal rat ration)	48·0	22·6	47·2
Hexoestrol			
0·15 μg./day	44·6	25·4	56·2
0·30 μg./day	44·4	29·1	65·5
0·60 μg./day	45·9	45·3	100·2
Kale extract (fresh kale equivalent)			
5·0 g./day	45·6	21·4	47·0
10·0 g./day	45·6	21·1	46·3
20·0 g./day	47·1	22·5	48·2

Although successive doses of hexoestrol produced increases in uterine weight, amounts of kale extract equivalent to 5·0, 10·0 and 20·0 g. fresh kale/day produced no such response.

EXPERIMENT II: MATERIALS AND METHODS

Kale

The kale used was again of the marrow stem type, grown on the farm of the University of Nottingham School of Agriculture, Sutton Bonington. The kale was stored in a polythene bag at —15°C for 28 days prior to assay.

Extraction procedure

The extraction procedure employed was identical to that in Experiment I. The final ether extract was fractionated on an alumina column (Peter Spence

Type H). The fractions were eluted with successively more polar solvents. This was done to provide information on the types of compound present. The fractions obtained were:

Fraction 1 eluted with petroleum spirit (boiling range 60°C–80°C)
Fraction 2 eluted with 10 per cent ether/90 per cent petroleum spirit
Fraction 3 eluted with 30 per cent ether/70 per cent petroleum spirit
Fraction 4 eluted with acetone.

Assay method

The fractions were assayed separately by the method of Lauson, Heller, Golden & Severingham (1932). Twenty-five intact, immature female Wistar rats, 21 to 23 days old and weighing 30 to 45 g. were divided randomly into five groups. One group was designated control and received arachis oil only. Each of the remaining four was allocated to one of the fractions listed above. Each fraction was taken up in 12·0 ml. arachis oil and the oil was injected subcutaneously at the rate of 0·6 ml./day in two equal doses 12 hours apart for three days. The rats were killed in ether 24 hours after the last injection and the uteri were removed, cleaned, blotted dry and weighed on a torsion balance.

RESULTS

TABLE II

UTERINE WEIGHT RESPONSE TO INJECTIONS OF FRACTIONS OF KALE EXTRACT IN ARACHIS OIL

Treatment	*Mean body weight (g.)*	*Mean uterine weight (mg.)*	*Mean uterine weight (mg./100 g. body weight)*
Control (arachis oil)	49·0	23·2	47·4
Fraction 1	47·7	24·5	49·5
Fraction 2	48·2	21·7	45·4
Fraction 3	50·9	22·5	44·2
Fraction 4	46·6	20·6	44·3

None of the four extracts produced significant increases in uterine weight relative to the control.

EXPERIMENT III: MATERIALS AND METHODS

Kale

Since the data from the first two experiments showed that the samples of kale tested contained no oestrogenic activity as judged by the criterion of increased rat uterine weight, it was decided to obtain a sample of kale from a farm where there was evidence of lowered fertility associated with the feeding of large quantities of kale. With the co-operation of the Ministry of Agriculture Veterinary Investigation Laboratory at Sutton Bonington such a farm was located and a sample of kale obtained for assay. The kale was again of the marrow stem type grown on a farm in the county of Rutland. It was stored in a polythene bag at —15°C for almost three months prior to assay.

Extraction procedure

It was decided to incorporate into Experiment III a comparison of the methods of extraction of plant oestrogens proposed by Robinson (1949) on the one hand and by Bickoff, Booth, Livingston, Hendrickson & Lyman (1959) on the other, since Chury (1960) used the former method when he observed an oestrogenic effect of kale. Eight hundred grams of kale leaves were available for assay. One half of this was treated in a manner identical to that in Experiment II but omitting the fractionation steps. The amounts of solvents used were in proportion to the quantity of plant material.

The remaining 400 g. of kale were treated according to the method outlined by Robinson (1949). The dry extract was taken up in 4·0 ml. ethyl alcohol and added to 16·0 ml. of arachis oil. The alcohol was then removed in a stream of warm air.

Assay method

The assay method used was again that of Lauson, Heller, Golden & Severingham (1932). Twenty-four intact, immature female Wistar rats, 21 to 23 days old and weighing 35 to 45 g. were divided randomly into three groups. One group was designated control and received arachis oil only. The other two groups were allocated to the alcoholic extract and the acetone extract respectively. All other details of the assay were as in Experiment II.

RESULTS

TABLE III

UTERINE WEIGHT RESPONSE TO INJECTIONS OF TWO KALE EXTRACTS IN ARACHIS OIL

Treatment	*Mean body weight (g.)*	*Mean uterine weight (mg.)*	*Mean uterine weight (mg./100 g. body weight)*
Control (arachis oil)	51·2	20·5	40·2
Alcoholic extract (Robinson, 1948)	46·9	18·7	39·4
Acetone extract (Bickoff *et al.*, 1959)	46·2	19·6	42·3

Neither method of extraction produced an extract which caused a significant increase in uterine weight relative to the control.

DISCUSSION

The amount of oestrogenic activity found within a plant species varies from area to area, from season to season and from year to year (Legg, Curnow & Simpson, 1950; Kitts, Swierstra, Brink & Wood, 1959). The factors controlling this variation are not understood fully, but the stage of maturity is known to be important in many of the species studied. For example, clover is most actively oestrogenic in the early growing stages (Kitts, Swierstra, Brink & Wood, 1959), whereas grasses show most activity just prior to the flowering

stage (Legg, Curnow & Simpson, 1950). The effects of climate, soil type and fertilizer treatment are not known.

The fact that oestrogenic activity was not found in any of the samples of kale tested in the present work but was detected in the samples assayed by Chury (1960) may be due to any of the factors quoted above or may be connected with any of the following:

There may be a difference in the variety of kale assayed since Chury made no reference to the type he used. The assay methods employed in the two studies were also different, in that Chury employed a vaginal cornification method in spayed rats using intravaginal administration, and an ovarian weight method in immature mice using oral administration of the extract in oil. The method of extraction used by Chury was that of Robinson (1949) which has since been shown to be unreliable for many plant species. However, the use of this method in Experiment III of the present work provided no different result from that obtained by the extraction method of Bickoff and co-workers (1959).

Failure to demonstrate oestrogenic activity by assay in rodents does not preclude the possibility of an oestrogenic effect in ruminants. It has been shown that certain plant oestrogens are made more active after incubation with rumen fluid (Nillson, 1961*a*) and Moule, Braden & Lamond (1963) have emphasized that whenever possible the assay of plant oestrogens should be carried out in the species of animal showing signs of infertility. The common plant oestrogens, genistein and coumestrol, have been shown to be pro-oestrogens incapable of direct local action (Biggers & Curnow, 1954). The sites of transformation into oestrogens are thought to be the rumen and the liver (Nillson, 1961*a*, *b*). The use of sheep (wethers or ovariectomized ewes) for the assay of plant material for oestrogenic activity liable to cause infertility in ruminants might therefore be advisable, to obtain a final answer to the question.

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Investigation de la possibilite d'une activite oestrogène du chou fourrager (Pickard et Crighton)

Résumé. La possibilité d'un effet oestrogène du chou fourrager a été étudiée par essai biologique d'extraits de chou chez les rats. Aucune activité oestrogène ne fut décélable dans les extraits acétoniques des échantillons de chou administrés par voie orale ou sous-cutanée. Aucune différence ne fut observée entre la réponse à l'extrait acétonique et la réponse à l'extrait obtenu par distillation en alcool alcalin, tous les deux administrés par voie sous-cutanée.

Untersuchungen über die Möglichkeit einer oestrogenen Wirkung von Kohl (Brassica oleracea) (Pickard und Crighton)

Zusammenfassung. Die perorale und subkutane Verabreichung von Azeton-Futterkohlauszügen an Ratten zeigte keinen oestrogenen Effekt. Es gab auch keinen Wirkungsunterschied zwischen der subkutanen Verabreichung von Azetonauszug und subkutaner Verabreichung von alkalischalkoholischem Destillat.

Investigación de la possibilidad de activadad estrógena en la col forrajera (Pickard y Crighton)

Resumen. La posibilidad de un efecto estrógeno de la col ha sido investigado por ensayo biológico de extractos de col en ratas. Los extractos acetónicos de las muestras de col administrados por via orale o subcutanea no produciron evecto estrógeno. Ninguna diferencia en respuesta fué observada entre el extracto acetónico y el obtenido por destilación en alcohol alcalino administrados por via subcutanea.

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MODIFICATION OF THE OESTROUS CYCLE OF THE UNDER-FED RAT INDUCED BY THE PROXIMITY OF A MALE

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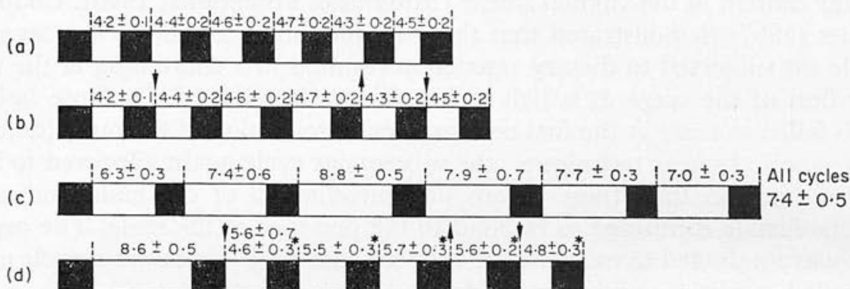
Dietary restriction in the rat has been shown to affect the oestrous cycle, increasing the length of the di-oestrous period and producing a reduction in the cellular content of the vaginal smear (Mulinos & Pomerantz, 1940). Cooper & Haynes (1967) demonstrated that the introduction of a male to the cage of a female rat subjected to dietary restriction resulted in a shortening of the cycle regardless of the stage at which the male was introduced. In those animals which failed to mate at the first oestrus after introduction of the male (detected by the vaginal smear technique), the subsequent cycle again appeared to be of shorter duration than those before the introduction of the male, indicating that the female continued to respond to the presence of the male. The present work was conducted to examine the induced changes in the oestrous cycle under controlled conditions and in more detail.

Eighty nulliparous female rats from a closed colony of Wistar origin were allocated to the experiment. They were 12 weeks old, weighed 120 to 160 g and had completed at least two normal cycles. The rats were caged individually under a lighting regimen of 12 hr light/12 hr dark, the period of light being from 08.00 to 20.00 hours. The females were allocated randomly into two groups, one of twenty rats on unrestricted feeding and one of sixty rats fed 65% of the previous mean daily intake of the group on unrestricted feeding. Each group was divided into two equal sub-groups, to one of which males were to be introduced (Text-fig. 1). Daily vaginal smears were prepared from all females, the cycle being classified into the stages, pro-oestrus, oestrus, metoestrus and di-oestrus. Of the animals subjected to dietary restriction, five became anoestrous and were discarded. Males selected for introduction to the cages of the experimental females were of the same strain as the females, 6 to 9 months old and of proven fertility. In order to prevent mating and to differentiate the possible effects of copulatory behaviour from those of male proximity, physical contact was minimized by dividing each cage in half by a ½-in. wire mesh barrier Males were in close proximity to the experimental females for periods of 16 hr in each 24 hr from 17.00 to 09.00 hours. During the day, the males were caged in groups. Each male was marked so that the same individual could be introduced each night into the cage of the same experimental female.

Introduction of the male to the female under dietary restriction was carried

out as soon as a metoestrous smear was detected in the first cycle to occur following a di-oestrous period of at least 5 days. In the group on unrestricted feeding, introduction of the male was carried out as soon as metoestrus was detected in a normal cycle. Introduction of males to both groups was synchronized as far as possible. After three cycles, daily introduction of the male was terminated for a period of one cycle, following which a second male of the same strain was introduced as before for a period of one cycle. The changes in mean cycle lengths for each group are shown in Text-fig. 1. The significance of differences was assessed by the t test. The introduction of the first male, its removal for one cycle and the subsequent introduction of a second male had no significant effect on the mean cycle lengths of animals on unrestricted feeding (Text-fig. 1, a and b). The introduction of the first male to the females on restricted diet, however, resulted in a marked reduction in mean cycle length when the mean for the first cycle after introduction in all twenty-seven females to which the male was introduced was compared with the overall mean for



TEXT-FIG. 1. Cycle lengths (mean ± S.E.) of rats on the various treatments. (a) Unrestricted feeding, control; (b) unrestricted, male introduced; (c) restricted, control; (d) restricted, male introduced. Arrows indicate male introduced (↓) and removed (↑) Shaded areas represent pro-oestrus + oestrus + metoestrus, unshaded areas represent di-oestrus. Asterisks indicate that the mean and S.E. were calculated from those animals which showed a reduction in cycle length in response to the introduction of the first male.

control animals (i.e. of six cycles from each of twenty animals) ($P < 0·05$). When the mean cycle length immediately after introduction of the male was compared with the mean length of the last cycle immediately before introduction, there was also a marked reduction ($P < 0·001$). This response occurred in twenty-four out of twenty-seven animals. Of three cycles following the introduction of the male to these twenty-four animals, the third cycle was significantly longer than the first ($P < 0·01$) although all three showed the effect of the proximity of the male in that they were of a shorter duration than those before the introduction of the male. Removal of the male for one cycle did not result in any significant change in cycle length. The introduction of the second male again significantly reduced the mean cycle length compared with the cycle immediately before that introduction ($P < 0·05$) (Text-fig. 1, c and d) although this response was only seen in seventeen out of the twenty-four animals which had shown a shortening of the cycle in response to the introduction of the first male.

The present work confirms and extends the findings of Cooper & Haynes

(1967) in that an immediate and marked reduction in the mean oestrous cycle length of female rats subjected to dietary restriction was induced by the proximity of the male. Copulatory behaviour was not a necessary factor in the response. Although the response was less apparent in subsequent cycles, the female appearing to become less sensitive to the proximity of the male, the imposition of a period during which the male was not introduced daily, followed by the random re-introduction of males, resulted in a second demonstration of the response. The effects of physical contact were not completely excluded in this experiment and it is not yet possible to state which factors associated with the presence of the male were responsible for inducing these changes in the cycle.

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THE EFFECTS OF CASTRATION AND ANDROGEN REPLACEMENT THERAPY ON THE ABILITY OF THE MALE TO INFLUENCE THE OESTROUS CYCLE OF THE UNDERFED RAT

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It is established that stimuli associated with the presence of a sexually mature male can influence the oestrous cycle of the underfed rat (Cooper & Haynes, 1967). This effect is still present when physical contact between the sexes is prevented, the lengthened and irregular cycles characteristic of undernutrition becoming significantly shorter (McNeilly, Cooper & Crighton, 1970; Purvis, Cooper & Haynes, 1971). A similar response to the proximity of the male has been demonstrated in the mouse, the lengthened and irregular oestrous cycles usually observed when mice are grouped not being found in the presence of a male (Whitten, 1957). Castration of the male prevented the acceleration of oestrous behaviour, but the stimulus value of the male for the female returned following androgen therapy (Bronson & Whitten, 1968). The object of the work described here was to determine whether the reduction in oestrous cycle duration induced in underfed rats by the proximity of a male was also androgen-dependent.

All the rats used were weaned at 21 days of age and housed in groups of ten to twelve in large communal cages until selection. The female rats were allocated to the experiments when they were approximately 5 months of age and weighed 150 to 200 g. The male rats were selected at approximately 6 months of age and weighed 250 to 300 g. Castration of a number of males was carried out when they were 28 days old. During the experimental period, all animals were housed individually in metal battery cages. Each cage was divided into two equal parts by means of a wire barrier as described by McNeilly *et al.* (1970) in order to minimize direct physical contact between animals.

Two experiments were carried out. In Exp. 1, forty female rats were allocated at random to the following treatment groups: ten animals were allowed unrestricted access to food. The remaining thirty animals were subjected to a restricted dietary regimen consisting of 65% by weight of the mean daily food consumption of the unrestricted group. Ten of these animals were fed this restricted diet only, ten were underfed in proximity to intact males and ten were underfed in proximity to castrate males. For Exp. 2, twenty female rats were allocated at random to two treatment groups. Ten animals were fed a restricted diet as described in Exp. 1 in proximity to an intact male and the

remaining ten animals were underfed in proximity to a castrate male which had been given a subcutaneous injection of testosterone propionate (30 μg/100 g body weight) each day for a period of 14 days preceding introduction to the cages in which the females were housed.

From the time of the first vaginal smear, all females were allowed to complete two oestrous cycles on an unrestricted feed intake and the underfed females completed either two or three (Exp. 1) or three (Exp. 2) oestrous cycles when subjected to dietary restriction. Thus, changes in dietary regimen were made

Table 1

DURATIONS OF OESTROUS CYCLE IN RATS (EXPERIMENT 1)

Sequential treatments	*Duration of oestrous cycle (days)*			
	Exposed to males		*Controls*	
			Underfed throughout	*Unrestricted throughout*
Unrestricted feeding for two oestrous cycles	4·2±0·40 4·6±0·18	3·6±0·24 4·1±0·20	4·6±0·18 4·0±0·24	4·1±0·24 4·8±0·20
Underfeeding for two oestrous cycles	5·3±0·29 5·0±0·34	5·0±0·41 5·3±0·29	4·9±0·35 4·6±0·31	4·0±0·21 4·6±0·26
	Intact males	*Castrate males*		
Underfeeding + male for one oestrous cycle	3·7±0·29	5·6±0·41	5·8±0·43	4·3±0·24 4·5±0·34

Table 2

DURATIONS OF OESTROUS CYCLE IN RATS (EXPERIMENT 2)

Sequential treatments	*Duration of oestrous cycle (days)*	
Unrestricted feeding for two oestrous cycles	4·0±0·15 4·2±0·26	4·5±0·22 4·4±0·43
Underfeeding for three oestrous cycles	4·9±0·46 5·0±0·71 5·0±0·56	5·9±0·71 7·2±0·75 6·9±0·51
	Intact males	*Castrate males after androgen therapy*
Underfeeding + male for one oestrous cycle	3·8±0·28	4·3±0·33

at different stages in the cycle in different individuals. The males were placed in proximity to the females following this period of four or five oestrous cycles for the duration of one cycle, introduction being made on the day of metoestrus in each case, following which the experiment was terminated. The males were introduced into the battery cages overnight for a total of 15 hr (from 18.00 to 09.00 hours) in each 24 hr, the pattern previously established by Cooper & Haynes (1967) and McNeilly *et al.* (1970). During the intervening period, all males were housed in communal cages and selected at random for introduction to the cages of the females each evening since previous work showed that females

appeared to become less sensitive to the presence of a particular male with time (McNeilly *et al.*, 1970). Oestrous cycles were monitored by the examination of daily vaginal smears.

The results of the two experiments are summarized in Tables 1 and 2. Statistical evaluations were by analysis of variance. Underfeeding for two oestrous cycles caused an overall significant increase in cycle length ($P<0{\cdot}05$). The proximity of intact males for one oestrous cycle significantly shortened this cycle length to a value more closely associated with that found in unrestricted animals ($P<0{\cdot}01$). This response to the male was not observed when underfed females were in proximity to castrate males, but was obtained with castrate males following the period of androgen replacement therapy ($P<0{\cdot}01$).

The experiments described confirm the observation of Cooper & Haynes (1967) and McNeilly *et al.* (1970) that a marked shortening of the duration of the oestrous cycle occurs when an underfed rat is placed in proximity to a male. The ability of the male to induce the acceleration of oestrus is dependent upon the presence of testes, the response not being apparent after castration. The finding that androgen replacement therapy is able to restore the stimulus value of the male for the female is similar to that in experiments carried out on mice (Bronson & Whitten, 1968) and indicates that whatever mechanism is responsible for the normal expression of male sexual stimuli, it is probably to a large extent dependent upon testicular steroid secretion. It is not possible to state with certainty the exact nature of the stimulus which can be transmitted from one animal to another under conditions where physical contact was not completely excluded but it seems likely, on the basis of comparable experiments carried out with mice, that this is largely pheromonal.

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PROCEEDINGS OF THE SOCIETY FOR THE STUDY OF FERTILITY
ABSTRACT OF PAPER PRESENTED AT ANNUAL CONFERENCE,
13th TO 17th JULY 1971

Peripheral steroid hormone levels and nitrogen retention in the gilt from prepuberty to parturition. I. J. SHEARER, N. B. HAYNES and D. B. CRIGHTON, *Department of Physiology and Environmental Studies, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leics. LE*12 5*RD*.

This paper describes an investigation into the control of nitrogen retention by progesterone and oestradiol-17β in the female pig. It was instigated in view of the proposed anabolic action of progesterone during pregancy in this species (Rombauts & du Mesnil du Buisson, 1964) and the opposite catabolic influence in man during the normal menstrual cycle (Landau & Lugibihl, 1961).

Daily plasma progesterone determinations and nitrogen retention studies were performed on gilts at various stages of the reproductive process. In addition, plasma oestradiol-17β concentrations were measured in some animals. Blood was collected from in-dwelling cannulae and competitive protein binding assays were used for steroid estimations.

Prepubertal nitrogen retention was 245·7±2·8 mg/kg body wt/day and mean progesterone concentrations of 2·4 to 0·2 ng/ml plasma were found. During oestrous cycles, the nitrogen retention (124·5±8·8 mg/kg body wt/day) was significantly lower than that found during prepuberty and showed more pronounced fluctuation. There were no correlations between nitrogen retention data and progesterone changes (30 to 50 ng/ml during the luteal phase and 2 to 5 ng/ml around oestrus) or oestradiol-17β changes (10 to 90 pg/ml on the day before oestrus). Through early pregnancy, nitrogen retention (73·5±5·9 mg/kg body wt/day) remained relatively constant despite progesterone changes from luteal phase levels to 15 to 20 ng/ml by Day 26. By Day 90 of pregnancy nitrogen retention had risen (90·0±5·8 mg/kg body wt/day) and remained constant to Day 110. During this time progesterone levels rose from 15 ng/ml to 40 ng/ml, fell to 12 ng/ml during parturition and remained low (2 to 5 ng/ml) during lactation. Plasma oestradiol-17β concentration rose from 20 pg/ml on Day 112 to a value greater than 200 pg/ml on Day 114 of pregnancy and fell to 25 pg/ml by the day of parturition (Day 116). A further peak of the same magnitude occurred during Days 6 and 7 of lactation. In two abnormal animals, one having an abortion on Day 23 and one an anovulatory oestrus, there were no correlations between nitrogen retention and fluctuations in progesterone concentration.

In summary, the significant fall in nitrogen retention and the greater variability in the cyclic animal compared to the prepubertal state suggests a hormonal control of nitrogen retention triggered at puberty which is catabolic. However, the lack of correlation between nitrogen retention and progesterone levels throughout implies that progesterone in fact, is not mainly responsible for the hormonal control of protein metabolism in the gilt, and if a relationship between hormone output and nitrogen retention exists, it is a complex one.

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