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**The Assay of Growth Hormone and Gonadotrophins
in Relation to Clinical Problems**

by

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ABSTRACT OF THESIS

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clinical problems.

Since the early 1900's, many investigators have studied the effects of pituitary ablation and the mode of action of the hypophyseal hormones. Initially, work was mainly directed towards the purification and bioassay of the various hormones, although the effects of hormone administration were also studied. Recently, attempts have been made to synthesise some of the hormones. The aim of this thesis is to describe a series of studies undertaken in an attempt to develop new assay methods for growth hormone and the gonadotrophins and the application of these procedures. (89)

An investigation of the bioassay for growth hormone depending on the increase in tibial epiphyseal cartilage width in immature hypophysectomised rats has shown that the method is not specific and is of low sensitivity. The procedure has been used to compare the potency of pituitary extracts from different species and to provide a measure of the effect on body growth and on cartilage width of two synthetic compounds and of nerve section. A dithiocarbamoylhydrazine derivative, Compound 33, 828 (I.C.I.) was found to have a markedly inhibitory effect on general body growth and cartilage width, possibly due to the toxicity of the compound. A synthetic polypeptide, Ciba 30920-Ba which is claimed to have an adrenocorticotrophic hormone-like action on the adrenal, had no marked effect on cartilage growth. It has also been shown that the artificial induction of muscular atrophy in young rats by section of the sciatic nerve did not interfere with cartilage growth and that the administration of pituitary hormones to animals treated in this way was without effect. (166)

A haemagglutination-inhibition method has been developed for the assay of growth hormone and has proved to be sensitive and highly specific. When estimates of the growth hormone potency of standard pituitary preparations were made by both the bioassay described above and the immunological method, similar results were obtained. The immunological procedure was, however, found not to be sufficiently sensitive for clinical application. A latex particle agglutination-inhibition method for the quantitative determination of human chorionic gonadotrophin has also been developed.



This again proved to be unsuitable for clinical application. (88)

The pyruvic acid content of the immature rat ovary, both prior to and following gonadotrophic stimulation, was estimated by two different methods. A marked rise in pyruvic acid was noted following initial stimulation with pregnant mare serum gonadotrophin, but this rapidly fell to a low level which could not be altered by further gonadotrophic stimulation. The relationship between pyruvic acid and gonadotrophic stimulation is discussed. (65)

The studies reported in this thesis have shown that the methods available for the quantitative determination of growth hormone and gonadotrophins are not entirely satisfactory because of poor sensitivity or lack of specificity. However, despite these limitations, useful information can be obtained by these procedures although it is clear that they are not suitable for clinical application. The development of more sensitive and specific methods for the estimation of these hormones is therefore necessary and it is suggested that future work in this field should be directed towards this end. (90)

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CHAPTER I

GENERAL INTRODUCTION.

Growth Hormone

As far back as 1886, Marie defined the disease state of acromegaly and Minkowski, (1887) noted that it was usually associated with an enlargement of the pituitary. It was, however, not until Ashner (1909, 1910 & 1912) observed that hypophysectomy in young animals resulted in dwarfism, that the existence of a pituitary secretion which enhanced body growth was recognised; at the same time, atrophy of the genital organs in hypophysectomised dogs established the presence of a gonadotrophic secretion from the pituitary (Ashner, 1912). The first definite proof of the existence of this growth hormone came in 1921 when Evans and Long produced giant rats by daily injections, over long periods, of an extract of ox pituitary. In 1922, these workers reported that in their crude pituitary extracts, the growth promoting factor was chemically distinct from the gonadotrophic factor.

Over the next 30 - 40 years work was mainly directed towards the development of assay methods and to the purification of growth hormone. During the last 5 - 10 years attempts have also been made to synthesise the hormone and to develop more sensitive methods of assay which could be applied to its measurement in body fluids.

Assays Dependant on Weight Gain

The classical methods for the assay of growth hormone

depend on its ability to cause growth. Thus, as early as 1931, Evans and Simpson suggested that a possible procedure was to measure the increase in body weight of normal 'plateaued' rats following the administration of growth hormone. Details of sensitivity and precision were not, however, reported. Full methodological details were later given by Marx, Simpson and Evans in 1942. They reported that good results were obtained when young adult rats of between 5 - 6 months in age and weighing 220 - 280 g. were used. The hormone was administered daily for 15 - 20 days and the minimum effective dose was 250 μ g./day. It was shown that the 20-day injection period gave a slightly steeper dose response curve, but figures for the index of precision (λ) were not quoted. The method was subsequently shown to be reasonably satisfactory with regard to its precision and specificity (Fónss-Bech, 1947; Greenspan, Li, Simpson and Evans, 1950). The chief disadvantages of the procedure are its high degree of insensitivity and the lengthy injection period required, while its main advantage is that the assay requires intact rather than hypophysectomised rats.

In 1942, Marx and his co-workers reported that the weight gain test as described by Evans and Simpson (1931) could be made more sensitive by the use of immature hypophysectomised rats instead of adult animals. In this procedure approximately 28 day old rats were hypophysectomised, rested for 10-12 days and then given daily intraperitoneal injections of growth hormone for 15-20

days. The minimum effective dose was 30 $\mu\text{g.}/\text{day}$ and as with the original technique the 20-day injection period was found to give a steeper dose response curve than did the 15-day period. Figures for the index of precision were not quoted, but the method was considered to be somewhat less precise than that using plateaued rats. Its main disadvantages lie firstly in the use of hypophysectomised animals and secondly, in its lack of sensitivity which makes it unsuitable for clinical application.

Attempts have also been made to develop a similar assay method using intact dwarf mice by Fønss-Bech (1947) and Kemp (1948). It is difficult to estimate the sensitivity of the procedure because the results were expressed in terms of animal units. It is clear, however, that thyroid-stimulating hormone (TSH) interferes with the method. A 2-3 week injection period is required.

More recently, Lostroh and Li (1957) noted that the hypophysectomised mouse increased in weight when growth hormone was administered daily for 17 days. The minimum effective dose was 15 $\mu\text{g.}/\text{day}$. No attempt has, however, been made to develop this as an assay method.

Increase in Tail Length

In 1938, Freud and Levie suggested that the increase in tail length of the hypophysectomised rat might form the basis of an assay for growth hormone. In this procedure the animal was X-rayed before and after the administration of the hormone and from the resultant skiagrams the increase in tail length, from the

beginning of the first caudal vertebra to the tip of the tail was measured. Later, Fønss-Bech (1947) made a careful study of this method and Dingemans, Freud and Uyldert (1948) used it to measure the 'chondotrophin' (i. e. growth hormone) content of human serum. From these studies it is difficult to estimate the sensitivity of the method, it would, however, appear to be low and there is every indication that it is not specific. More recently, de Groot (1963) has developed a modified method based on the synergistic effects of thyroxine and growth hormone. She claimed that using a 3 week injection period, the effective dose range was 5 - 20 µg. GH/day when administered together with 6 µg. thyroxine/day.

Tibial Test

In 1941 Kibrick, Becks, Marx and Evans noted that if growth hormone was administered to hypophysectomised immature female rats at dose levels which did not have a significant effect on body weight, a significant increase in the width of the epiphyseal cartilage could be detected. It was, however, not until 1943 that this method which is known as the 'tibial test' was first cited as a possible bioassay for growth hormone by Evans, Simpson, Marx and Kibrick. At this time it was considered to be more specific, sensitive and practicable than the weight gain test in plateaued or hypophysectomised rats. It was also shown that with a 4-day injection period the minimum sensitivity was approximately 40 µg./

rat. A full investigation of the assay was not carried out until 1949 when Greenspan, Li, Simpson and Evans studied the specificity of the procedure and attempted to define the limits within which the assay could be successfully used. In the original method a lengthy staining technique was employed prior to the measurement of the epiphyseal cartilage width. This was successfully simplified by Greenspan et al. (1950) and their method is still the procedure of choice.

By 1953, it had become apparent that the tibial test was too insensitive for clinical application and was probably not specific. Li (1953) suggested that the ideal test animal should be thyroidectomised, adrenalectomised, gonadectomised and hypophysectomised. Such animals are, however, extremely difficult to obtain and to keep alive and although the precision of the assay was greatly improved, its sensitivity was considerably reduced. For this reason animals of this type have not been used for routine clinical studies.

Work in this field continued in the hope that greater sensitivity and precision would be obtained if the conditions of assay and parameters measured could be correctly adjusted. In 1957, Lostroh and Li reported a non-specific assay method using hypophysectomised mice in which greater sensitivity ($< 1 \mu\text{g./day}$) was obtained, but only at the expense of a much longer injection period. The procedure has, however, not been widely used. The response was still affected by other hormone preparations.

Denko and Bergenstal (1955) explored the possibility of measuring the uptake of S-35 into the chondroitin sulphate of the tibial epiphyseal cartilage, and in 1960, Collins and Baker described an assay method for growth hormone depending on the uptake of S-35 by the costal cartilage. Collins, Lyster and Carpenter (1961), in a comparison of their S-35 uptake method and the tibial test, claimed that the former technique was more sensitive, precise and specific. The effective dose range was 3 - 20 $\mu\text{g.}/\text{day}$ and 5 - 30 $\mu\text{g.}/\text{day}$ respectively; the index of precision was 0.25 and 0.45 respectively. It is, however, of interest to note that Tweed and McCullagh (1962) in a careful study of the same two assay techniques found very poor correlation between the results obtained by the tibial test and the method dependant on S-35 uptake.

A further modification of the tibial test is the P-32 uptake technique of Hamori and Mess (1962). This autoradiographic procedure appears to be somewhat simpler to carry out than the S-35 method. It is claimed to be specific and to be three times more sensitive than the tibial test. Its great advantage lies in the use of intact neo-natal rats instead of hypophysectomised animals. Rats of this age have virtually no calcified bone cells in the epiphyses and a comparison of the conventional tibial test and the P-32 method cannot be undertaken in the same animal.

In the first section of this thesis are presented the results of a study on the tibial test (Chapter 2). The specificity of the

method has been reappraised with particular reference to the effect of the other anterior pituitary hormones, to adrenalectomy and to the food intake of the test animals. The procedure has been applied to the quantitative estimation of the potency of various pituitary extracts obtained from different species, to assessing the biological effects of two new synthetic compounds, and to a study of the effects of certain surgical procedures on growth of the tibial cartilage.

Cellular Growth

In 1962, Moon, Jentoft and Li showed that when human liver cells were grown in a culture medium, they multiplied more rapidly in the presence of human growth hormone, HGH, and that the increase in the number of cell nuclei was proportional to the amount of growth hormone used; the minimum dose being 75 $\mu\text{g.}$ /culture flask. The increase in nitrogen which was also measured did not provide such a good index.

The procedure was said to be highly specific for HGH, growth hormone from other species being ineffective. Attempts to repeat this work in this laboratory were unsuccessful due to the failure to establish a viable culture of human liver cells. Although highly insensitive it was hoped that this method might have provided a means of standardising HGH preparations which would have obviated the use of hypophysectomised animals.

Chemical Methods

Several workers have attempted to develop chemical methods

of assaying growth hormone. These have all depended on the ability of the hormone to alter certain metabolic reactions which could be measured chemically. The most frequently studied have been :

- a) the retention of nitrogen,
- b) the reduction in blood - non - protein - nitrogen,
- c) the rise in plasma non-esterified fatty acids (NEFA),
- d) the hypoglycaemic effect.

None of these procedures has provided a sensitive, accurate or specific measure of growth hormone. Some of the methods have, however, been used clinically to ascertain the effectiveness of administered growth hormone. Thus it has been assumed that a patient receiving growth hormone in whom a rise in plasma NEFA or a nitrogen retention has been noted, showed an anabolic response to the administered hormone.

It can be seen that the detection and assay of growth hormone by biological methods is usually a long process involving laborious and often non-specific techniques, and is quite unsuitable for application to the measurement of the hormone in body fluids. However, in recent years the development of new and sensitive immunological techniques has opened up a new and promising field of investigation. For these reasons attempts have been made to develop sensitive immunological assays for growth hormone some of which will be discussed below.

f Production of Antisera to Pituitary Extracts

During the 1930's, Witebsky and Behrens (1932) and Kestner (1938) attempted to obtain antisera to anterior lobe pituitary extracts. They were successful in obtaining low titre antisera which were, however, not specific. This lack of specificity was primarily due to the use of crude pituitary homogenates as the antigen. Work of this type has been continued in recent years by Cruickshank and Currie (1958) who also failed to obtain specific antisera and by Anigstein, Whitney and Rennels (1958) and by Anigstein, Rennels and Anigstein (1960) who succeeded in characterising some of the antibodies present in an antiserum prepared against the anterior lobe of the rat pituitary. In particular, they found antibodies specific to growth hormone and to gonadotrophins, and suggested that weaker antibodies to ACTH and TSH might be present.

Apart from this work on crude pituitary extracts, antisera have also been prepared to highly purified pituitary extracts, in order to ascertain the immunological homogeneity of such preparations and as an index of the structural similarity of extracts from different species.

Immunological Assay of Growth Hormone

Hayashida and Li (1958 abc) carried out anaphylactic shock experiments, precipitin tests and an antihormone experiment based on the tibial test, in an attempt to study the immunological properties of beef and human growth hormone antisera. These

workers suggested that the development of a precipitin test might provide an alternative assay method for growth hormone which would be more sensitive and specific than the tibial test. They claimed to be able to detect as little as 1 μ g. beef growth hormone and 0.1 μ g. human growth hormone by such a procedure.

At the same time Read and Stone (1958) developed an immunological method based on a haemagglutination-inhibition technique, using the ultra-sensitive antigen-tagged red cells prepared according to the method of Boyden (1951). They claimed to be able to detect as little as 0.001 μ g. of a human growth hormone preparation supplied by Raben (Boston, U.S.A.). Further papers by Read and Bryan (1960 ab) gave details of the method, the statistical method used to evaluate the results, specificity tests employed and the values for human sera obtained.

Read and Stone's preliminary report proved to be a great impetus to other workers, and in the years immediately following there have been an increasing number of reports on the immunological assay of growth hormone. Initially, the method used was Read's haemagglutination-inhibition assay and serum growth hormone levels estimated in this way have been reported by Boucher and Mason (1961), Dominguez and Pearson (1962), Ehrlich and Randle (1961 abc), Hartog and Russell Fraser (1961), Hayashida (1962) and Kaplan and Grumbach (1962). The results obtained by these workers, while in general agreement, show considerable variability, the actual values obtained are quoted in Chapter 3

Table 29. It is of interest that all the workers who used this procedure find it to be unsatisfactory.

When it became apparent that the haemagglutination-inhibition method was not satisfactory for the estimation of growth hormone in biological fluids, other immunological techniques were tried. In 1961, Trenkle, Moudgal, Sadri and Li reported that human growth hormone could be estimated by a complement fixation technique. This did not, however, prove sensitive enough for clinical application.

More recently, radio-immunological assays have been developed. In these assays, radio-isotopes are used to define the end-point of antigen/antibody reactions instead of the more conventional immunological end-points such as the formation of a visible precipitate, agglutination or haemolysin reactions. The principle behind this assay lies in the fact that the antibodies present in antiserum react quantitatively with the appropriate antigen. If the antigen present comes from more than one source the antigens from the different sources will compete with each other for the binding sites in proportion to the amounts originally present. A source of antigen is therefore labelled with a radio-isotope (usually I-131), and the amount of this required to give 80 - 100% binding of the antibodies ascertained. This amount of activated antigen is then incubated with the test sample and a predetermined amount of antiserum. At the end of the incubation period the free and bound antigen fractions are separated and counted. The amount of

added unactivated antigen can then be calculated by reference to a standard curve.

Using this basic theory two different methods of assay have been developed. The first to be described were those of Greenspan, Cofell, Lew and Peng (1962) and Utiger, Parker and Daughaday (1962) in which the end-point is an activated precipitin reaction. The bound antigen is obtained in the form of a precipitin complex, while the free antigen remains in the supernatant. This method has a minimum sensitivity of about 3 mug. and a working range of up to 400 mug.; it has been applied to clinical problems. The second method is derived from the Yalow and Berson (1960) technique for insulin. In this method the free and bound antigens are separated from each other by electrophoresis on cellulose acetate. It has been developed independantly by Glick, Roth, Yalow and Berson (1963) and Hunter and Greenwood (1962). A more detailed report by the later workers was published two years later (Greenwood, Hunter and Klopper, 1964). This procedure has a minimum sensitivity of about 1 mug. and a working range of between 1 - 20 mug.; it gives results which correspond well with the tibial test and has been applied to clinical problems.

Application of Immunological Techniques to Endocrinology.

Immunological techniques were first applied to the field of endocrinology by Stavitsky and Arquilla (1953) who were trying to establish the presence of insulin antibodies in patients exhibiting insulin resistance, and subsequently developed the first hormone

immunological assay. In the years following the publication of Read's method for the immunological assay for growth hormone, in addition to the work on growth hormone and insulin many workers have reported the successful production of antisera to other hormones. Thus, antisera have been prepared to ACTH (Fishman, McGarry and Beck, 1959; McGarry, Ballantyne and Beck, 1962), Prolactin (Laron and Asa, 1963; Levy and Sampliner, 1961, 1962), TSH (Fishman et al., 1959; Hayashida, Rankin, McClelland and Contopoulos, 1961) and the gonadotrophins (Gold, Castillo, Baram and Scommegna, 1962 and Segal, Laurence, Perlbachs and Hakim, 1962 who worked with NIH - FSH & LH; Moudgal and Li, 1961 abc - sheep and human LH; McGarry and Beck, 1963 - human FSH; Butt, Crooke, Cunningham and Gell, 1960 and Butt, Crooke and Cunningham, 1961 - HMG and HCG; Brody and Carlstrom, 1960, 1961 & 1962, Lunenfield, Isersky and Shelesnyak, 1962, Taymor, Goss and Buytendorp, 1964, Wide and Gemzell, 1960 and Wide, 1962 who all worked with HCG).

Immunological Aspects of Human Chorionic Gonadotrophin

This hormone is produced in large quantities during early pregnancy and the immunological work in this field has been concentrated on firstly, the production of a rapid qualitative test for pregnancy and secondly, the development of a rapid quantitative method for the measurement of HCG in normal and abnormal pregnancy.

In 1959, Got, Levy and Bourrillon published their findings on the preparation of antisera to HCG. Three preparations exhibiting different levels of purity were used and it appeared that the antigenic impurities present (in the antisera formed) were of human protein origin and could be eliminated by prior absorption of the antisera with normal human serum.

Since 1960, Brody and Carlstrom have published a series of papers dealing with the immunological assay of HCG by a modified complement fixation test. These workers have estimated (1962) the serum HCG in 474 subjects at intervals from the 5 - 40th week of pregnancy, and claim that their values are in agreement with those of Loraine (1957). It would appear, however, that the results obtained were in general higher than those of Loraine (1957). In 18 patients with threatened abortions who subsequently aborted and 5 subjects with ectopic pregnancies abnormally low HCG levels were found, while in 15 patients with threatened abortions but good prognosis and subsequent normal pregnancies, HCG levels which were within the normal range were observed.

Over the same period of time, Wide and Gemzell, (1960, 1962) have attempted to prepare an antiserum to HCG and to estimate HCG in urine using a haemagglutination-inhibition technique. Their antiserum was not as pure as that of Brody and Carlstrom and the results obtained by these workers, which are probably inaccurate, appear to be marked over estimates. Their immunological values are four times higher than the value obtained by

bioassay, although the overall pattern of their results is similar to that of other workers, with peak levels of excretion occurring between the seventh and the twelfth week of pregnancy. They reported values below the normal range in 33 cases of spontaneous abortion and levels within the normal range in 39 cases of threatened abortion.

Unlike Wide and Gemzell, Keele, Remple, Bean and Webster (1962) were able to prepare a very pure HCG antiserum which showed no cross reactions with human FSH or LH, although it did show a cross reaction with human seromucoid. This antiserum was capable of neutralising the biological activity of HCG both in vivo and in vitro. When used to diagnose pregnancy, 26 cases were studied and all gave positive reactions when tested between the 6th and 14th weeks.

At the same period of time, Lunenfeld and his co-workers studied the immunological properties of HCG. In 1962, they reported the preparation of an antiserum to HCG which was capable of neutralising the biological activity of HCG both in vitro and also in vivo. This antiserum contained antibodies to serum proteins which were serologically active but incapable of neutralising the biological activity of HCG. It was suggested that this contamination could cause the results obtained by immunological means to be considerable overestimates of the true potency. In a later study (Issersky, Lunenfeld and Shelesnyak, 1963) they prepared an HCG antiserum which was capable of neutralising the follicle-stimulating activity of

a human post-menopausal urine gonadotrophin (HMG) preparation and an HMG antiserum which was able to neutralise the follicle-stimulating activity of HCG. This means that both HCG and HMG have a common follicle-stimulating antibody but whether this is due to a contamination of both these preparations by FSH or indicates the presence of a common follicle-stimulating principle inherent in the molecule was not established by the experiments reported.

Immunological Pregnancy Tests

At least three such tests are now commercially produced. A test based on the agglutination of latex particles is marketed by Ortho Pharmaceutical Ltd. while tests based on the agglutination of prepared red cells are marketed by Organon Laboratories Ltd. under the name 'Pregnosticon' and by Burrough - Welcome under the name 'Puerpuerin'.

The Ortho Company claim an accuracy of $97.77 \pm 0.8\%$ in a series of 1,390 cases, which agrees well with the figures of 97.99% and 94.80% obtained by the Xenopus toad test (1,193 cases) and the rabbit (328 cases) respectively. Unlike the toad and rabbit tests, the incorrect results were mainly due to false negatives (2.09%). This test has been used by a number of groups in the U.S.A. and found to be a satisfactory method for the diagnosis of pregnancy. Goldin (1962) reports 94.9% accuracy in 39 cases; Henry and Little (1962) 95% accuracy in 40 cases, the latter workers got no false positive results in 48 non-pregnant

women ranging in age from under 18 to over 50, while in a series of 54 men, the 2 subjects presenting false positive results had scrotal abscesses.

Raj, Bayron, Waltman and Green (1963) claimed 100% accuracy using the 'Pregnosticon' test as against 93.5% accuracy using the rat ovarian hyperaemia test. They reported that in a series of 545 pregnant subjects and in 560 non-pregnant subjects no inaccurate results were obtained by the immunological diagnostic test while only 422 correct pregnancy results and 506 correct non-pregnancy results were obtained by the rat ovarian hyperaemia test in the same subjects.

While immunological pregnancy tests appear to be an inexpensive, rapid, simple to perform and reasonably accurate method of diagnosis; it must, however, be emphasised that in their present form only qualitative and no quantitative results can be obtained.

In the second part of this thesis is presented the results of an immunological study of growth hormone and of the gonadotrophins. Attempts were made to raise antisera to these hormones. The haemagglutination-inhibition assay was established for growth hormone and applied to the estimation of the growth hormone potency of human pituitary fractions and human serum. The result of an attempt to modify the qualitative latex agglutination-inhibition pregnancy diagnosis test to form a quantitative assay for HCG are also presented.

Immunological Assay of FSH and LH

A number of workers have attempted to prepare antisera to FSH and LH of both human and ovine origin. The work in this field has been badly hampered by the lack of pure human or ovine FSH and LH preparations.

Moudgal and Li, (1961 abc) have prepared specific antisera to highly purified preparations of human and ovine LH, while Segal et al. (1962) have prepared reasonably specific antisera to NIH-FSH-SI and NIH-LH-SI. In spite of the preparation of these highly specific antisera, reliable immunological assay methods for FSH and LH have not yet been established, although Goss and Taymor (1962) claim to be able to measure human LH quantitatively using the Ortho Pregnancy Kit. They report good agreement between the results obtained by the ventral prostate and immunological methods when pituitary extracts are assayed, but not with urinary extracts; they also claim that human FSH and ovine LH do not interfere in this system.

At the present time, the most sensitive and specific methods of estimating FSH and LH are biological in nature. The most satisfactory of these are the ovarian augmentation test in intact weanling mice (Brown, 1955) which is claimed to be specific for FSH (Simpson, 1961) and has a sensitivity of approximately 15 μ g. NIH-FSH-SI, the ovarian ascorbic acid depletion test (Parlow, 1958, 1961) and the ovarian cholesterol depletion test (Bell, Mukerji and Loraine, 1964) which are both performed in intact immature rats

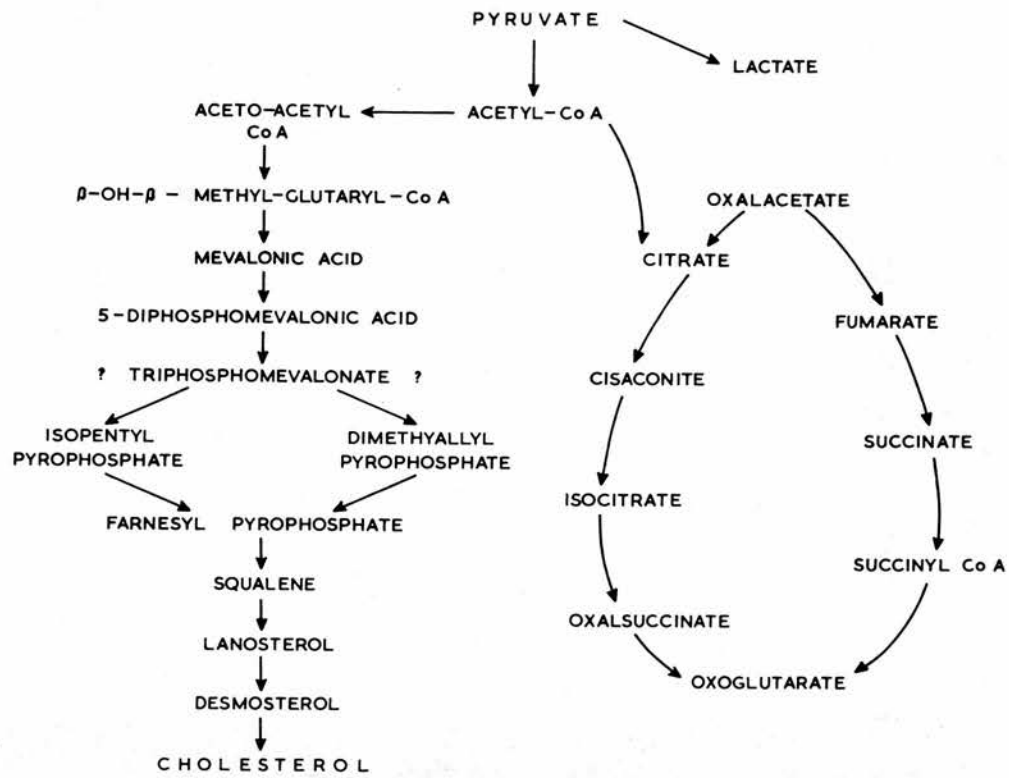
and claimed to be specific for LH, they have an effective dose range of 0.4 - 2.0 μ g. and 0.06 - 0.3 μ g. NIH-LH-S3 respectively.

Biosynthesis of Cholesterol

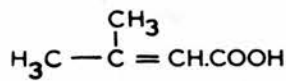
By 1960, the biosynthetic pathway for cholesterol had been established (see Cornforth, 1960 and fig. 1). Most of this work had been done in vitro, by following the metabolism of labelled (C-13, C-14) substrates by yeast cultures and liver slices (amongst other references see Cornforth, Hunter and Popjack, 1953 ab; Cornforth, Gore and Popjack, 1957; Gosselin and Lynen, 1964 and Woodford and Bloch, 1955). Claesson and Hillarp had demonstrated the presence of a cholesterol-like oestrogen precursor in the ovarian tissue of rabbits (1947a) and of rats and guinea-pigs (1947b) by histochemical means, while quantitative estimations of the cholesterol content of the ovaries of immature rats both before and after gonadotrophic stimulation have been made by Mukerji, Bell and Loraine (1965). Since the production and metabolism of cholesterol had proved to be highly sensitive to the administration of exogenous gonadotrophin (Bell et al., 1964; Mukerji et al., 1965) it was thought that an investigation of some of the earlier steps of the biosynthetic pathway for cholesterol might prove rewarding. It was hoped that as well as obtaining quantitative information about the presence of cholesterol precursors in the ovary, new assay methods for the quantitative measurement of the gonadotrophic hormones might be developed.

The method of Searcy and Berquist (1960) is not specific for

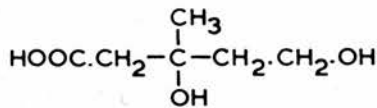
**Fig. 1:- THE BIOSYNTHETIC PATHWAY OF CHOLESTEROL, TOGETHER
WITH THE FORMULAE OF SOME OF THE COMPOUNDS.**



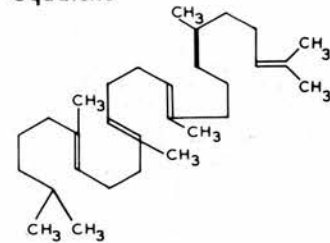
Dimethylacrylic acid



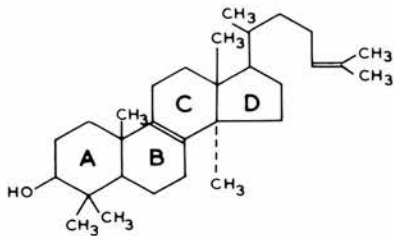
Mevalonic acid



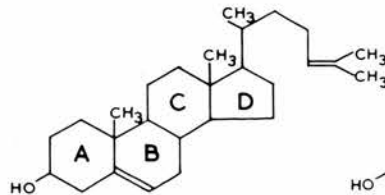
Squalene



Lanosterol



Desmosterol



Cholesterol

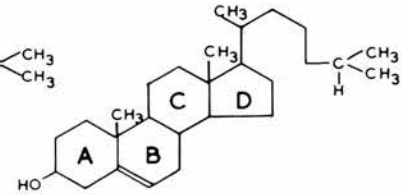


Fig.1.

cholesterol, but is a colour reaction which gives an estimate of all compounds present which have a similar ring A and ring B structure to cholesterol. In view of the fact that this method of cholesterol estimation was used by Bell and his colleagues, it was thought that a specific investigation into the two immediate precursors, lanosterol and desmosterol would not yield any further information.

It had been suggested (Robinson, 1932) that the straight chain, isoprenoid compound squalene might provide the carbon skeleton of the cholesterol molecule. Langdon and Bloch (1953) showed that acetate could be metabolically converted to squalene and squalene to cholesterol, while the work of Cornforth and Popjack (1954) appeared to confirm the role of squalene as an intermediate in the production of cholesterol. Initially it was thought that the most active biological precursor of cholesterol was the isoprenoid compound, the C₆ acid - dimethylacrylic acid. However isotopic experiments have shown that the C₅ compound, mevalonic acid is a much better precursor of cholesterol than dimethylacrylic acid (Tavormina, Gibbs and Huff, 1956). While it would have been interesting to investigate the mevalonic acid content of the ovaries, it was found that the specific methods of assay were too laborious and time-consuming for inclusion into a bioassay procedure and no attempts were made to measure the mevalonic acid content of ovarian material.

The acetyl co-enzyme A used in cholesterol biosynthesis can be derived from either the break down of fatty acids or from

pyruvic acid. Pyruvic acid is a general metabolic precursor with a very rapid rate of turnover and destruction following the removal of tissues from the body (Neish, 1957). It was thought, however, that an investigation into the ovarian content of pyruvic acid particularly following gonadotrophic stimulation might yield interesting information about the general metabolic effects of the gonadotrophins on ovarian material; especially as the most sensitive available methods (Katsuki, Kawano, Yoshida, Kanayuki and Tanaka, 1961; Bücher, Czok, Lamprecht and Latzko, 1963) have a sensitivity of about 0.1 - 0.2 μ moles pyruvic acid. In the final section of this thesis is described a study of the pyruvic acid content of ovarian tissue from immature rats and of the effects of gonadotrophin administration on the level of pyruvic acid.

CHAPTER II

TIBIAL TEST

INTRODUCTION

The first bioassay method for growth hormone was developed in 1931 by Evans and Simpson and depended on the increase in total body weight of plateaued rats, i. e. adult animals which had virtually ceased to grow. Later various modifications such as the use of hypophysectomised immature rats were introduced in an attempt to increase the sensitivity of the procedure. In the early 1940's a test based on the increase in width of the proximal epiphyseal cartilage of the tibia in hypophysectomised immature rats was devised by Evans and his co-workers (1943). This method was about three times more sensitive, was considerably more practicable than previously described assays, then became the assay of choice for growth hormone. When first developed, this assay was thought to be specific for growth hormone, but as purer hormone preparations were made available it became apparent that this was not the case, although little quantitative data on the subject has been quoted in the literature. In the present study, conditions of assay such as the length of the injection period, the minimum permissible post-hypophysectomy rest period and the specificity of the method were reinvestigated, with a view to using the assay for the quantitative determination of growth hormone.

The assay method is however, neither sensitive nor specific enough, to be used for the direct measurement of growth hormone

activity in body fluids. Nevertheless it provides an excellent method for comparing the potency of different pituitary growth hormone preparations and the best method of comparing growth hormones from different animal species. It also appeared possible that it could be used as an index of body growth and in this form the assay was applied to the assessment of two new synthetic compounds. It was also used to investigate the condition of the epiphyseal cartilage in animals suffering from artificially induced muscular atrophy.

MATERIALS AND METHODS

1. Hormones and Synthetics

The preparations used in this study will be discussed under three headings, a) growth hormone standards, b) test preparations including synthetics and c) the hormones used in specificity tests. All the hormones were of pituitary origin.

a) Standards

The international standard replaced the Armour material and both are of bovine origin. M. R. C. standard 'A' is a first attempt to supply a standard growth hormone preparation of human origin.

1. The International Standard - This is a growth hormone preparation of bovine origin supplied by the Department of Biological Standards, National Institute for Medical Research (N. I. M. R.) London. This preparation consists of three batches of hormone each of 25 g.; two obtained from workers in the U.S.A. and one from Canadian manufacturers. Mixing was carried out by dissolving all the material together and then lyophilising the resultant

solution. Ampoules, which each contain 30 mg. of lyophilised material were filled with dry nitrogen, prior to sealing and are maintained at -10°C .

By definition of the Expert Committee on Biological Standardization of the World Health Organisation (W.H.O.) the growth hormone activity represented by 1 i.u. is contained in 1 mg. of this material. Contamination is as follows:-

TSH - 0.034 i.u./mg.

ACTH - < 0.01 i.u./mg.

Prolactin - < 0.25 i.u./mg.

2. The Armour House Standard 'Somar 'A' - a growth hormone preparation of bovine origin prepared by the Armour Pharmaceutical Co. Ltd. and distributed by the Endocrinology Study Section of the National Institutes of Health (N.I.H.), Bethesda, Maryland, U.S.A. This is a lyophilised preparation and each sterile ampoule contains 50 mg. The estimated potency of this preparation is about 1.3 i.u./mg.

Contamination is as follows:-

TSH - < 0.06 i.u./mg.

ACTH - < 0.02 i.u./mg.

Prolactin - < 0.5 i.u./mg.

Gonadotrophin - < 10.0 units/mg.

3. M.R.C. - Standard 'A' - a growth hormone preparation of human origin supplied by N.I.M.R. Preliminary estimations of potency suggest that each ampoule has an activity equal to

about 0.14 i.u. (increase in body weight in hypophysectomised rats).

4. NIH - Beef and Sheep Pituitary Growth Hormones - Three such preparations were supplied at different times by the N. I. H. ; two were of bovine and one of ovine pituitary origin. They are:-

NIH - GH - B2)) beef
NIH - GH - B4)	
NIH - GH - S3	sheep

Each preparation is claimed to be approximately equipotent with the International Standard and the contamination data is as follows:-

TSH	-	< 0.01 i.u./mg.
ACTH	-	< 0.001 i.u./mg.
Prolactin	-	< 0.05 i.u./mg.
LH	-	< 0.001 i.u./mg.
FSH	-	no detectable activity could be found in 1.8 mg. of the growth hormone preparation.

b) Test Preparations

The test preparations studied herein may be divided into three groups. The first consists of a growth hormone of bovine pituitary origin. The second of eight growth hormone fractions prepared from human pituitaries, and the third of two synthetic compounds.

1. 'KWA' Growth Hormone - this preparation was extracted from bovine pituitaries by Dr. Kwa of the Antoni van Leeuwenhoek-

Huis, Amsterdam. It is prepared from a sub-cellular pituitary fraction according to the method of Tuynman, Kwa and Bloemendal (1962).

2. Human Growth Hormone Preparations - All the human growth hormones used were prepared by initial extraction with hot glacial acetic acid according to the method of Raben (1957, 1959).

Four of these were prepared and supplied by Dr. Raben; they are-

Raben 6

Raben 9

Raben 11

Raben 12

One, designated the Cambridge preparation, was prepared in the Department of Biochemistry, Cambridge, U.K. The three remaining preparations were supplied by the Imperial Cancer Research Fund, and are:-

ICRF 6

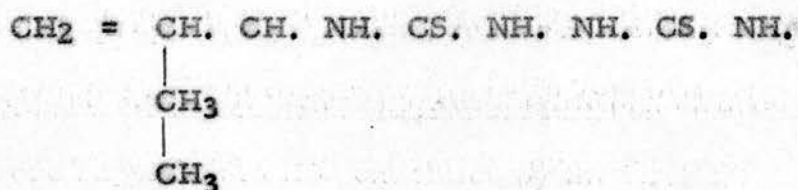
ICRF 6, peak 1

ICRF 6, peak 2

The two ICRF sub-fractions were obtained by fractionation of a Raben-type growth hormone preparation (ICRF 6) on a G200 Sephadex Column; buffer 0.05 M borate, 0.05 M KCl, pH 8.6.

3. Synthetics -

- a) Compound 33,828 (I.C.I.) - a Dithiocarbamoylhydrazine derivative 1- α -methyl-allythiocarbamoyl-2-methyl-thiocarbamoyl-hydrazine



b) Synacthen - Ciba 30920 - Ba - a synthetic polypeptide.

This synthetic compound consists of the β 1-24 amino acid sequence of natural ACTH. It has a molecular weight of 3,294 and an ACTH - like activity of about 100 i.u./mg. (Schuler, Schär and Desaulles, (1963).

c) Hormones Used in Specificity Tests

Only hormones derived from the anterior lobe of the pituitary were used in the specificity studies. These were as follows:-

1. Adrenocorticotrophic Hormone - Two commercial preparations available for clinical use were used in this study:

a) 'Zinc corticotrophin' supplied by Organon Laboratories Ltd.

This preparation had an activity of 100 i.u./ampoule.

b) 'Acthar', manufactured by Armour Pharmaceutical Co., Ltd.

Two preparations with activities of 40 and 70 i.u./ampoule were used.

2. Follicle-Stimulating Hormone (Ovine NIH - FSH - SI) -

This is distributed by the Endocrinology Study Section of the National Institutes of Health (Bethesda, U.S.A.) and is contaminated with 0.6% LH as estimated by the ovarian ascorbic acid depletion test (Parlow, unpublished data).

Other contamination data:-

ACTH - 0.00044 i.u./mg. (adrenal ascorbic acid depletion method).

Prolactin - < 0.15 i.u./mg. (pigeon crop assay)

TSH - < 0.01 U.S.P. units/mg. (P^{32} uptake)

GH - no detectable growth hormone activity in ten-day weight tests following daily administration of 2.5mg.

3. Luteinising Hormone (ovine NIH - LH - SI) - This preparation is distributed by the N.I.H. and total doses of up to 6 mg. show no significant FSH contamination (increase in ovarian weight assay).

Other contamination data :-

ACTH - 0.00025 i.u./mg.

Prolactin - no detectable activity following administration of 25 mg. total dose.

TSH - 0.015 U.S.P. units/mg.

GH - no detectable activity in ten-day weight tests following daily administration of 1.0 mg.

4. Prolactin (ovine - NIH - P - S3) - This preparation, distributed by the N.I.H. is said to have a potency of 15 i.u./mg.

Contamination data is as follows:-

ACTH - 0.00025 i.u./mg.

GH - > 0.1 and < 1.0%, i.e. less than 0.01 i.u./mg.

No detectable FSH and LH activity in amounts of up to 8 mg. and 10 mg. respectively.

5. Thyroid-Stimulating Hormone - The following two preparations were used in this study :-

- a) NIH - TSH - BI. A preparation of bovine origin distributed by the NIH for experimental purposes. It contains approximately 0.07 i.u./mg.
- b) Ambinon. A preparation supplied by Organon for clinical use, with an activity of 10 u.s.p. units/ampoule.

2. Animals

Two separate strains of rats were used in these studies. Animals of the Wistar strain were bred in a closed colony maintained in the laboratory in Edinburgh, while the Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories Inc., Boston, Mass., U.S.A.

All hypophysectomised animals were of the Sprague-Dawley strain and were hypophysectomised in the U.S.A. before dispatch to Edinburgh

3. Method in Detail

The tibial assay used was based on the method described by Evans, et al. (1943), together with the modifications suggested by Greenspan, et al. (1950). The assays were of a 4 - or 6 - point design and the results were analysed according to the simplified method of Gaddum (1953b) (See Appendix).

i) Procedure

Immature female rats were hypophysectomised at age 21 -

25 days when weighing 50 ± 2.0 g. The rats were rested for between 10 and 12 days after hypophysectomy before receiving four daily subcutaneous injections of the test preparation (dissolved and made up to the required concentration in 0.9% saline). Twenty-four hours after receiving the last injection the rats were killed with ether and the tibiae dissected free. The pituitary fossa was examined macroscopically for signs of pituitary remnants; the tibiae from any rats exhibiting signs of pituitary remnants were discarded. The tibiae were stored in 10% formol saline (10% formaldehyde solution w/v in 0.9% saline) until cartilage width measurements could be made.

ii) Staining Procedure

- a. Each tibia was halved longitudinally through the condylar groove and down the tibial crest.
- b. Cut halves were soaked in water for 10 minutes.
- c. Sections transferred to acetone and left for 6 minutes.
- d. Sections washed in water for 3 minutes.
- e. Sections transferred to freshly prepared 2% silver nitrate solution and left cut surface uppermost for 2 minutes.
- f. Sections were then rinsed in water before being exposed under water to a strong light for about 3 - 5 minutes (until calcified portions were suitably darkened).
- g. The sections were then thinned by further longitudinal sectioning and the cartilage area between the stained calcified portions measured with a Watson binocular microscope. A x 10 objec-

tive and a x6 micrometer eyepiece were used. The mean epiphyseal cartilage width was calculated for each section from the ten estimations made across each section .

iii) Histological Procedures

Histological sections were prepared in the following manner. The tibiae were decalcified; median longitudinal sections were cut and stained with haematoxylin|eosin to show the condition of the cartilage plate and the surrounding bone. Photomicrographs of the tibiae have been mounted, together with an arbitrary scale to emphasise the difference in cartilage widths.

iv) Comment

In the majority of the experiments reported herein the experimental conditions were identical to the above, however, in some of the experiments described, the conditions have been slightly altered. In particular, the sex, age and weight range of the rats, and the injection period, have been adjusted to meet the requirements of particular experiments and these modifications are detailed in the appropriate places.

RESULTS

The results presented herein will be considered under the following headings, A) Tibial Test, B) Hormone Assays and C) Applications of the Procedure.

The first section comprises the results of a typical growth hormone assay by the tibial test, some experiments in which the conditions of assay, i. e. the length of the post-hypophysectomy

rest period and the length of the injection period, were examined and the results of specificity tests which included some experiments on feeding.

In the second section are reported the results obtained from some experiments in which the potency of a number of growth hormone preparations from different animal species was estimated in terms of the International Standard.

The final section comprises the results of experiments in which two new synthetic compounds were tested for effects on body growth and the effect of artificially induced muscular atrophy on cartilage width was also examined.

A) Tibial Test

1) Typical growth hormone assay.

The results from a typical 6-point assay of growth hormone are shown in Table 1. It will be seen that a four-fold dose interval gives a reasonable inter-dose increase in cartilage width.

As will be seen from these results, each mean width shows a very high standard deviation. This is the inevitable consequence of the immense variability in inter-animal response and is the major cause of the lack of precision in this assay.

It can also be seen that this assay method is highly insensitive. In the standard curve shown here a daily injection of 12.5 μ g. for 4 days causes an increase in cartilage width which is not significantly greater than the mean control width. This indicates that samples with concentrations of less than 50 μ g. cannot be estimated

TABLE 1

RESULTS OF A TYPICAL GROWTH HORMONE ASSAY

Dose	International Standard B.G.H.				KWA Preparation BGH				0.9% Saline 0.5 mls./rat/ day for 4 days
	12.5 µg./rat/ day for 4 days	50 µg./rat/ day for 4 days	200 µg./rat/ day for 4 days	8.16 µg./rat/ day for 4 days	33.3 µg./rat/ day for 4 days	133.3 µg./rat/ day for 4 days	317.85 µ 331.99 281.77 355.87 288.17		
Epiphyseal Cartilage Width	284.21 μ 187.69 269.10 206.70 195.00	224.74 μ 340.27 300.79 290.06 238.87	311.51 μ 369.04 366.60 554.90	279.34 μ 287.62 218.40 241.80 260.81	307.61 μ 296.40 238.39 337.84	317.85 μ 331.99 281.77 355.87 288.17	204.75 207.67 214.50 195.00 202.80 206.21 173.06 179.89		
Mean Width	228.54 μ	278.95 μ	350.51 μ	257.59 μ	295.06 μ	315.12 μ	197.98 μ		
S.D. S.E.	\pm 44.76 \pm 20.02	\pm 47.20 \pm 21.10	\pm 26.72 \pm 11.96	\pm 28.15 \pm 12.60	\pm 41.64 \pm 20.82	\pm 30.80 \pm 13.78	\pm 14.44 \pm 5.11		
No. of animals	5	5	4	5	4	5	8		

Assay Caddum 6-point design.

Relative potency 110.7% (Fid. limits 40-322%), λ 0.515The two dose response curves show NO significant curvature. F in both bases lies bet. 0.6 & 0.7
The two curves show NO significant deviation from parallelism. F lies between 0.1 and 0.2

accurately. None of the tibial assays carried out has proved to be statistically invalid and so, where large quantities of material are available, reliable estimations of potency can be made.

2) Modifications to the method

The method, as described previously, is the standard procedure used by most workers in this field. It was felt that some of the conditions - in particular, the length of the post-hypophysectomy rest period, i.e. the period between hypophysectomy and commencement of growth hormone treatment; and the actual length of the injection period - required investigation in order to establish the optimal conditions for the assay in the colony of animals used.

a) The Effect on Tibial Epiphyseal Cartilage Width of Altering the Length of the Post-Hypophysectomy Rest Period

Introduction :-

The rest period of 10 - 12 days advocated by Evans et al. (1943) in their original paper on the tibial assay has been generally adopted by all subsequent workers in this field (e.g. Greenspan et al., 1949; Li, 1953; Lestroh and Li, 1957). The rest period not only allows the cartilage time to regress, but also enables one to detect animals in which the removal of the pituitary has not been complete.

The present study was undertaken in order to determine how quickly cartilage regression starts, and the earliest period after hypophysectomy that cartilage regression can be said to be complete.

Method:-

Rats were sampled at approximately daily intervals after hypophysectomy, and cartilage width measurements made in the usual way.

Results :-

The results of this experiment are shown in Table 2.

From the results it can be seen that there is a steady depression of cartilage width over the first 6 days after hypophysectomy, thereafter no marked changes occur after the 8th day. The width ($228.51 \pm 8.55 \mu$) noted on the fourth day after hypophysectomy is still significantly higher ($P < 0.01$) than the width ($197.98 \pm 5.11 \mu$) of the 'control' animals which were killed on the fifteenth day. The cartilage widths obtained from the killed 6 days and more (except animals killed on day 9) after hypophysectomy are not significantly different from those killed on the fifteenth day. The animals killed on day 9 had cartilage widths which were significantly lower than those of the control animals.

Discussion:-

From this experiment it would appear that cartilage regression is complete by 6 - 8 days after hypophysectomy, and that the 12-day rest period recommended by Evans and his co-workers (1943) is not really essential. If, however, the material to be assayed is scarce, use of the 12-day rest period permits one to reject with some certainty unsuitable animals such as weaklings and incompletely hypophysectomised rats. However, if one is confident

TABLE 2.

The effect of hypophysectomy on the width of the tibial epiphyseal cartilage of immature female rats.

Days after Hypophysectomy	No. of Observations	Epiphyseal Cartilage Width in μ mean \pm S.E.	Difference in Cartilage Widths P
Intact ¹	6	304.27 \pm 13.59	< 0.001
1	2	297.86 \pm 5.85	< 0.001
3	2	263.00 \pm 2.69	< 0.001
4	4	228.51 \pm 8.55	< 0.01
6	2	174.52 \pm 3.90	> 0.05 ^o
8	2	205.24 \pm 4.39	> 0.50
9	2	169.16 \pm 8.29	< 0.05
10	2	191.59 \pm 12.19	> 0.50
11	6	180.86 \pm 9.09	> 0.10
15* (control)	8	197.98 \pm 5.11	

¹ Animals set aside before hypophysectomy and killed immediately on arrival in Edinburgh i.e. equivalent to the animals killed 1 day after hypophysectomy.

* Standard control animals injected with 0.5 ml./day 0.9% saline from day 11 to 14 inclusive and killed on day 15.

^o Significantly lower than the control value.

that all the animals will prove to be satisfactory, and time is at a premium, a shorter rest period can be used. This observation has been used to reduce the experimental period of some long-term studies reported hereafter.

b) The Effect of Length of the Injection Period on Cartilage Width

Introduction:-

Greenspan et al. (1949) found that when the same total dose was given over a three or four day period either intraperitoneally, subcutaneously or intravenously, there was no significant difference in the cartilage width response noted. If however this total dose was given as a single injection or over a two day period, maximal response by the cartilage was not attained. Increasing the frequency of administration to two injections per day did not affect the response.

In the present experiment the daily dose was kept constant, but injection periods of four and fourteen days, with consequent differences in total dose, were compared.

Method:-

The injection schedule is shown in Table 3. Animals were killed one day after the final injection and cartilage width measurements made in the usual manner.

Results :-

The results obtained are shown in Table 4. It will be seen that there was no significant difference in the cartilage widths of animals which had received four or fourteen daily injections, although

TABLE 3

Injection schedule showing dose / day and the total dose received over a 4 and 14 day period.

Growth Hormone Dose/Day	Total Dose over 4 days	Total Dose over 14 days
5 $\mu\text{g.}$	20 $\mu\text{g.}$	70 $\mu\text{g.}$
10 $\mu\text{g.}$	40 $\mu\text{g.}$	140 $\mu\text{g.}$
20 $\mu\text{g.}$	80 $\mu\text{g.}$	280 $\mu\text{g.}$

TABLE 4

The epiphyseal cartilage response to daily injections of growth hormone over 4 and 14 day periods and the significance figures for the 4 day v. 14 day period.

Growth Hormone	Tibial Cartilage Width Mean \pm S.E.		Degrees of Freedom	t	P
	4 days	14 days			
5 $\mu\text{g/day}$	198.41 \pm 22.8	206.37 \pm 7.3	8	0.1244	> 0.9
10 $\mu\text{g/day}$	242.45 \pm 17.6	235.62 \pm 6.1	7	0.4675	> 0.6
20 $\mu\text{g/day}$	269.71 \pm 12.3	280.74 \pm 14.5	8	0.538	> 0.6

the total amount of growth hormone administered in each case was widely different.

Discussion:-

From this experiment it would seem that a maximal increase in cartilage width is attained after a four day injection period, a finding in agreement with that of Greenspan et al., (1949). These workers, however, had kept the total dose administered constant, so it was somewhat surprising to find, that, when in fact the total dose administered was more than trebled during the 14 day injection period, no significant difference in cartilage width was noted. This implies that the cartilage responds to a daily stimulus, rather than to a total stimulus.

It is possible that over a long period, the growth promoting effect of the hormone diminishes and thus an apparently stable width is obtained. It is also possible that continuous injections of the hormone cause the formation of weak antibodies and that these inhibit slightly the growth promoting activity of the hormone, thereby producing an apparently constant cartilage width.

A four day injection period is therefore sufficient for assay work. If, however, the injection period is extended to fourteen days, it is possible that, while the growth hormone response remains constant, hidden growth promoting contaminants may appear in unknown substances. It may thus be advisable to prolong the injection period when examining substances for non-specific effects on cartilage width.

3. Specificity of the Method

Introduction:

Following the development of increasingly pure hormone preparations it became apparent that this test was not as specific for growth hormone as had originally been thought. It was also known that a reduction in food intake of growing animals will prevent an increase in weight, and even cause a weight loss if the reduction in feeding is very severe. Becks, Simpson, Li and Evans (1944) and Wyman and Tum-Suden (1945) have observed that in normal rats a reduction in cartilage width followed a period of reduced feeding.

It has been suggested that the ideal test animal for the tibial assay is one which is not only hypophysectomised but also adrenalectomised, thyroidectomised and gonadectomised (Li, 1953). ACTH is known to inhibit cartilage width (Becks, Simpson, Li and Evans, 1944; but the effect of adrenalectomy per se is still in dispute.

- The experiments in this group were designed therefore to
- a) compare the effect of the anterior pituitary hormones ACTH, FSH, LH, prolactin and TSH either alone or in combination with growth hormone on the epiphyseal cartilage width of the tibia. The effect of these hormones on total body weight during the experimental period was also noted.
 - b) compare the effect of starvation with the effect of hypophysectomy on the proximal epiphyseal cartilage of the tibia of both sexually immature and mature rats; the histological appearance

of the cartilage was also examined.

- c) study the effect on cartilage width of adrenalectomising both intact and hypophysectomised rats. The effect of adrenalectomy on body weight was also noted and a comparison of the effects made.

a. The Effect of Anterior Pituitary Hormone

Method:-

In this experiment slightly older hypophysectomised male rats weighing 100 ± 10 g. were used. The hormones were administered twice daily according to the schedule contained in Table 5 and the injection period lengthened to fourteen days. Animals were weighed at the commencement of the injection period and again on the final day of the experiment. The alteration in body weight during the treatment period was noted. Cartilage width measurements and histological sections were prepared as previously described.

Results:-

The effect of hormone administration on both body weight and on the width of the tibial epiphyseal cartilage are shown in Tables 6 and 7 and figure 2. The histological sections are compared in Figs. 3 and 4.

Control Rats

A group of hypophysectomised rats treated with saline was used as controls. The final mean cartilage width was $132 \pm 5.34 \mu$ and there was a mean increase of 3.75 ± 1.7 g. in body weight during the injection period (Table 6).

TABLE 5.

Injection schedule for experiment to determine the effect of anterior pituitary hormones on the width of the tibial epiphyseal cartilage.

Material administered	Dose/ Injection	Total dose over 14 days
1. Saline 0.9%	1.0 ml.	28.0 ml.
2. Growth Hormone (GH) NIH-B2	0.5 mg.	14.0 mg.
3. Prolactin (P) NIH-P-S3	0.5 mg.	14.0 mg.
4. Mixed gonadotrophins		
FSH NIH-FSH-S1	0.5 mg.	14.0 mg.
LH NIH-LH-S1	0.005 mg.	0.14 mg.
5. TSH 'Ambinon'	0.5 i.u.	14.0 i.u.
6. ACTH 'Zinc Corticotrophin' 'Acther'	1.0 i.u.	28.0 i.u.
7. Combined hormones I		
GH	0.5 mg.	14.0 mg.
P	0.5 mg.	14.0 mg.
FSH	0.5 mg.	14.0 mg.
LH	0.005 mg.	0.14 mg.
TSH	0.5 i.u.	14.0 i.u.
ACTH	1.0 i.u.	28.0 i.u.
8. Combined hormones II		
GH	0.25 mg.	7.0 mg.
P	0.25 mg.	7.0 mg.
FSH	0.25 mg.	7.0 mg.
LH	0.0025 mg.	0.07 mg.
TSH	0.25 i.u.	7.0 i.u.
ACTH	0.5 i.u.	14.0 i.u.

TABLE 6.

The effect of hormone administration on tibial epiphysis and total body weight in hypophysectomised male rats.

Treatment	No. of Animals	Width of Tibial Epiphyseal Cartilage Plate (micra)	Increase or Decrease in Total Body Weight (gm.)
0.9% Saline	4	132.0 \pm 5.34*	3.75 \pm 1.7*
ACTH	6	85.0 \pm 4.09*	- 3.83 \pm 1.68*
TSH	7	177.4 \pm 9.24*	17.0 \pm 1.23*
Prolactin	7	192.8 \pm 5.34*	15.0 \pm 1.17*
LH) +) FSH)	6	137.7 \pm 5.08*	9.5 \pm 1.57*
Growth Hormone	7	292.0 \pm 6.18*	47.14 \pm 6.6*
Combined I	6	268.3 \pm 10.47*	70.33 \pm 2.79*
Combined II	7	282.7 \pm 9.15*	67.43 \pm 2.72*

* Mean \pm Standard Error

TABLE 7

Effect of ACTH on the width of the Tibial Epiphyseal Cartilage Plate and Body Weight of Immature Hypophysectomised Rats.

Treatment	No. of Animals	Width of Tibial Epiphyseal Cartilage Plate in μ Mean \pm S.D.	Decrease in Total body weight Mean \pm S.D.
0.9% saline	11	122.26 \pm 4.94	- 4.0 \pm 3.15
ACTH	12	100.30 \pm 7.24	- 5.0 \pm 3.49

**Fig. 2:- THE EFFECT OF HORMONE TREATMENT ON TIBIAL EPIPHYSEAL
CARTILAGE WIDTH AND ON BODY WEIGHT IN THE INTACT AND
HYPOPHYSECTOMISED RAT.**

EFFECT OF HORMONE TREATMENT IN THE INTACT AND HYPOPHYSECTOMIZED RAT

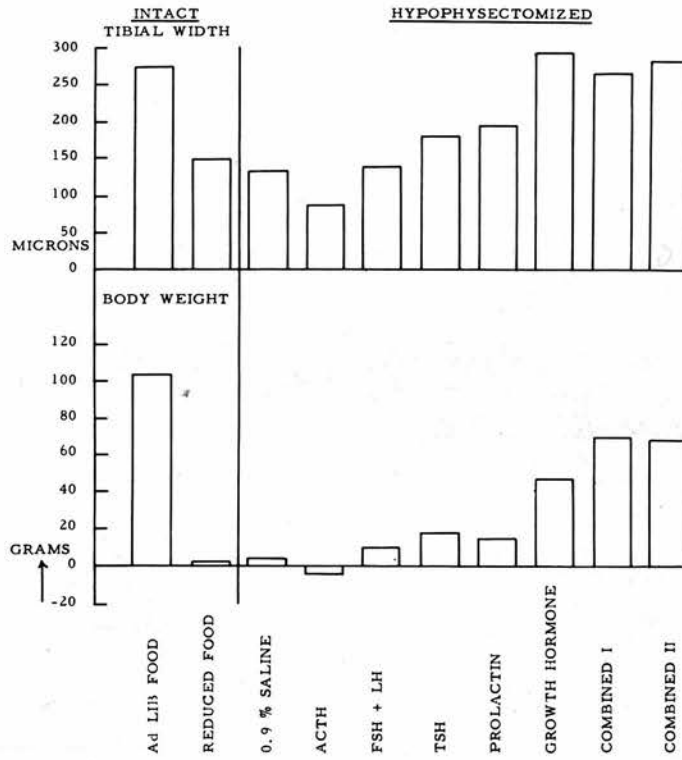


Fig. 2.

Adrenocorticotrophin

The administration of ACTH caused a reduction in cartilage width ($P < 0.001$) in two experiments but in body weight ($P < 0.02$) only in the animals which had been treated with the 'zinc corticotrophin'.

Thyroid-stimulating hormone

The administration of TSH caused significant increases in body weight ($P < 0.001$) and in cartilage width ($P < 0.01$).

Gonadotrophins

When LH and FSH were administered together a significant increase in body weight ($P < 0.001$) was observed. Cartilage width was however unaffected.

Growth Hormone

The animals treated with growth hormone alone showed an increase in body weight of 47.14 ± 6.6 g. and a mean cartilage width ($292.0 \pm 6.18 \mu$) which is more than twice the mean control width.

Combined Hormones

When the hormones were administered together, the increase in body weight was greater ($P < 0.02$) than the increase in the group treated with growth hormone alone. There was no significant difference between the two groups receiving the combined hormones although in one group of animals the dose was double that in the other.

The combined hormones also produced a marked increase in

cartilage width; the effect observed was approximately the same at both dose levels. No significant difference was found when the mean cartilage width of the animals receiving the combined hormone was compared with that of intact animals and animals receiving growth hormone alone.

Histology

Figure 3 shows that in the hypophysectomised rat, ACTH caused a considerable reduction in cartilage width (reading 2 - 3 scale units). Growth hormone caused a marked increase over the saline-treated control animals, the readings were respectively 10 - 11 and 4 - 5 scale units.

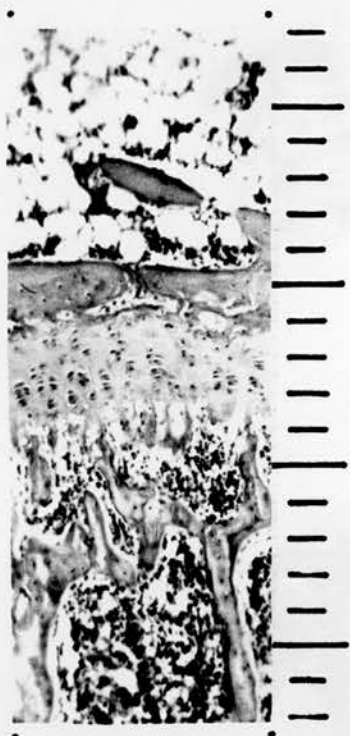
Figure 4 shows the effect of the administration of 'combined hormones' at two dose levels to hypophysectomised rats (see Tables 5 and 6). It will be noted that although the dosage in one group of animals was half that in the other group, the effect on cartilage width was approximately the same (reading 6 scale units in each case).

Discussion

It was interesting to note that significant alterations in cartilage width occurred following the administration of all the hormones except the gonadotrophins LH and FSH. Except for ACTH which caused a significant depression in cartilage width, this alteration took the form of an increase in cartilage width. When the experiment with ACTH was repeated using a completely different preparation (ARMOUR's - ACTHAR), a marked depression of

**Fig. 3:- PHOTOMICROGRAPH SHOWING THE EFFECT OF GROWTH HORMONE
AND ACTH ADMINISTRATION ON THE CARTILAGE WIDTH OF THE
TIBIAE OF HYPOPHYSECTOMISED RATS.**

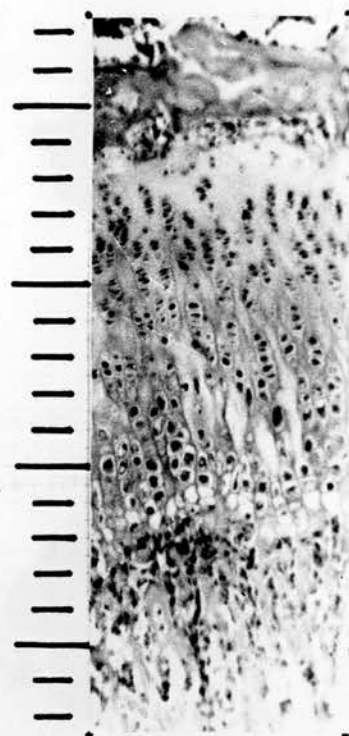
TIBIAL EPIPHYSEAL CARTILAGE HYPOPHYSECTOMIZED ANIMALS



ACTH
2 iu./day.



SALINE
1 mL/day.

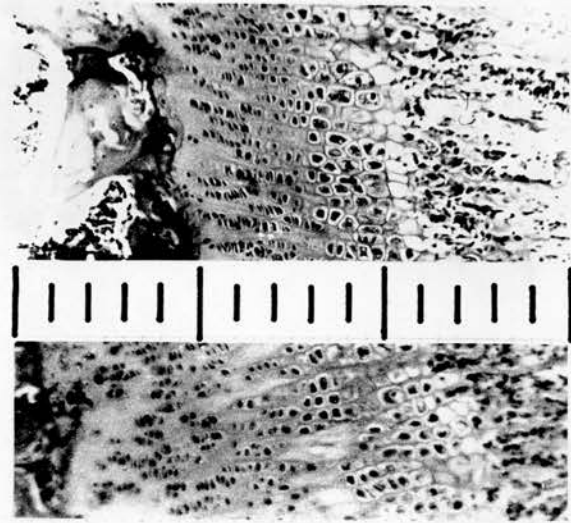
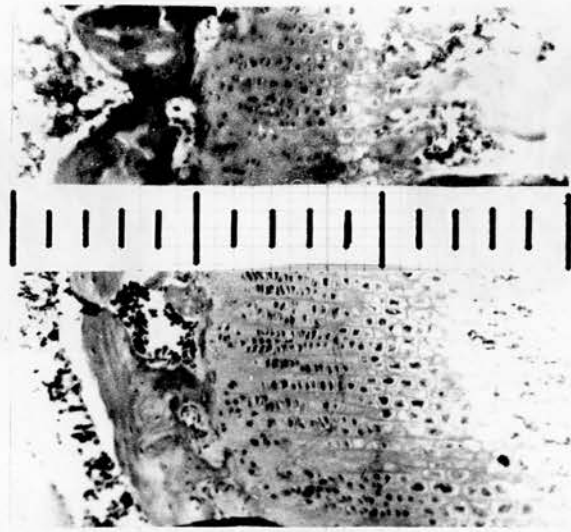


GROWTH H.
1 mg./day.

Fig.3.

**Fig. 4.- PHOTOMICROGRAPHS SHOWING THE EFFECT OF GROWTH HORMONE
AND COMBINED HORMONE ADMINISTRATION ON THE CARTILAGE
WIDTH OF THE TIBIAE OF HYPOPHYSECTOMISED RATS.**

TIBIAL EPIPHYSEAL CARTILAGE
HYPOPHYSECTOMIZED ANIMALS



COMBINED SALINE GROWTH COMBINED
HORMONES HORMONE HORMONES
I II

Fig.4.

cartilage width was again seen. In this repeat experiment, however, the loss in total body weight noted was not significantly different from the control value; this is probably due to the fact that the saline-treated control group of hypophysectomised animals also showed a slight loss in weight during the experimental period. A significant loss in weight had been noted in the first experiment when Organon's Zinc Corticotrophin was used, but in this study the control animals showed a slight gain in weight during the experimental period. Cortisone is known to have a marked depressant effect on actively growing tissues (Ragan, Howed, Plotz, Meyer and Blunt, 1949; Sobel 1958; and Sayeed, Blumenthal and Blumenthal, 1962, amongst others) and it is possible that ACTH acts on cartilage growth by stimulating cortisone production from the adrenal cortex; Angervall and Lundin (1962) have noted an increase in adrenal weight following the administration of ACTH to hypophysectomised pregnant rats; they also observed a slight loss in body weight during the experimental period.

The TSH used was a commercial preparation and not of a particularly high standard of purity, so that its stimulating effect on cartilage and body growth may be partly ascribed to this. Li (1953), however, has found that thyroxine caused a considerable increase in cartilage width (which also had a synergistic effect with growth hormone) so it is possible that the effect noted here was caused by increased liberation of thyroxine following stimulation of the thyroid gland by the administered TSH.

The percentage growth hormone contamination for the prolactin preparation used is known and claimed to be more than 0.1% but less than 1%, i. e. in this study more than 1 $\mu\text{g.}$ but less than 10 $\mu\text{g./day.}$ In a previous experiment (see length of injection period) it had been found that while a daily injection of 10 $\mu\text{g./day}$ growth hormone caused an increase in cartilage width, this was not, however, significantly different from the control value ($P > 0.1$). It would seem, therefore, that the marked effect of prolactin on cartilage width cannot be entirely ascribed to growth hormone contamination, but must be, in part, an intrinsic property of the hormone. This would appear to be a feasible hypothesis in view of the present controversy as to whether human prolactin exists as a separate entity or as an additional biological effect of growth hormone (Chadwick, Foley and Gemzell, 1961; Wallace and Ferguson, 1961. Recently, however, Apostolakis (1965) has claimed that he has isolated human prolactin and shown it to be a separate hormone. He has prepared human pituitary fractions which have a relatively high prolactin activity and a low growth hormone activity (about 0.3 i. u./mg.) but he considers that the growth hormone activity is due to contamination of the fraction rather than an intrinsic growth promoting activity of the prolactin hormone.

When the two gonadotrophins were administered together, they had no effect on cartilage width although they caused a small but significant rise in body weight. These were the only hormones

to show different effects in the two assay methods and it was interesting to see that they had a positive effect in the body weight test which has been claimed to be the more specific and precise, if less sensitive of the two bioassays for growth hormone (11, 1953).

It would appear, therefore, that all the anterior pituitary hormones cause a significant alteration in body weight. It was interesting to note that when the hormones were administered separately, the animals receiving growth hormone showed the greatest increase in body weight, approximately three times that seen in the animals treated with TSH and Prolactin. If, however, the hormones were administered jointly there was a marked synergistic effect, the body weight increase being approximately 50% greater than that seen in animals treated with growth hormone alone, even when the growth hormone content of the mixture was half the amount given to growth hormone treated animals. Synergistic effects of anterior pituitary hormones upon body weight in the pigeon have also been noted by Bates, Miller and Garrison (1962).

Similarly, significant alterations in cartilage width were noted following the administration of all the hormones except the gonadotrophins LH and FSH. A synergistic effect was not noted as a maximal tibial response appears to have been obtained with the dose of growth hormone administered. While the conditions of this experiment would mask the appearance of a synergistic effect

on tibial cartilage width, the occurrence of such an effect is still possible.

b. Feeding.

Method:

Two groups of Sprague-Dawley rats with mean body weights of 100 g. and 250 g. respectively were used. The rats in the lower weight range were then sub-divided into three groups, one group was hypophysectomised and maintained on an unrestricted diet, the remaining intact animals were divided into two groups, one of which was allowed to feed ad libitum, the other group was placed on reduced feeding so that the body weight was maintained at the same level as that of the hypophysectomised rats. The older rats were also sub-divided into two groups. One group consisted of hypophysectomised animals fed ad libitum; the other, of intact animals, was fed sufficient to maintain the body weight at the same levels as that of the hypophysectomised animals.

One tibia from each animal was prepared for cartilage width measurements as previously described. For the histological examination, sections were made from tibiae selected at random from each of the subgroups and prepared as described earlier.

Results:

The results are shown in Table 8 and in Fig. 5.

Effect on body weight and cartilage width

Where the body weight of intact animals was maintained at the same level as that of the comparable hypophysectomised group,

TABLE 8

The effect of feeding on the tibial epiphysis and body weight in hypophysectomised and intact rats.

	Feeding	No. of animals	Width of Tibial Epiphyseal Cartilage Plate (micra)	Increase in Total Body Weight (gm).
Initial Weight 90-110 g.				
Hypophysectomised	Unlimited	4	132.0 \pm 5.34 *	3.75 \pm 1.7 *
Intact	Reduced food	6	149.2 \pm 10.25 *	3.0 \pm 1.9 *
Intact	Unlimited	6	276.3 \pm 11.64 *	104.1 \pm 3.8 *
Initial Weight 250 g.				
Completely hypophysectomised	Unlimited	5	81.9 \pm 4.39 *	-16.4 \pm 5.3 *
Partially hypophysectomised	Unlimited	2	149.3 \pm 15.97 *	+49.0 - 3.0 *
Intact	Reduced	7	97.9 \pm 3.43 *	-12.9 \pm 2.6 *

* Mean \pm Standard Error

the cartilage width of the intact animals was found to be of the same order as that of the hypophysectomised rats. There was no difference at the lower weight range, although a slight but insignificant difference ($P > 0.1$) was apparent in the animals from the higher weight range.

Histology

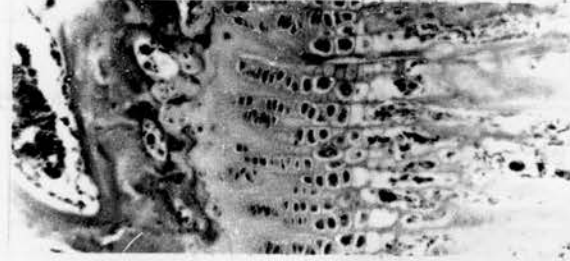
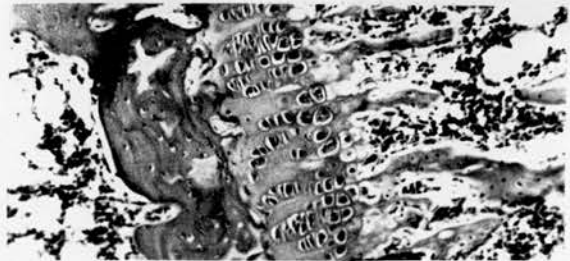
The sections shown in Fig. 5 were taken 1) from an intact rat on reduced food, 2) from a partially, and 3) a completely hypophysectomised rat; the latter two animals were on unlimited food. The sections show that although the widths of the intact and of the completely hypophysectomised cartilages were almost the same (readings approximately 3 scale units), the cartilage cells from the hypophysectomised rat appeared to be much more atrophied than those from the intact animal. The section from the partially hypophysectomised rat showed a similar atrophy of the cartilage cells but an increase in plate width (4 - 5 scale units) suggesting that the pituitary was still partially functional.

Discussion

Both Becks et al. (1944) and Wyman and Tum-Suden (1945) had noted a reduction in cartilage width in animals following reduced feeding. This observation was confirmed in the present study where in both intact immature and intact mature rats fed sufficient to maintain, but not to increase the body weight, the cartilage width was reduced to the same level as that seen in the hypophysectomised group, whose growth had been arrested by removal of the pituitary.

**Fig. 5:- PHOTOMICROGRAPH SHOWING THE EFFECT OF FEEDING ON THE
CARTILAGE WIDTH OF THE TIBIAE OF PITUITARY INTACT
AND HYPOPHYSECTOMISED RATS.**

TIBIAL EPIPHYSEAL CARTILAGE



INTACT

HYPOPHYSECTOMIZED

PARTIAL

COMPLETE

REDUCED FOOD

AD LIB FOOD

AD LIB FOOD

Fig. 5.

However, it was observed in the present experiments that the cartilage cells of the hypophysectomised group showed a considerable degree of atrophy not seen in the 'starved' group, suggesting that the 'growth arrest' of these animals was only temporary and could be reversed again by unlimited feeding.

It would appear, therefore, that over - or under-feeding of rats being used for a tibial assay might materially affect the final result obtained. It is to be hoped that if animals have unlimited access to a balanced diet and an adequate fluid supply (as in these experiments), they will ingest the correct amount for their needs.

c. Adrenalectomy

Method

Young male rats (100 ± 10 g.) hypophysectomised 24 hours before air transport and intact animals of a similar weight range imported at the same time, were unilaterally nephrectomised 4 - 5 days after hypophysectomy. The animals were unilaterally nephrectomised in order to study the effect of hypophysectomy and adrenalectomy on kidney growth, but as this procedure appeared to have no effect on tibial cartilage growth it can be dismissed from further consideration. Half the intact and half the hypophysectomised rats were bilaterally adrenalectomised at the same time. The rats were killed 14 days after adrenalectomy and the tibiae treated in the usual manner.

Results

From Table 9 it would appear that adrenalectomy per se had no effect on the cartilage width of intact animals although the weight gain observed during the experimental period was less ($P < 0.01$) than that of the intact animals. A group of hypophysectomised rats was also adrenalectomised, but only two out of twelve survived the double operation. The difference in cartilage width between this latter group and the hypophysectomised group is not significant, but whether the result is meaningful, in view of the extensive mortality of the adrenalectomised animals, is questionable.

Discussion

The effects of adrenalectomy and ACTH on cartilage width and body weight noted by various workers are highly contradictory. Buffet and Wyman (1955) observed that there was a significant increase in cartilage width following adrenalectomy in intact rats (ages 80 - 200 days), a finding which supported that of Maasen (1951) who considered that adrenalectomy stimulated epiphyseal growth. The claims of Maasen were based on somewhat flimsy evidence gathered from measurements of tibial length; not cartilage width measurements. On the other hand Wyman and Tum-Suden (1945) and Ingalls and Hayes (1941) observed a reduction in cartilage width following adrenalectomy. Wyman and Tum-Suden ascribed this to 'self-starvation' in their young animals as cartilage width was restored to control levels following salt treatment and restoration

TABLE 9

Effect of bilateral adrenalectomy and hypophysectomy and ACTH administration on the body weight and the width of the tibial epiphyseal cartilage of unilaterally nephrectomised immature male rats.

Treatment	No. of animals	Mean width of tibial epiphyseal cartilage plate in μ Mean \pm S.E.	Mean increase or decrease in total body weight in g. Mean \pm S.E.
Pituitary intact	12	301.27 \pm 12.09	62.05 \pm 5.29
Pituitary intact/ adrenalectomised	11	296.42 \pm 11.52	41.18 \pm 3.46
Hypophysectomised/ adrenalectomised	2	152.47 \pm 20.84	1.0 \pm 0
Hypophysectomised	11	122.26 \pm 4.94	-4.0 \pm 3.15
Hypophysectomised, 2 i.u. ACTH/day	12	100.30 \pm 7.24	-5.0 \pm 3.49
Hypophysectomised/ adrenalectomised, 2 i.u. ACTH/day	3	119.06 \pm 8.25	-10.6 \pm 6.44

of appetite. The observations of Wyman and Tum-Suden appear to be confirmed in this experiment in which adrenalectomy per se did not appear to affect cartilage growth in either intact or hypophysectomised rats. Adrenalectomy did, however, appear to have a deleterious effect on the general health of hypophysectomised rats, and few survived the double operation.

It would seem, therefore, that routine tibial assays would not appreciably benefit by the use of test animals which had been both hypophysectomised and adrenalectomised.

B. Hormone Assays

Comparison of the Potency of Various Growth Hormone

Preparations.

Introduction

The growth hormone content of all but one of the preparations listed at the beginning of the chapter have been estimated in terms of the international standard. Wherever possible 6 - point assays were performed, but in some cases of duplication and shortage of material 4 - point assays were used.

Results

The results of sixteen such assays are shown in Tables 10, 11 and 12. All assays were proved to be valid; the five 4-point assays showed no significant deviation from parallelism, while the remaining eleven 6-point assays showed, in addition, no significant curvature of the dose response curve.

TABLE 10

Comparison of International Standard with Armour
Standard Beef Growth Hormone

Assay Design	Relative Potency	Fiducial Limits (P = 0.95)	λ	Laboratory
3 + 3	0.744	40 - 125%	0.235	Edinburgh
	0.75	52 - 98%		Standards* Committee

*The assay used is described as depending on the growth rate of hyper-thyroid mixed animals. It probably means the increase in body weight rather than the tibial assay.

While the International Standard therefore has a potency of only 75% of that of the Armour Standard, it was decided that 1 i.u. Growth Hormone represents the activity contained in 1 mg. of the International Standard.

TABLE 11.

Estimation of the Growth Hormone Content of a number of Preparations of Growth Hormone from Species Other Than Human.

Preparation	Assay Design	Growth Hormone content	\bar{x}	Fiducial Limits ($P = 0.95$)
ARMOUR	2 + 2	1.34 i.u./mg.	0.235	0.80 - 2.48 i.u.
'Kwa'	3 + 3	1.68 i.u./mg.	0.513	0.61 - 4.92 i.u.
NIH-B2	(3 + 3)	(0.72 i.u./mg.	0.820	0.17 - 3.08 i.u.
	(2 + 2)	(0.79 i.u./mg.	0.740	0.34 - 4.96 i.u.
NIH-B4				
NIH-S3	3 + 3	0.79 i.u./mg.	0.870	0.15 - 3.68 i.u.

TABLE 12.

Estimation of the Growth Hormone Content of a Number of Preparations
of Human Growth Hormone

Preparation	Assay Design	Growth Hormone content	λ	Fiducial Limits ($P = 0.95$)
Raben 6	2 + 2	0.371 i.u./mg	0.197	0.21 - 0.79 i.u.
Raben 9	(3 + 3) (2 + 2)	(0.415 i.u./mg. 0.455 i.u./mg.)	0.975 0.747	0.09 - 2.04 i.u. 0.07 - 1.98 i.u.
Raben 11	3 + 3	0.353 i.u./mg.	0.882	0.07 - 1.51 i.u.
Raben 12	3 + 3	0.146 i.u./mg.	0.889	0.01 - 0.47 i.u.
Cambridge	3 + 3	1.17 i.u./mg.	1.09	0.24 - 4.96 i.u.
MRC Standard A	3 + 3	0.11 i.u./Ampoule	0.520	0.03 - 0.26 i.u.
ICRF 6	(2 + 2) (3 + 3)	(0.80 i.u./mg. 0.92 i.u./mg.)	0.383 0.354	0.56 - 1.14 i.u. 0.72 - 1.43 i.u.
ICRF 6 Peak 1	2 + 2	0.16 i.u./mg.	0.395	0.11 - 0.24 i.u.
ICRF 6 Peak 2	3 + 3	1.14 i.u./mg.	0.446	0.44 - 2.69 i.u.

Armour House Standard - Somar A

A comparison of the International Growth Hormone Standard with the Armour House Standard (Somar A) shows that the International Standard has an activity of only 74.4% (fiducial limits 40 - 125%) of that of the Armour material (Table 6). This result agrees with the findings of the biological standards committee which accepted a final figure of 75% (95% confidence limits 52 - 98%) obtained using the growth hormone assay based on the increase in body weight of hypophysectomised immature rats. As the Committee agreed to define the International Unit of Growth Hormone as the activity contained in 1 mg. of the prepared International Standard, this means that 1 mg. of Armour Standard has an activity equal to 1.34 i.u. growth hormone.

Beef and Sheep Pituitary Growth Hormone Preparations

As will be seen from Tables 10 and 11, except for the recently prepared Kwa 'soft' preparation of growth hormone this makes the Armour material the most potent preparation of growth hormone tested. It is interesting to note the very high level (1.68 i.u./mg.) of activity obtained in material which has not been subjected to harsh chemical procedures.

Using a preparative system based on the Cohn fractionation technique, which involves alcohol precipitation at low temperatures, the beef and sheep growth hormones supplied by the National Institute of Health, U.S.A., were approximately equipotent with each other, but had a somewhat lower activity (approximately 0.7 -

0.8 i.u./mg.) than the International Standard.

Human Pituitary Growth Hormone Preparations

The even harsher technique of pituitary extraction used by Raben, which involves extraction of the pituitary material with glacial acetic acid at 70°C, has resulted in preparations with a relatively low activity (about 0.4 i.u./mg.). It is possible that a high proportion of the growth hormone material consists of denatured protein formed during extraction by the Raben method and therefore biologically inactive.

The ICRF material which was also prepared according to the Raben method is approximately twice as active (0.8 i.u./mg.), the result of further purification procedures aimed at producing a highly purified preparation. It was interesting to note that when this preparation was subjected to further separation on a G 200 Sephadex column (buffer 0.05 M borate, 0.05 M KCl, pH 8.6) two distinct fractions were obtained. These were designated Peak 1 and Peak 2. Peak 2 appeared to have a high biological activity (1.14 i.u./mg) while Peak 1 had a very low activity (0.16 i.u./mg.). The estimates of biological activity in each fraction agree with the estimates of immunological potency made by Dr. Hunter, who found that the respective immunological estimates of the growth hormone activity of Peaks 2 and 1 were one-and-a-half and one-fifth the activity of the initial material. It is not yet clear as to whether Peak 1 is mainly denatured protein or a 'prolactin' fraction with only a low biological activity.

The material supplied by the Medical Research Council - MRC Standard A - also shows a high activity (0.11 i.u./ampoule, i.e. approximately 1.0 i.u./mg.). It also has been subjected to stringent purification procedures, as it has been prepared for clinical use and as a U.K. standard for human growth hormone.

The Cambridge human growth hormone was prepared for immunological work, and therefore submitted to stringent purification procedures before assay. This material was also found to have a high biological activity (1.17 i.u./mg.).

Discussion

The fiducial limits of the tibial test are usually wide and the lambda figures frequently high. Lambda figures of 0.225 (Dingemans *et al.*, (1948) to 0.330 (Gemzell and Heijkenskjold, 1956) have been reported in the literature; the lambda figures obtained in these experiments have usually been much higher (range 0.197 - 1.09). High lambda figures are usually taken to indicate a lack of precision, but here they simply reflect the combination of a high standard deviation and a relatively shallow slope. In the tibial assay, high standard deviations are the inevitable consequence of the variability of animal response and groups of 5 - 6 animals have been used wherever possible, in order to minimise this effect.

As the fiducial limits of all the assays overlap, it seems reasonable to conclude that the test preparations have common identity and are in fact growth hormone.



C. Applications of the Tibial Test

Effect of Dithiocarbamoylhydrazine derivative - Compound

33,828 (I. C. I.) on Cartilage Growth

Introduction

This dithiocarbamoyl derivative, compound 33,828 (Imperial Chemical Industries) had been shown to inhibit pituitary gonadotrophic function in rats (Paget, Walpole and Richardson, 1961). Brown (1963) considers that compound 33,828 reduces the formation of pituitary gonadotrophin, but whether it acts as a selective or total pituitary depressant was not however determined.

In this study, the effect of the compound, when administered alone or in combination with growth hormone (NIH - B - 2), on growth-promoting activity as judged by the tibial test in hypophysectomised rats was examined.

Materials and Methods

1. Compound 33,828
2. Growth hormone - beef - (NIH-B-2).

Both compounds were dissolved in 0.1N NaOH and made up to the desired concentration with 0.9% saline. The injection schedule is shown in Table 13. The dose of growth hormone (50 µg/day) used was selected because it was known to have a marked effect on cartilage width. The doses of compound 33,828 (1.5, 6.0 and 24.0 mg./kg.) administered to the rats were comparable to those given to human subjects (Bell, Brown, Fotherby, Loraine and Robson, 1962). A standard four-day tibial assay was carried out.

The effect of Compound 33,828 alone and together with beef growth hormone on tibial epiphyseal width and on body weight.

Treatment showing - dose/injection	No. of Animals	Total dose over 4 days (ug)	Width of tibial epiphyseal cartilage ₊ plate - micra (Mean - S.E.)	Increase in body wt. during treatment - g (Mean - S.E.)
0.9% saline	6	0.80 mL.	212.58 ± 10.1	1.16 ± 0.6
33,828 (75 ug)	5	300	214.93 ± 9.8	1.2 ± 1.1
33,828 (300 ug)	5	1200	196.61 ± 8.8	- 0.4 ± 0.75
33,828 (1200 ug)	5	4800	187.28 ± 10.95	- 2.4 ± 0.51
(33,828 (75 ug) (Growth Hormone (50 ug)	4	300 200	255.57 ± 13.6	5.75 ± 1.3
(33,828 (300 ug) (Growth Hormone (50 ug)	4	1200 200	253.97 ± 13.5	5.75 ± 2.3
(33,828 (1200 ug) (Growth Hormone (50 ug)	5	4800 200	237.0 ± 9.1	1.2 ± 0.86
Growth Hormone (50 ug)	5	200	270.61 ± 9.6	7.8 ± 0.97

Results

The effects of growth hormone and compound 33,828 separately and in combination on the width of the tibial epiphyseal cartilage and on body weight of hypophysectomised immature female rats are shown in Table 13. It can be seen that the lowest dose of the compound had no effect ($P > 0.1$) on cartilage width, although the two higher doses caused considerable reductions in cartilage width ($P < 0.001$ in both cases). When administered together with beef growth hormone, all three doses inhibited the action of the hormone ($P < 0.001$ in all cases), the degree of inhibition depending on the amount of the compound administered.

Although only the short four day assay period was used, the effects on body weight show a similar trend. Only the animals treated with the highest dose of the compound showed significant differences. A small overall loss in weight (mean - 2.4 g.) was observed in the treated animals, and when compared with the animals receiving saline this loss was significant ($P < 0.01$). Weight gain in the rats treated with the compound plus growth hormone was no different ($P > 0.1$) from that of the control animals, but was significantly less than that of animals treated with hormone alone ($P < 0.001$).

Discussion

These results indicate that in the rat compound 33,828 is capable of inhibiting growth. While its action on body growth is

similar to that of ACTH (see page), weight loss and cartilage regression also occur during reduced feeding experiments. From this experiment, it was not possible to decide whether the growth-inhibiting effect of this compound was mediated through the adrenal gland (although it was noted that the mean adrenal gland weight - Table 14 - of animals treated with 1200 $\mu\text{g.}/\text{day}$ of the compound together with 50 $\mu\text{g.}/\text{day}$ of growth hormone was significantly greater - $P < 0.001$ in both cases - than that of either the saline or growth hormone treated animals, implying increased adrenal activity in this group) or simply a self-imposed starvation effect resulting from a general toxicity inherent in the compound. Brown (1963) had observed general toxic effects with high doses of the compound, but considered that the depression of pituitary FSH noted could not be entirely ascribed to toxicity. It is probable therefore that some, if not all, of the growth inhibition seen in this study reflects a true biological effect of this dithiocarbamoylhydrazine derivative.

6. Effect of a Synthetic Polypeptide - CIBA 30920 - BA. on Cartilage Width.

Introduction

This synthetic polypeptide is a straight chain compound consisting of the β 1-24 amino acids found in natural ACTH. It has been shown to have ACTH-like activity and it is claimed that 0.01 mg. of this compound has an activity comparable to 1 i.u. of natural ACTH (Schuler, Schär and Desaulles, 1963).

TABLE 14

Effect of Compound 33,828 Alone and in Combination with
Beef Growth Hormone on the Adrenal Weight of Immature
Hypophysectomised Rats.

Treatment	No. of Animals	Adrenal Weight in Mgs.	
		Mean	± S.E.
0.9% saline	6	9.16	± 0.60
33,828 (75 µg)	5	8.60	± 0.81
33,828 (300 µg)	5	8.80	± 0.73
33,828 (1200 µg)	5	10.00	± 0.45
(33,828 (75 µg) (Growth Hormone (50 µg)	4	9.00	± 0.82
(33,828 (300 µg) (Growth Hormone (50 µg)	4	9.25	± 0.48
(33,828 (1200 µg) (Growth Hormone (50 µg)	5	12.00	± 0.32
Growth Hormone (50 µg)	5	9.60	± 0.19

In this experiment, the effect of the compound in a dose said to be equivalent to 1 i.u./day (which in the natural ACTH was known to have a marked depressant activity on cartilage width) was studied.

Results

The results of this experiment are shown in Table 15. It will be seen that, while there is apparently a slight increase in the cartilage width of animals treated with the Ciba synthetic, there is no significant difference in the cartilage response of this group and the saline control group.

Discussion

The results of this experiment were completely unexpected. Schuler et al., (1963) had shown that the material depleted adrenal ascorbic acid (Sayer's test for ACTH) and had melanocyte activity. By 1962 Li had shown that natural ACTH consisted of a 39-amino acid polypeptide; the 25 - 39 amino acid sequence being variable between species and considered by him to be non-essential for hormonal activity. He had also examined a synthetic polypeptide consisting of the β 1-19 amino acids and shown it to have both high ACTH and melanocyte activity. Thus it was expected that the Ciba synthetic compound would have had a marked depressing effect on cartilage growth.

Several explanations are available. The first and simplest, is that not enough of the compound was administered. In the two experiments in which natural ACTH was used a total dose of 28 i.u./animal over a 14-day period was administered as against 4 i.u./

TABLE 15.

A comparison of the effect of saline and the Ciba synthetic compound 30920-Ba on the width of the tibial epiphyseal cartilage.

Treatment	No. of Animals	Epiphyseal Cartilage Width in μ mean \pm S.E.	t	P
0.9% saline	8	197.98 \pm 5.11	1.1846	> 0.2
Ciba 30920 - Ba	8	214.98 \pm 13.42		

animal over a 4-day period here. This would imply that ACTH requires a longer period of time than growth hormone to make its effect observable.

A second explanation could lie in the fact that this compound is more rapidly destroyed than natural ACTH and therefore may have been rendered inactive before it could depress cartilage growth.

The third explanation could lie in the structure of the compound. The synthetic molecule is much smaller than natural ACTH, and consists only of the 1 - 24 amino acids, which, however, it is claimed are the active components of the natural ACTH. It is, however, possible that the growth inhibiting properties may be associated with the amino acids sequence 25 - 39, which has up till now been regarded as being concerned simply with species specificity and not to be essential for the biological activity of the hormone.

The first and second hypotheses could be proved or disproved by repeating the experiment. If, a higher total dose, administered more frequently over a prolonged period did not diminish cartilage width, it would seem that the third hypothesis was the most feasible.

7. The effect of anterior pituitary hormones on the tibial epiphyseal cartilage of rats in which atrophy of the gastrocnemius muscle has been experimentally induced.

Introduction

There is practically no information available at present on the relationships between muscular atrophy, bone growth and

hormone treatment although Simpson, Evans and Li (1949) observed muscular hypertrophy accompanied by bone growth in hypophysectomised rats following treatment with growth hormone.

The present study was undertaken in order to determine the action of individual anterior pituitary hormones on the tibiae of rats in which muscular atrophy had been induced by section of the nerve. In this way it was hoped to ascertain whether muscular atrophy would modify the tibial response to hormone administration, and whether this response would be affected by the time at which the hormone was administered. In the series of experiments recorded here in non-hypophysectomised animals, the hormones were administered either prior to or immediately following nerve section, or after muscular atrophy had occurred.

Materials and Methods

The rats were of the Wistar strain and had a mean initial weight of 56 g (range 49 - 72 g); male and female rats were used in equal numbers wherever possible. In all groups apart from the unoperated animals (killed on day of operation), atrophy of the gastrocnemius muscle was produced by cutting the nerve on one side. The nerves of right and left legs were sectioned in equal numbers. In order to prevent reinnervation of the muscle during the post-operative period a portion of the nerve (mean length 15 mm.) was removed.

The animals were subdivided into three main groups which were treated in the following way:-

Group A - animals were treated for 9 days prior to and 2 days after operation and killed on the 12th post-operative day.

Group B - animals were treated for the 11 days immediately post-operative and killed on the 12th day.

Group C - animals were rested immediately after operation and treated from the 9th - 21st day, being killed on 22nd day.

Apart from the unoperated animals, rats were injected with one of the following:-

1. 0.9% saline - 0.5 mls/day
2. Growth hormone - NIH - GH - B₄ - 2 µg/day
3. Adrenocorticotrophic hormone (ACTH) - 'Zinc Corticotrophin' Organon - 2 i.u./day
4. Thyroid-stimulating hormone (TSH) - NIH - TSH - B1 - 1 i.u./day
5. Follicle-stimulating hormone (FSH) - NIH - FSH - S1 - 1 mg./day

At death the tibiae were dissected out and stored in 10% formol saline until the cartilage width could be measured. All cartilage width measurements were made according to the modified method of Greenspan, et al. (1950).

Results

The results have been summarised in Tables 16 and 17 and Fig. 6.

TABLE 16.

Effect of hormone treatment on the tibiae of rats with artificially induced atrophy of the gastrocnemius muscle

	No. of Animals/ Group	DENERVATED Tibial Width in Micra			INNERVATED Tibial Width in Micra			P
		Mean	S.D.	S.E.	Mean	S.D.	S.E.	
Controls -								
Unoperated	8				242	83	29	
Group A	10	246	41	13	260	29	9	N.S.
Group B	5	250	20	9	246	35	16	N.S.
Group C	5	195	36	16	216	56	25	N.S.
ACTH -								
Group A	10	190	61	19	184	61	19	N.S.
Group B	10	251	32	10	274	43	13	N.S.
Group C	10	218	50	16	234	60	19	N.S.
G.H. -								
Group A	10	202	33	10	209	31	10	N.S.
Group B	10	228	31	10	240	29	9	N.S.
Group C	9	193	56	19	222	50	17	N.S.
TSH -								
Group A	5	202	67	30	189	61	27	N.S.
Group B	5	214	24	11	251	12	5	<0.01
Group C	5	220	33	15	223	42	19	N.S.
FSH -								
Group A	10	192	54	18	196	45	14	N.S.
Group B	10	276	59	19	279	39	12	N.S.
Group C	7	199	30	12	207	28	11	N.S.

Unoperated = animals killed on day of operation.

Group A = animals treated for 9 days prior to, and 2 days after, operation and killed on 12th post-operative day.

Group B = animals treated for 11 days immediately post-operative and killed on 12th day.

Group C = animals treated from the 9th to 21st day post-operatively and killed on 22nd day.

TABLE 17.

Effect of hormone treatment on the tibiae of rats with artificially induced atrophy of the gastrocnemius muscle -
Significance figures for treated groups versus the matching control groups.

	DENERVATED			INNERVATED			
	Group A	Group B	Group C	Unoperated	Group A	Group B	
<u>CONTROL</u>							
Group A				N.S.			
Group B				N.S.			
Group C				N.S.			
<u>ACTH</u>							
Group A	<0.05			<0.10	<0.01		
Group B		N.S.		N.S.		N.S.	
Group C			N.S.	N.S.			N.S.
<u>G.H.</u>							
Group A	<0.02			N.S.	<0.001		
Group B		N.S.		N.S.		N.S.	
Group C			N.S.	N.S.			N.S.
<u>TSH</u>							
Group A	N.S.			N.S.	<0.01		
Group B		<0.05		N.S.		N.S.	
Group C			N.S.	N.S.			N.S.
<u>FSH</u>							
Group A	<0.05			<0.001	<0.001		
Group B		N.S.		N.S.		N.S.	
Group C			N.S.	N.S.			N.S.

Unoperated = animals killed on day of operation.

Group A = animals treated for nine days prior to and 2 days after operation and killed on 12th post-operative day.

Group B = animals treated for 11 days immediately post-operative and killed on 12th day.

Group C = animals treated from the 9th-21st day post-operatively and killed on 22nd day.

Fig. 6:- EFFECT OF HORMONE TREATMENT ON THE TIBIAE OF RATS WITH ARTIFICIALLY INDUCED ATROPHY OF THE GASTROCNEMIUS MUSCLE.

UNOPERATED i.e. ANIMALS KILLED ON DAY OF OPERATION.

GROUP A i.e. ANIMALS TREATED FOR 9 DAYS PRIOR TO, AND FOR 2 DAYS AFTER OPERATION AND KILLED ON THE 12TH POSTOPERATIVE DAY.

GROUP B i.e. ANIMALS TREATED FOR 11 DAYS IMMEDIATELY POSTOPERATIVE AND KILLED ON THE 12TH POSTOPERATIVE DAY.

GROUP C i.e. ANIMALS TREATED FROM THE 9TH - 21ST POSTOPERATIVE DAYS AND KILLED ON THE 22ND DAY.

EFFECT OF HORMONE TREATMENT ON THE TIBIAE OF RATS WITH ARTIFICIALLY INDUCED ATROPHY OF THE GASTROCNEMIUS MUSCLE.

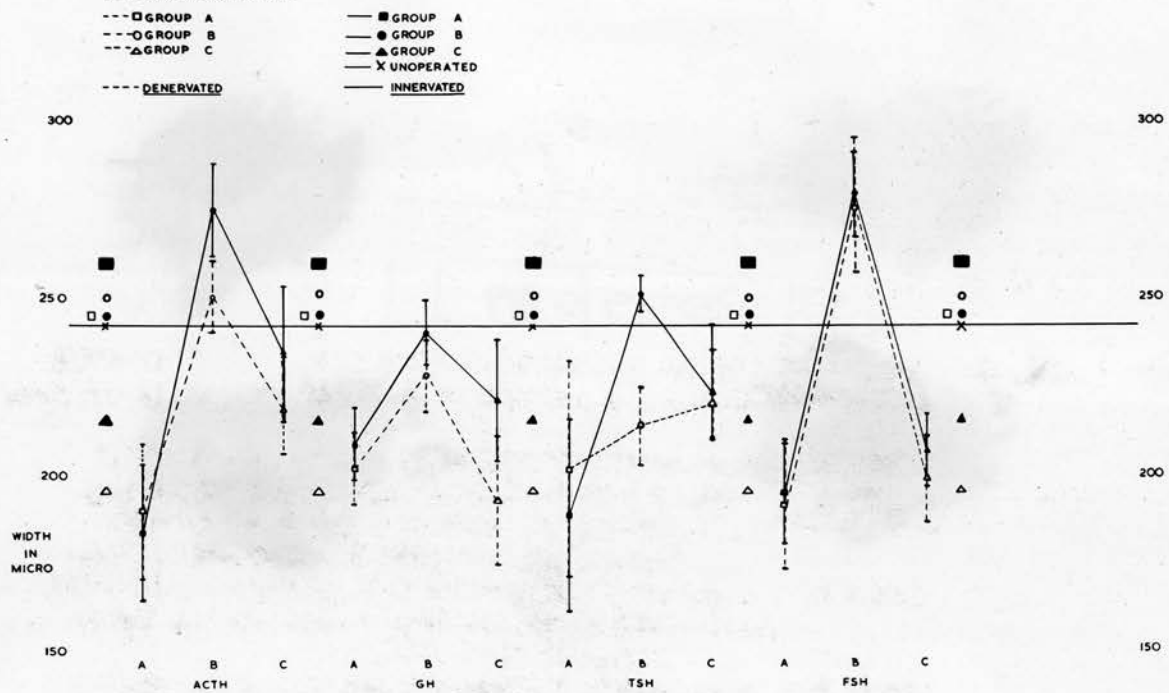


Fig.6.

Controls

The unoperated animals, i. e. those killed on the day of operation, have a mean cartilage width of $242 \pm 29 \mu$. The mean width (innervated leg) of none of the sub-groups is significantly different from that of the unoperated group; neither can a difference between the cartilage widths of the innervated and denervated limbs be observed within any of the sub-groups.

ACTH, Growth Hormone, and FSH

The results observed following treatment with ACTH, Growth Hormone and FSH were surprisingly similar. In spite of the apparent difference in the cartilage widths of innervated and denervated limbs observed in ACTH group A and group B, and in Growth Hormone group A and group B, none of the groups showed a significant difference. The cartilage widths of the ACTH group A animals were narrower than those of the matching controls ($P < 0.05$ denervated leg; $P < 0.01$ innervated leg and $P < 0.1$ innervated leg v. unoperated limbs); as were also those of the growth hormone group A treated animals ($P < 0.02$ denervated leg and $P < 0.001$ innervated leg.) A similar reduction in cartilage width can be seen in the FSH treated group A animals ($P < 0.05$ denervated leg; $P < 0.001$ innervated leg and $P < 0.001$ innervated leg v. unoperated limbs).

TSH

No difference in epiphyseal cartilage width was observed between the innervated and the denervated legs of the TSH treated

group A and C animals, although the cartilage width of the denervated leg was considerably less than that of the innervated leg in the group B. When the TSH treated groups are compared with their matching controls, the cartilage width of the TSH group B - denervated legs is less than that of the appropriate control ($P < 0.05$), as is also that of the TSH - group A - innervated leg ($P < 0.01$).

Discussion

The experiment was designed in order to ascertain whether atrophy of the gastrocnemius muscle affected tibial epiphyseal cartilage width, and whether the response was modified in any way by treatment with individual anterior pituitary hormones. Hormone treatment was given i) prior to nerve section ii) in the immediate post-operative period, and iii) after atrophy was established, in order to fix in time any interaction of hormone treatment and the establishment of muscular atrophy, i. e. to find out if the epiphyseal cartilage of the tibia is more sensitive to hormone treatment in the days immediately prior to nerve section, during the period of atrophy induction, or after atrophy is well established.

From the results reported here it would appear that section of the sciatic nerve, with resulting atrophy of the muscle, did not modify the width of the tibial epiphyseal cartilage in normal rats treated with saline in the above manner when examined 12 and 22 days after nerve section, although muscular atrophy was by this

time well established.

The general lack of response amongst the rats treated with hormones was somewhat surprising, especially when compared with the results obtained on muscle weight. The reduction in cartilage width noted in all but one of the hormone treated group A type animals is extremely difficult to explain. In this connection it is of interest to note that the administration of both ACTH and growth hormone caused a marked diminution in cartilage width of both innervated and denervated limbs. This catabolic effect is quite contrary to the more usual marked increase in cartilage width following growth hormone treatment (Becks et al 1941), although it has been observed following the administration of ACTH (Becks et al 1944). In fact a lack of response by these animals could have been explained more easily, as it could have been ascribed to the treatment schedule: in these animals, hormone treatment was discontinued 9 days before death. Any effect, therefore, could have worn off by the time the tibiae were removed. On the other hand this suggestion does not explain the non-response observed in groups B & C, as in these groups hormone treatment was not discontinued until the day preceding death. It is possible, however, that despite the massive doses of hormone used in this experiment, these were not sufficiently large to superimpose their usually marked effects on rats with intact hypophyses.

Finally, it is just possible that positive responses may have been masked by the large standard errors encountered; the wide

range observed is probably due to the fact that rats with intact hypophyses were used in this study, and while the initial weight range was not excessively wide, it is very possible that the growth rate, and perforce the cartilage width of individual rats, was very different.

GENERAL DISCUSSION

Over the last two decades the tibial test has been the most widely accepted bioassay method for growth hormone. In spite of various modifications, the procedure has not been applicable to clinical problems. Gemzell, Heijkenskjöld and Ström (1955) and Gemzell (1959) have however, reported the presence of a growth promoting substance, which they considered to be growth hormone, in fractionated plasma samples from acromegalic subjects. While, one would expect growth hormone to be present in the blood of active acromegalic, recent work has shown that it would be most unlikely for the levels to be high enough for it to be detected by the tibial test. In the work presented herein, it has been shown that a daily injection of 5 $\mu\text{g.}$ /rat for 4 days produces a final mean cartilage width which is not significantly greater than that shown by animals have received only 0.9% saline. This figure for the sensitivity of the tibial test should be compared with the work using radio-immuno-assay methods which have shown the level of growth hormone in acromegalic blood to be in the region of 0.1 $\mu\text{g.}$ /ml. plasma (Glick, et al., 1963). As one would expect the levels in

normal subjects and other disease states to be rather less than the acromegalic figure, this means that the tibial assay is virtually useless for clinical estimations.

The assay is however of value in the comparison of different preparations of pituitary growth hormone, in particular of preparations from different species. It has been found that the epiphyseal cartilage of the rat appears to respond to growth hormone of widely divergent origin, including a wide variety of mammals (li, Papkoff and Jordan, 1959) and other vertebrates such as a fish (the shad), frogs and chickens (Solomon and Greep, 1959). Although the rat tibial epiphyseal cartilage plate apparently exhibits a non-specific response to growth hormone, Solomon and Greep (1959) claim that when equal weights of crude pituitary extracts were tested, the rat cartilage was apparently more sensitive to amphibian and mammalian extracts than to those obtained from piscine, avian and reptilian pituitaries. This is only to be expected and an interphylur difference has also been shown for prolactin, Forsyth (1964) claims that a human growth hormone preparation which appeared to have a very low prolactin concentration (about 4 i. u. /mg.) when tested by the pigeon crop assay proved to have a much higher concentration (about 15 i. u. /mg.) when tested in the rabbit. It is probable, that, in the experiments reported here, the very marked variation in potency of the different pituitary extracts noted, is due, not so much to inter-species differences as to variations in the methods of extraction and

purification used. It has been noted that the material (all of human origin) prepared and obtained direct from Raben (Boston) had a consistently low activity, although material (also of human origin) initially prepared by his method and then further purified for use in clinical and immunological work had a consistently high biological activity (Cambridge, ICRF & MRC). Similar differences in the potencies of the bovine and ovine pituitary growth hormone preparations were also observed; it was interesting to note the very high biological activity of Kwa's 'soft' bovine preparation, which was simply prepared by submitting a pituitary homogenate to high speed centrifugation and separation on a Sephadex column. It is tempting to contrast this very high activity with the generally low activity of the Raben preparations and to ascribe the difference to the extraction methods. It is probable, that, the Raben method, which includes extraction of the pituitaries with hot glacial acetic acid (70°C.), is a very harsh chemical procedure when compared to the relatively gentle Kwa method, and that it is accompanied by a certain amount of denaturation of protein which will result in a material with a relatively low biological activity.

In 1959, Li et al. observed that when the same weight (5 µg./day) of growth hormone from a number of mammals was administered to the hypophysectomised rat, there was a marked difference in the cartilage width and body weight responses to the different growth hormones. After 4 days the tibial widths were approximately the same (indicating apparent equipotency) for all the

growth hormones, but after about 10 days a divergence in the body weight response became noticeable. At about this time the body weight of animals receiving primate, i. e. human and monkey, growth hormone appeared to reach a plateau, and although treatment was continued for 90 days failed to show any further response, while the animals receiving bovine and whale growth hormone continued to respond i. e. increase in weight, throughout the entire period. Differences between the body weight and cartilage width responses were also observed in the experiments reported here. The difference was most marked following the administration of combined LH and FSH, these hormones had no effect on cartilage width i. e. bone growth, but caused a marked stimulation of whole body growth as measured by an increase in total body weight. While, the difference noted, following the administration of growth hormone and the two levels of combined hormones, could be ascribed to a differential response, it is more probable that a synergistic effect of the combined hormones on tibial width has been masked. From the results obtained it is obvious that a near maximal tibial stimulation was produced by a daily dose of 1 mg. growth hormone, but that the body weight increase was by no means maximal. It was also interesting to note that, in the experiment designed to test the optimal injection period, that while maximal cartilage width stimulation occurred within 4 days, body weight was still increasing after 14 days.

The tibial test has also been applied to the assessment of the

general growth properties of two new compounds. It was interesting to note that, while the synthetic polypeptide Ciba 30920 - Ba with known ACTH-like activity did not have an inhibitory effect on cartilage width, the pituitary inhibitor I. GI. 33,828 did show a marked depressant action on the cartilage. Since, Li (1962) had shown that a synthetic B 1 - 19 amino acid chain had ACTH-like effects and could stimulate cortisone production by the adrenal and similar properties had been shown for the B 1 - 24 synthetic polypeptide Ciba 30920 - Ba (Schuler et al., 1963) it was surprising to find that this product had no apparent effect on cartilage width. The mode of action of naturally occurring ACTH on cartilage growth is still somewhat obscure. It has been suggested that ACTH acts on the adrenal cortex to stimulate the production of cortisone (a known growth inhibitor, Ragan et al 1949; Sobel, 1958) which then acts on the cartilage (Becks et al. 1944). Becks and his co-workers found that in pituitary intact rats, ACTH did not cause cartilage regression in adrenalectomised animals, however, the results of an experiment in which marked cartilage depression was observed following the administration of ACTH to rats which had been both hypophysectomised and adrenalectomised were inconclusive due to the very high mortality encountered in both the control and treatment groups (see page and Table 9). It is, however, possible that this was a real effect, indicating that the inhibitory effect of ACTH on cartilage growth is a direct effect, rather than one mediated through stimulated adrenal production of cortisone; and that this

action is located somewhere in the β 25 - 39 amino acid chain which has so far, been considered to be concerned only with species specificity and is absent from the structure of the synthetic compound thus suggesting that the lack of depressant activity of this compound on cartilage growth is due to the absence of a specific growth inhibitor located somewhere in the β 25 - 39 position.

On the other hand it was equally surprising to find that the dithiocarbamoylhydrazine derivative (I. C. I. 33, 828) had a very marked depressant action on cartilage growth. It had been shown to inhibit pituitary gonadotrophic action (Paget et al., 1961; Bell et al., 1962), but it was not known whether the compound had a general or a specifically gonadotrophin inhibitory action on the pituitary. If it was a general inhibitor, it could act by inhibiting growth hormone release from the pituitary in much the same way as some of the monoamine oxidase inhibitors prevent the release of pituitary growth hormone (Zor, Didckstein and Sulman, 1965). If, however, its action on the pituitary was specifically one of gonadotrophin secretion inhibition, it would seem unlikely to have an effect on cartilage growth, unless it was one of general toxicity. Brown, (1963) observed that the compound produced marked signs of toxicity, and as the animals in the experiment presented here showed a tendency to lose weight it is possible that some of the effect of this compound on tibial epiphyseal cartilage width was simply a reflection of the general toxicity of Compound 33, 828. However, the experiments presented here do not elucidate the mechanism of action of

these two new products.

It has long been thought that a relationship existed between some muscular diseases and the pituitary gland (for references see Adams, Denny-Brown and Pearson, 1962; Herman, 1953 and Shy, 1960). Muscular atrophy is followed by a general withering of the affected limb and it was thought that application of the tibial test to an experiment in which muscular atrophy was artificially induced in rats might provide some useful information about bone growth in this condition. It appeared, however, that under the conditions of this experiment, bone growth was not affected, even though the animals became very lame. Hormone therapy in these animals did not appear to have an appreciable effect on cartilage growth.

It would appear, therefore, that the future use of the tibial assay lies in 1) the standardisation of new growth hormone preparations, 2) the assessment of 'growth' promoting or inhibiting properties of synthetic compounds and 3) its application to the elucidation of the physiological effects of protein hormones in the experimental animal.

SUMMARY

1. The tibial assay method for growth hormone has been established and some of the conditions of assay examined.
2. Specificity tests which included observations on the effect of the anterior pituitary hormones ACTH, FSH, LH, Prolactin and TSH feeding experiments and the effect of adrenalectomy on the width

of the tibial epiphyseal cartilage in hypophysectomised immature rats, have been studied.

3. The potency of 13 pituitary growth hormone preparations (3 of bovine, 1 of ovine and 9 of human origin) has been estimated in terms of the International Standard.
4. The effects of a dithiocarbamoylhydrazine derivative Compound 33,828 (ICI) and of a synthetic polypeptide Ciba 30920 - Ba on the cartilage width of hypophysectomised immature female rats have been investigated.
5. The effect of hormone administration on the condition of the tibial epiphyseal cartilage in pituitary intact immature animals suffering from artificially induced muscular atrophy has been studied.

CHAPTER III

IMMUNOLOGICAL ASSAYS

INTRODUCTION

The first suggestion that immunological techniques might be applied to the field of endocrinology was that of Stavitsky and Arquilla (1953) who were investigating the presence of insulin antibodies in the blood of patients showing a marked insulin resistance. In 1956, Arquilla and Stavitsky prepared an insulin antiserum in rabbits and applied immunological methods to the assay of purified insulin. Although Ferguson and Boyden (1953) recorded the formation of high titre antisera to BGH in sheep, which could be detected by the tanned cell technique, it was not until 1958 that Read and Stone reported, they had set up a haemagglutination system to beef growth hormone which might be used as a possible method of assay for the hormone. This was followed by a large number of papers recording attempts to prepare antisera to other hormones (for references see Chapter 1). The majority of these workers hoped to establish simple, specific quantitative assay methods for the hormone, but in addition many investigators on HCG were looking for a simple, quick and foolproof pregnancy test which would also be cheap to perform. In the early 1960's Ortho Pharmaceutical Ltd. brought out a commercial pregnancy diagnosis test based on an agglutination reaction between an HCG antiserum and suitably HCG-tagged latex particles.

The aim of the work reported herein, was firstly, to establish a sensitive assay method for human growth hormone which could be applied to clinical problems, and secondly, to establish a quick, specific and sensitive assay method for HCG which could be applied to the quantitative measurement of the hormone in urine and blood samples.

MATERIALS AND METHODS

1. Hormones

The hormones employed in this section were used for three purposes, namely a) raising antisera in rabbits, b) specificity tests and c) assay experiments.

a) Hormones used for raising antisera.

Bovine growth hormone - Armour 'Somar A'.

Human growth hormone - Raben 6.

Follicle-Stimulating hormone - NIH - S1.

Luteinising hormone - NIH - S1.

Human Chorionic Gonadotrophin - Organon 'Pregnyl'

Pregnant mare serum gonadotrophin - Organon 'Gestyl'.

All the above hormones except for the two Organon preparations (Pregnyl and Gestyl) have been described in the Tibial Test Section (see pages 23 - 29). Both the Organon preparations had been standardised in terms of the appropriate International Standard prior to distribution.

b) Hormones used in specificity tests.

In addition to the hormones quoted above, the following

hormones which have also been described in the Tibial Test Section (see pages 23-29) were also used in specificity tests:-

Bovine growth hormone - NIH - B2

Ovine growth hormone - NIH - S3

Thyroid-stimulating hormone - NIH - B1

Adreno-corticotrophic hormone - Armour 'Acthar'

c) Hormones used in assays.

i. - Standard Preparations

Beef growth hormone - Armour 'Somar A'

Human growth hormone - Raben 6

Human chorionic gonadotrophin - International Standard

ii - Test Materials

Beef growth hormone - NIH - B2

Human growth hormone - Raben 6

Human pituitary extract - a crude pituitary extract

from which four subfractions designated :-

Fraction 1

Fraction 2

Fraction 3

Fraction 4

have been obtained by further separation of the extract on a DEAE-cellulose column.

Human chorionic gonadotrophin - Organon 'Pregnyl'

2. Preparation of Diluent

In view of the fact that serum proteins from different animal species may crossreact, it was considered necessary to restrict the number of species involved as far as possible. It was therefore, decided, in view of the fact that the antiserum was prepared in rabbits, a phosphate buffered saline (1/15M: 0.9% N-Ce; pH 6.4) containing 1% normal rabbit serum (NRS) should be used as the diluent.

In early pilot experiments the NRS used was prepared in the laboratory, however, in later experiments the DIFCO preparation was employed. Following exhaustive tests with a number of red cell preparations, both control and antigen-coated, it was found that in no case was the addition of the cells to a phosphate buffered saline containing 1% NRS (Difco) followed by either spontaneous agglutination or haemolysis of the cells even when the tubes were left for 2 - 3 days at +4°C. The Difco NRS preparation was, therefore, considered to be satisfactory for routine work.

3. Preparation of Antisera in Rabbits

A specific antiserum can only be prepared from a pure antigen. The application of immunological techniques to hormone assay has therefore, only become possible since the preparation of pituitary hormones of high purity.

Protein hormones are of themselves only weakly antigenic and in order to obtain an antiserum of high titre, they have to be injected in a suitable adjuvant. The adjuvant is used to assist in the formation of antibodies, firstly, by slowing down the rate of

absorption and therefore of destruction of the antigen, and secondly, by stimulating the antibody producing mechanisms. It was found that the light mineral oil mixture known as Freund's Incomplete Adjuvant (F.I.A.) recommended by Read did not give a measurable antibody titre following the administration of growth hormone to rabbits. With a commercial preparation (Difco) of Freund's Complete Adjuvant (F.C.A.) which, however, consists of F.I.A. + killed mycobacteria, reasonably high titres could be obtained after a series of 3 or 4 injections.

In view of the fact that hormone preparations are seldom completely pure, there is a danger of selective fixation of the antibody, i. e. the production of antibodies in greater concentrations to the contaminants than to the main antigen present. Lack of pure antigens is the main factor affecting the specificity of the antiserum. However, if contaminating antibodies are present, it is possible by simple absorption techniques to reduce their concentration considerably. The cleanliness and specificity of the antiserum can then be confirmed by immuno-diffusion and immuno-electrophoretic techniques.

a) Immunisation with beef and human growth hormone.

Healthy young male or female rabbits of about 9 - 12 months, preferably with long pendulous ears and large ear veins, were chosen. In all cases they were acclimatised to their new surroundings for 3 - 4 weeks, prior to the commencement of the injection schedule.

An emulsion consisting of 2.5 mg. hormone dissolved in 2.5 ml. 0.9% saline and 2.5 ml. F.C.A. was prepared. Half of this preparation was injected intraperitoneally and the remainder subcutaneously in the back of the neck. The injections were repeated at intervals of 2 weeks. Fourteen days after the third injection, approximately 5 ml. blood were removed from the ear vein, and tested by the double dilution method in order to obtain a rough estimate of the antibody titre. If the titre was above 1 : 3,000, the animal was bled again; 30 - 40 ml. being withdrawn from a 2.5 kg. rabbit. If the titre was below 1 : 3,000 injections were continued at 14 - day intervals until a satisfactory level was reached.

b) Immunisation with Human Chorionic Gonadotrophin (HCG).

The preparation of HCG used was 'Pregnyl'; 300 i.u. were dissolved in 2.5 ml. 0.9% saline and an emulsion prepared using an equal volume of F.C.A. Animals were then treated as described for growth hormone.

c) Immunisation with Pregnant Mare Serum Gonadotrophin (PMSG).

The preparation used was 'Gestyl'; 3,000 i.u. were dissolved in 2.5 ml. 0.9% saline and an emulsion prepared using an equal volume of F.C.A. Rabbits were then treated as described above.

d) Immunisation with Follicle-Stimulating Hormone (FSH).

The preparation used was NIH - FSH - S - 1; 25 mg. were dissolved in 2.5 ml. 0.9% saline and an emulsion prepared using an equal volume of F.C.A. The rabbit was then treated as described above. Injections were discontinued, when 3 months after commencement of treatment, no antibodies could be detected.

e) Immunisation with Luteinising Hormone (LH).

The preparation used was NIH-LH-S-1 ; 10 mg. were dissolved in 2.5 ml. 0.9% saline and an emulsion prepared using an equal volume of F.C.A. The rabbit was then treated as above. Injections were discontinued, when 3 months after commencement of treatment, no antibodies could be detected.

4. Bleeding

The rabbit was firmly wrapped in a warm towel to prevent struggling. The ear was closely shaved, washed with alcohol and wiped with liquid paraffin. The ear was warmed, rubbed and the median vein dabbed with xylol to increase the flow of blood through the ear. The marginal vein was clamped with a bull-dog clip and a small longitudinal cut made in it with a Haggendorn needle. The blood was collected in a 50 ml. centrifuge tube, allowed to clot and then left for some hours at + 4°C. for retraction of the clot.

5. Purification of antisera. (Hayashida)

As much serum as possible was pipetted off into a clean centrifuge tube, and both this tube and the original tube containing

the clot were spun in the cold for 30 min. at approximately 2,500 rcf. The serum from each tube was pipetted off into clean containers; the serum from the tube containing the clot being respun. If this serum was not badly haemolyised the two batches were combined before further purification; otherwise the two samples were treated separately.

The antiserum was then incubated at 56°C. for 30 minutes to destroy the complement (a thermolabile factor which causes the lysis of red cells) and cooled.

The non-specific serum protein antibodies were then absorbed out. The antiserum was mixed with normal human serum (or normal serum from the species of origin of the antigen) diluted with 0.9% saline in the proportion 0.05 ml. 10% normal serum to 6.0 ml. antiserum, incubated at 37°C. for 1 - 2 hours, and left at +4°C. overnight, centrifuged in the cold at 2,500 ref. for 20 minutes, and the supernatant collected. This was repeated until no further visible precipitate was formed.

Other hormone antibodies can be absorbed out in the same way, using the appropriate absorbants.

Finally, non-specific cell antibodies were absorbed out by mixing and standing for 30 minutes at room temperature with an equal volume of washed, packed red cells (of the type to be used in the agglutination reaction).

Serum was finally stored in 0.5 ml. aliquots at -20°C.

6. Immuno - Diffusion (Kohn)

Oxoid 5.0 cm. cellulose acetate discs were used as the supporting medium. Spots were marked on the discs with a soft pencil to indicate the pattern of antigen and antibody application. The usual antigen - antibody distance was 1 - 1.5 cm.

The discs were soaked in a veronal buffer solution, by first floating the disc the buffer surface until the lower surface of the disc was completely wetted and then submerging the whole disc. (Quick immersion, without floatation causes airpockets which spoil the discs.) The discs were then blotted to remove excess moisture and placed on pin supports in a moist chamber.

A drop of antigen or antibody solution was applied to the appropriate spot with a fine capillary tube and allowed to dry into the surface of the disc. When all the samples had been applied and allowed to dry in, the discs were immersed in a bath of Whitmore White Oil 120 using the flotation technique described above.

The discs were left in the oilbath (at room temperature) until the diffusion reaction was completed, usually between 1 - 3 days. The discs were then removed from the oil bath washed free of oil with a detergent such as Teepol, and stained with either 0.2% Ponceau S or 0.001% Nigrosin.

The discs can be stored dry or cleared by flotation in the White Oil, which leaves the strip glass clear and in a suitable condition for photographic purposes.

7. Preparation of Antigen-Tagged Red Cells.

Boyden's report in 1951 that proteins could be absorbed onto the surface of sheep red cells which had been pretreated with tannic acid, and that these protein-conjugated cells could be agglutinated by highly diluted specific antisera, opened up a new area in which immunological techniques could be used. Over the next decade more methods of preparing protein-conjugated cells were developed.

It was reported by Renwick (1959) that Boyden's method was equally applicable to human red cells. As sheep erythrocytes were not available from a guaranteed constant source, or easily obtainable, human erythrocytes of an O Rh +ve nature were used in all experiments.

The various methods of conjugating growth hormone with red cells used in this work are detailed below.

a) Tannic Acid Method - Boyden, S.V. (1951)

Blood was collected under sterile conditions into an equal volume of Alsever's solution (a citrate/dextrose anticoagulant). While the blood was not used until at least 3 days after collection, it could be kept in this solution at +4°C. for 3 - 4 weeks without undue haemolysis.

The cells were then washed three times with 0.85% saline, and packed by light centrifugation. To each millilitre of packed erythrocytes was added 40 ml. pH 7.2 phosphate buffered saline. Equal volumes of this red cell suspension and 1:20,000 tannic acid

solution were incubated for 10 - 15 minutes at 37°C. The cells were then lightly (c. 500 rcf) packed and the supernatant poured off. The cells were washed once and resuspended in the original volume of pH 7.2 buffered saline. (Cells can be stored at this point for 2 - 3 days at +4°C.) Tubes containing 0.1 mg. growth hormone in 25 ml. pH 6.4 phosphate buffered saline were made up and 5.0 ml. tannic acid red cell suspension was added. The tubes were well mixed and allowed to stand at room temperature for 15 minutes. The cells were very lightly packed (c. 300 rcf.) and the supernatant discarded. The cells were washed once with 10 ml. 1% N.R.S. in pH 6.4 phosphate buffered saline (N.R.S. 1 : 100) and resuspended in 5.0 ml. N.R.S. 1 : 100.

Control cells were prepared in the same way using pH 6.4 buffered saline without hormone. Antigen-tagged cells were used on the day of preparation.

b) Tannic Acid - Formalinised Red Cells - Fulthorpe, A.J. (1957).

Blood was collected and the red cells washed, and tanned and sensitised to antigen according to the Boyden method.

After the final washing the sensitised cells were resuspended in a minimum volume of 0.85% saline and then added to an isotonic 0.05 M sodium borate /succinic acid buffer (pH 7.5) containing 1% N.R.S. and 20% formalin. The cell mixture was then left at +4°C. for 3 days to allow formalinisation to occur.

During this period the cell mixture was frequently shaken so as to minimise cell clumping. At the end of this period, the supernatant was removed and the cells resuspended in 1% N.R.S., this was repeated every 2 days for a week to remove all traces of free formalin.

Cells treated in this way are supposed to remain unchanged and with a constant sensitivity for about 4 months.

c) Formalin-Tannic Acid Treated Erythrocytes (FTTE)

Weinbach, R. (1958).

Blood was collected under sterile conditions into an equal volume of Alsever's solution. The cells were washed 3 times with normal saline.

Formalinisation of the cells was carried out by mixing equal volumes of an 8% v/v cell suspension and a 3% formalin solution which had been neutralised to pH 7.0. The mixture was shaken for 24 hours at 37°C. with two changes of formalin solution during this period. The cells were then thoroughly washed to remove all traces of formalin and then suspended in pH 7.2 phosphate buffered saline, 1.0 ml. packed cells to 9.0 ml. saline.

Equal volumes of the formalinised cell suspension and a 1 : 20,000 (w/v) tannic acid solution were mixed and incubated at 37°C. for 30 minutes. The cells were washed once with pH 6.4 phosphate buffered saline to remove any traces of tannic acid and made up to a 10% suspension with a pH 6.4 phosphate buffered saline.

Equal volumes of antigen solution (0.1 mg. growth hormone 25 ml. ; 100 i.u. HCG/1 ml.) in pH 6.4 phosphate buffered saline and the 10% tannic acid - formalinised red cell suspension were mixed and incubated at 56°C. for 1 hour. The cells were washed twice with the pH 6.4 buffered saline and made up to a 10% (v/v) suspension in normal saline containing 1% N.R.S. and 1 : 10,000 (w/v) merthiolate.

Control cells were prepared in the same way using pH 6.4 buffered saline without hormone.

In this form the cells will keep for several weeks at +4°C. Before use, the required amount should be washed and resuspended in fresh saline (with 1% N.R.S.) to form an approximately 2% cell suspension.

d) Formalinised Red Cells - Csizmas, L. (1960)

Blood was collected into an equal volume of Alsever's solution. The red cells were then washed 5 times with 10 volumes of normal saline. The washed packed red cells were resuspended in not less than 8 volumes of a pH 6.8 phosphate buffered saline.

Into a 2.5 cm. cellophane dialysis sac was poured 0.25 volumes (of the above cell suspension) 40% formaldehyde solution. This bag was placed in the bottom of a flask and the cell suspension poured over the formaldehyde sac. The flask was shaken gently at room temperature for about 2 hours. The sack was then opened and the contents mixed into the cell suspension. Shaking of the flask was continued for another 12 - 18 hours, care being

taken to prevent too much foaming of the flask contents.

The contents of the flask were then carefully decanted through a gauze filter to remove the cell debris. To the filtered cell suspension was then added 0.5 of its volume buffered saline. The cells were then washed 6 times with saline. The packed formalinised cells were then suspended in an equal volume of buffered saline and stored at +4°C.

e) Formalinised Erythrocytes - Fauconnier, B. (1958).

Blood was collected under sterile conditions into an equal volume of Alsever's solution. The cells were washed once and resuspended in a phosphate buffered saline to form a 25% cell suspension.

Coal gas (source of carbon monoxide) was passed through the cell suspension for 30 minutes. Following gassing, a 0.2 volume formol solution (30% formaldehyde : 2x concentrated buffer 1 : 1) was added to the suspension, mixed in by gentle shaking and incubated for 2 hours at 37°C. The cells were then washed 3 - 5 times with 10 volumes buffered saline to remove excess formalin and resuspended in buffer (25% cell concentration).

Treatment with carbon monoxide and formalin was repeated twice and the cells finally stored at +4°C. as a 25% cell suspension.

Before coating with antigen, the cells were lightly centrifuged and 2.0 ml. packed cells were resuspended in 8.0 ml. buffer. To this suspension was added 10.0 ml. buffer containing 0.1 mg. antigen and thoroughly well mixed. The antigen:cell mixture

was then stored at +4°C. in 1.0 ml. aliquots. Before use each 1.0 ml. aliquot was diluted to 20.0 ml. and the resultant cell suspension used in the haemagglutination reaction.

Control cells were prepared in the same way using, however, buffer without antigen.

f) Pyruvic Aldehyde Treated Erythrocytes - Ling, N. R.

Blood was collected into an equal volume of Alsever's solution. The cells were washed 7 - 10 times with 10 volumes of normal saline. After washing the packed cells were resuspended in an equal volume of saline.

Normal saline and 25% pyruvic aldehyde solution were mixed in the ratio 3 : 1.6 and the pH of the solution adjusted to approximately 7.0 with 10% sodium carbonate. To the neutralised pyruvic aldehyde solution were added 0.7 volumes of a 0.15 M phosphate buffer pH 8.0 and 1.0 volume of the 50% red cell suspension. The mixture was stored at +4°C. for 2 days with occasional stirring.

The cells were then thoroughly washed with normal saline to remove excess pyruvic aldehyde and stored as a 10% suspension (with 0.1% azide) at +4°C.

Antigen could then be attached to the cells by either the Boyden method or in the following manner. 1 vol. of the 10% cell suspension was centrifuged, washed once and resuspended in 0.6 vol. dilute buffer containing antigen (saline : water : 0.15 M phosphate buffer pH 6.0 10 : 10 : 1) and incubated for 1 hour at

50°C. After incubation the cells were washed 3 times with diluent (N.R.S. 1 : 100) and finally resuspended in 0.2 vol. diluent containing 0.1% sodium azide.

Control cells were prepared in the same manner except that buffer without antigen was used. Prepared cells were diluted 20 times before use.

3. Haemagglutination Reaction

It has long been known that blood cells will agglutinate when added to an alien serum. Since the first years of this century Landsteiner and his colleagues have applied this fact to the identification of the blood groups of human serum, and from this has developed the haemagglutination reaction as it is known today. It depends on the principle that

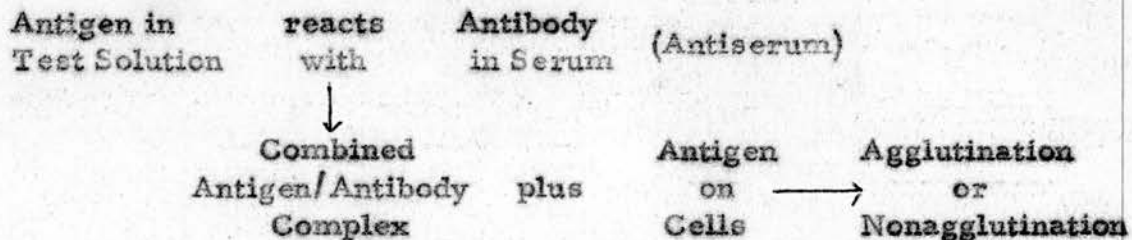
Antigen on Cells	will react with	Antibody In Serum	→	Agglutination of Cells
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Serial dilutions of antiserum were made using 1% normal rabbit serum as diluent. When all dilutions had been made 0.02 ml prepared antigen-coated red cells were added to each tube (containing 0.2 ml. diluted antiserum). The contents were mixed thoroughly by gentle shaking and the tubes allowed to stand at +4°C. overnight. The cell patterns were read the next morning. The titre of the antiserum was taken as the greatest dilution of the antiserum in which a positive agglutination pattern was observed.

The agglutination patterns observed in a typical haemagglutination reaction are recorded in Table 19.;

9. Haemagglutination - Inhibition Reaction

This modification of the haemagglutination reaction depends on the following principles:-



The end-point of the reaction depends on whether the amount of antigen in the test solution added to the antiserum is sufficient to inhibit all the antibody present. If this is the case, then the cells will not agglutinate; if, however, some antibody remains, this will react with the 'antigen on cells' to cause agglutination of the cells. The end-point of the assay was thus taken to be that dilution of antigen which just fails to inhibit agglutination.

Serial dilutions of standard antigen and the test solutions were prepared. An equal volume (0.2 ml.) of diluted antiserum was then added to each tube, the contents were mixed thoroughly by gentle shaking and allowed to stand for 1 - 2 hours at room temperature; 0.02 ml. prepared red cells were then added to each tube, the contents again mixed well and the tubes left to stand overnight at +4°C. The cell patterns were read next morning. These were the reverse of those seen in the haemagglutination reaction in that no agglutination was seen in the tubes containing high concentration of antigen.

When the agglutination - nonagglutination balance point had

been ascertained, the dilutions of antigen which will give a 0.1 log dose interval between these points were calculated. Ten tubes for each of these antigen dilutions were then set up and antiserum and prepared erythrocytes were added as described above. Following the overnight settling period the number of positive and negative results at each dilution were noted and the results analysed using a 4 - point quantal assay system (Gaddum, 1933 and 1953 b - see Appendix)

The agglutination patterns observed in a typical haemagglutination - inhibition reaction are recorded in Table 24.

10. Preparation of Latex Particles

Polystyrene latex particles 0.81 μ (Difco) were diluted 1 : 10 with water and filtered through Whatman No. 40 paper and stored at +4°C. The suspension was again filtered immediately before antigen coating.

2.0 μ g. antigen in 9.9 ml. 0.06 M Sorensen's phosphate buffer pH 7.4 were mixed with 0.1 ml. of the dilute latex suspension and allowed to stand for 15 - 30 minutes before use.

A commercial preparation of HCG-coated latex particles was also used. They were supplied by Ortho Pharmaceutical Ltd.

11. Latex Agglutination - Inhibition Reaction

The latex agglutination - inhibition reaction is based on the same principles as the haemagglutination - inhibition reaction. In this reaction, however, the end-point-turbidity of the supernatant - indicates the degree of latex particle agglutination and can be

estimated quantitatively with a spectrophotometer.

Serial dilutions of standard antigen and the test preparations were made. An equal volume (0.5 ml.) diluted antiserum was added to each tube, the contents well mixed and the tubes incubated in a water bath at 37°C. for 1 hour. Then 1.0 ml. thoroughly shaken prepared latex particle suspension was added to each tube; the tubes were reshaken and again incubated at 37°C. for 2 hours. Following incubation the tubes were centrifuged at 1500 rcf for 2 minutes, speed and time of centrifugation were critical.

Following centrifugation, the supernatant was carefully removed and an aliquot transferred to a glass micro-cuvette, the percentage light transmission at 650 μ was measured using a Unicam SP 600 spectrophotometer.

Results were calculated from a standard curve using the simplified 4 - or 6-point quantitative bioassay procedure (Gaddum, 1953). The percentage light transmissions observed in a typical standard curve and assay are shown in tables 30 and 31.

Results

The results presented herein will be considered under the four main headings 1) Antisera, 2) Red Cells, 3) Latex Particles and 4) Hormone Assays.

The first section comprises results pertaining to the production and preparation of the hormone antisera. It also records the levels of antibody titre reached and the results of specificity tests.

The results of experiments designed to establish the suitability of different preparations of red cells for use in haemagglutination reactions are contained in the second section.

In the third section are reported the results obtained from some preliminary experiments on latex particles.

The results of all the hormone assays performed by immune methods are grouped together under the fourth heading 'Hormone Assays'. This section has been further subdivided into

i) the results obtained using the haemagglutination - inhibition method for growth hormone and ii) results obtained with the latex particle agglutination - inhibition method for human chorionic gonadotrophin.

1. ANTISERA

A. Haemagglutination Titres

The final anti-hormone haemagglutination titres obtained are summarised in table 18, and a typical estimate of antibody titre is shown in table 19.

a) Anti - bovine pituitary growth hormone.

An antiserum to the bovine pituitary growth hormone (Armour - Somar A) was successfully raised in one rabbit. The haemagglutination titre reached was 1 : 51,200.

b) Anti - human pituitary growth hormone.

An antiserum to the human pituitary growth hormone (Raben 6) was successfully raised in four rabbits. The titres reached were 1 : 102,400 (1), 1 : 51,200 (1) and 1 : 25,600 (2)

TABLE 18

Pituitary hormone antibody titres (in rabbits)
obtained by the haemagglutination method.

Antigen	Antiserum Number	Haemagglutination Titre
Bovine Growth Hormone	BGH 1	1 : 51,200
Human Growth Hormone	HGH 1	1 : 25,600
	HGH 2	1 : 102,400
	HGH 3	1 : 25,600
	HGH 4	1 : 51,200
Human Chorionic Gonadotrophin	HCG 1	1 : 6,400
	HCG 2	1 : 51,200
	HCG 3	1 : 6,400
	HCG 4	1 : 6,400
	HCG 5	1 : 800
Ovine Follicle-Stimulating Hormone	FSH 1	Nil
Ovine Luteinising Hormone	LH 1	Nil

TABLE 19

Typical haemagglutination pattern observed during an estimation of antibody titre of an antiserus raised against human growth hormone.

Serum Dilution	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400
NRS with Antigen Cells	-	-	-	-	-	-	-	-	-	-	-
NGH 3 with Antigen Cells	+	+	+	+	+	+	+	+	+	-	-
HGH 3 with Antigen Cells	+	+	+	+	+	+	+	+	+	-	-
HGH 3 with Control Cells	-	-	-	-	-	-	-	-	-	-	-

+ indicates cells which have agglutinated

- indicates cells which have not agglutinated

respectively.

c) Anti-human chorionic gonadotrophin.

An antiserum to the human chorionic gonadotrophin (Pregnyl) was successfully raised in five rabbits. The titres reached were 1 : 51,200 (1), 1 : 6,400 (3) and 1 : 800 (1) respectively.

d) Other hormone preparations.

Attempts to raise antisera to luteinising hormone (NIH - LH-SI), follicle - stimulating hormone (NIH - FSH - SI) and pregnant mare serum gonadotrophin (Gestyl) were unsuccessful.

B. Specificity of Antisera.

All the specificity tests were carried out with absorbed antisera; immuno-diffusion on cellulose acetate discs was the method used for checking specificity.

a) Anti- bovine pituitary growth hormone.

Between 42 and 48 hours after application of the samples to the immuno-diffusion discs, a clear precipitin reaction between the anti - bovine (Armour - Somar A) growth hormone serum and the two bovine pituitary growth hormone preparations (Somar A and NIH - BGH - 2) could be distinguished. Even up to 96 hours after application of the samples, precipitin lines (indicating an immuno-reaction) did not materialise between the antiserum and the following hormones (used as antigens) :-

HGH - Raben 6 - 0.5 mg/ml.

HGH - Raben 12 - 0.5 mg/ml.

SGH - NIH - S3 - 0.5 mg./ml.

FSH - NIH - S1 - 0.5 mg./ml.

LH - NIH - S1 - 0.5 mg./ml.

TSH - NIH - B1 - 0.5 mg./ml.

ACTH - 'Acthar' - 70 i.u./ampoule.

b) Anti-human pituitary growth hormone.

A clear precipitin reaction between the anti-human growth hormone (Raben 6) serum and two pituitary extracts (Raben 6 - HGH and a crude human pituitary extract prepared in the laboratory in Edinburgh) was visible 48 hours after the commencement of the immuno-diffusion reaction. Even 96 hours after the application of the samples immuno-reactions had not apparently occurred between the antiserum and the following antigenic substances :-

Human serum

BGH - Armour, Somar A - 0.5 mg./ml.

FSH - NIH - S1 - 0.5 mg./ml.

LH - NIH - S1 - 0.5 mg./ml.

TSH - NIH - B1 - 0.5 mg./ml.

ACTH - 'Acthar' - 70 i.u./ampoule.

PMSG - 'Gestyl' - 1000 i.u./ml.

HCG - 'Pregnyl' - 4000 i.u./ml.

c) Anti - human chorionic gonadotrophin

By 48 hours after the commencement of the immuno-diffusion reaction a marked precipitin reaction between the antisera and the human chorionic gonadotrophin (Pregnyl) was visible. At least 2 - 3 (and possibly more) precipitin lines were visible. When

the five antisera were tested together with normal rabbit serum against the HCG preparation, a continuous precipitin band (showing that the antisera possessed a common antibody) lay between the antisera and the HCG, but did not extend in front of the NRS. There were no visible precipitin lines between the NRS point and the HCG position. None of the five antisera appeared to possess antibodies to the PMSG (Gestyl) 1500 i.u./ml.

2. CELLS

Cell preparations (both antigen-coated and control cells) were tested for spontaneous agglutination or haemolysis in buffer, diluent (i. e. buffer + 1% NRS) and normal human serum. Control cells were also tested for spontaneous agglutination and haemolysis in the diluted prepared antisera. The design of a typical test and the observations made therein are shown in table 20.

A. 'Boyden' Cells.

Spontaneous agglutination and/or haemolysis of either antigen-coated or control cells prepared according to the Boyden technique rarely occurred in tubes containing either buffer or diluent.

Agglutination of both coated and control cells frequently occurred when cells were added to tubes containing untreated human serum. In one experiment, 9 human sera (5 male and 4 female) were tested for their ability to cause spontaneous agglutination of the Boyden type cells. All the sera caused agglutination at a dilution of 1 : 10 while 2 sera diluted 1 : 80 were still able to cause agglutination of the cells. There appeared to be no relationship

TABLE 20

Results from a Typical Red Cell Test using Cells prepared according to the methods of Boyden and Fauconnier.

Cell Preparation	Buffer	Diluent	Antiserum
'Boyden' type Control Cells	- - - -	- - - -	- - - -
'Boyden' type Antigen-coated Cells	- - - -	- - - -	+ + + +
'Fauconnier' type Control Cells	H ± - H	- ± ± -	± H ± ±
'Fauconnier' type Antigen-coated Cells	+ + + +	+ + + +	+ + + +

H indicates presence of haemolysis
 + indicates presence of agglutinated cells
 - indicates no agglutinated cells present

Each type of cell was tested in quadruplicate; each symbol, therefore, indicates the result of a single tube.

between the known blood group of the serum and its ability to agglutinate the cells (see Table 21).

If, however, the test sera were first, incubated for at least 30 minutes (to destroy complement) and then absorbed with fresh untreated red cells, no agglutination or haemolysis of the cells was observed at a 1 : 5 dilution of the sera.

B. 'Fulthorpe' Cells.

No haemolysis was observed but haphazard spontaneous agglutination of both control and antigen-treated cells in both buffer and diluent occurred so frequently as to make this method of cell preparation unreliable.

C. 'Weinbach' Cells.

Similar results to those obtained with the Fulthorpe method were obtained. It appeared that this method of formalising cells was not suitable for use in haemagglutination reactions.

D. 'Cszimas' Cells.

Following formalinisation by the Cszimas method the cells were so crenallated as to be totally unusable.

E. 'Fauconnier' Cells.

Following formalinisation by the Fauconnier method the cells were of a good colour and not crenallated, but, both control and antigen-treated cells showed spontaneous agglutination in diluent.

F. 'Ling' Cells.

Cells prepared according to the Ling method were of good appearance and not crenallated. Control cells were not agglutinated

TABLE 21

Estimates of serum dilution titres at which spontaneous agglutination of antigentagged 'Boyden' type cells occurs in normal untreated sera.

Serum No.	Sex of Donor	Blood Group of Donor	Dilution at which Spontaneous Agglutination still occurs
1	M	B +ve	1:10
2	M	A -ve	1:10
3	F	O -ve	1:20
4	M	A -ve	1:20
5	M	O +ve	1:40
6	F	O -ve	1:40
7	F	B -ve	1:40
8	F	O+ ve	1:80
9	M	O +ve	1:80

M = male, F = female.

when added to buffer or diluent. Antigen-treated cells did not show spontaneous agglutination in buffer or diluent, neither did they agglutinate in antiserum. All attempts to coat the Ling cells with growth hormone failed.

G. Specificity of the Haemagglutination - Inhibition Reaction.

Using cells sensitised to HGH according to the Boyden technique, no cross reaction has been observed to the following hormones up to the concentrations quoted :-

BGH - Armour 'Somar A' - 100 μ g./tube

BGH - NIH - B2 - 100 μ g./tube

Prolactin - NIH - S3 - 100 μ g./tube

TSH - NIH - B1 - 100 μ g./tube

FSH - NIH - S1 - 100 μ g./tube

LH - NIH - S1 - 100 μ g./tube

HCG - 'Pregnyl' - 500 i.u./tube

PMSG - 'Gestyl' - 500 i.u./tube.

The addition of ACTH (Armour 'Acthar') to the reaction mixture caused marked haemolysis of the cells.

3. LATEX PARTICLES

A. Suspension Medium for Latex Particles.

As will be seen from Table 22 and fig. 7 there is a direct relationship between the concentration of latex particles and the percentage of light transmitted. For any given concentration of uncoated latex particles there is virtually no difference in the percentage of light transmitted whether the particles are suspended

TABLE 22

Estimations of the Percentage Light Transmission of different Concentrations of Latex Particles in Water and in Urine.

Percentage Latex Particle Concentration	Percentage Light Transmission at 650 mμ.		
	Water	Fresh Urine	3 month old urine
0.5	0	0	
0.25	0.8	0.1	1.3
0.125	3.1	4.3	3.9
0.0625	13.5	14.1	14.4
0.0312	35.8	35.3	34.8
0.016	59.3	57.1	58.0
0.008	77.0	75.0	74.0
0.004	87.5	85.0	84.8
0.002	93.3	93.0	92.1
0.001	96.7	98.0	93.8

**Fig. 7:- ESTIMATION OF THE PERCENTAGE LIGHT TRANSMISSION OF
DIFFERENT CONCENTRATIONS OF LATEX PARTICLES IN WATER
AND IN URINE.**

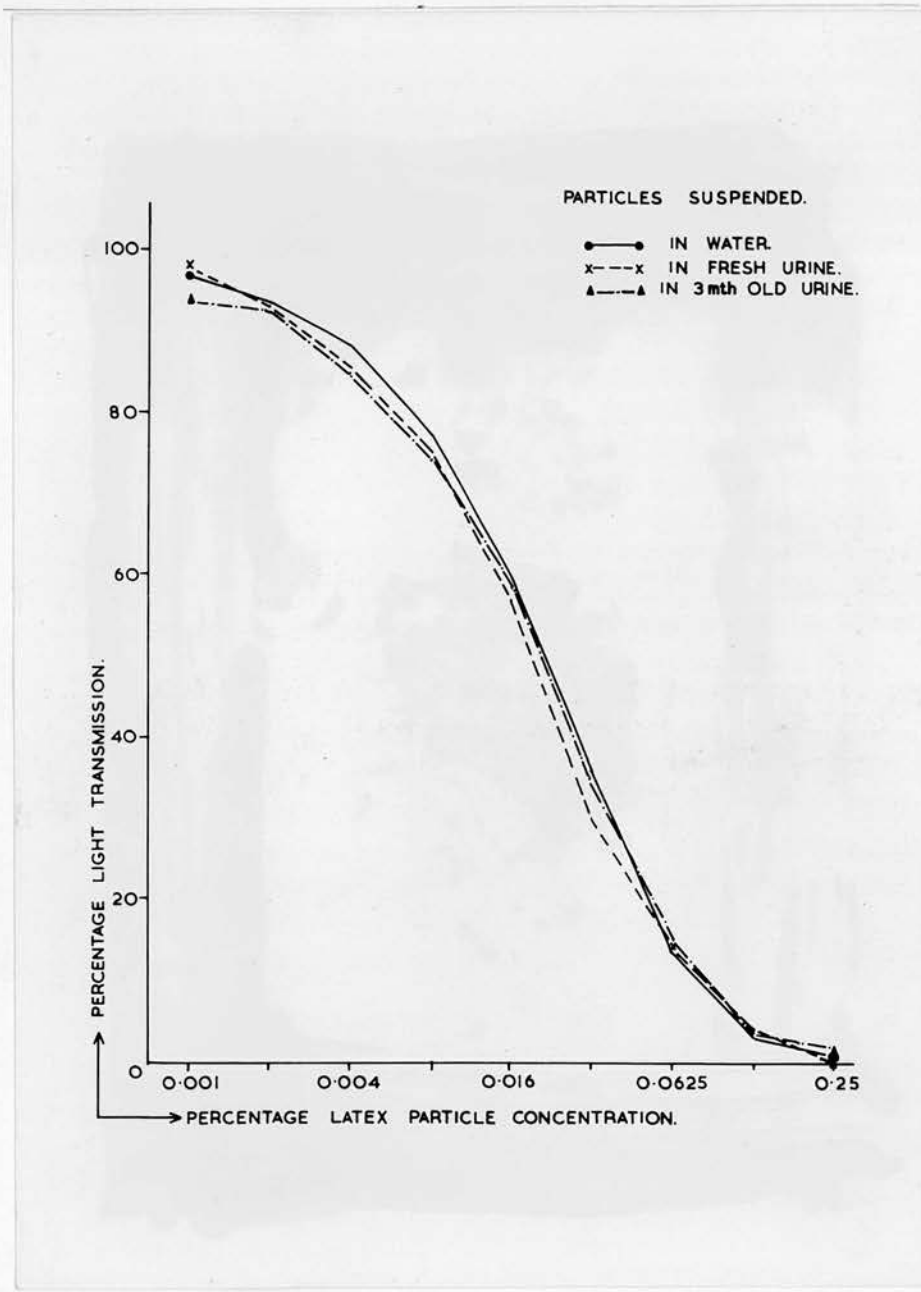


Fig.7.

in water or in fresh or 3 month old urine.

B. Specificity of the Latex Agglutination - Inhibition Reaction

The results of a specificity test are shown in Table 23 and fig. 8. It will be seen that the prior addition of between 3.1 and 200 ug./tube of FSH and LH to the anti-HCG serum did not inhibit the agglutination of the coated latex particles. This indicates that these two pituitary gonadotrophic hormones do not interfere with the assay and that the pituitary LH which has similar biological properties to HCG does not have common immuno-properties.

4. HORMONE ASSAYS

i) Haemagglutination-Inhibition Method

A. Typical Growth Hormone Assay

The results of a typical 4-point quantal assay are shown in Tables 24 and 25. The slope of the dose response curve is steep and a very narrow log. dose interval (0.1) can be used. The λ figure (0.043) is very low indicating a high degree of precision in the assay and the fiducial limits are relatively narrow (92 - 114%). There was no significant deviation from parallelism between the dose response curves of the standard and the unknown.

B. Growth Hormone Standards and Pituitary Extracts.

The results of 12 quantal assays are shown in tables 26 - 28. They were all of a 2 + 2 design and all were statistically valid i. e. the slopes of the standard and unknown curves were all parallel.

Using an antiserum to BGH, estimations of the potency of a growth hormone preparation NIH - B2 were made on three occasions

**Fig. 8:- THE LATEX PARTICLE AGGLUTINATION-INHIBITION ASSAY
FOR HUMAN CHORIONIC GONADOTROPHIN. DOSE RESPONSE
CURVES FOR HCG, NIH-FSH AND NIH-LH.**

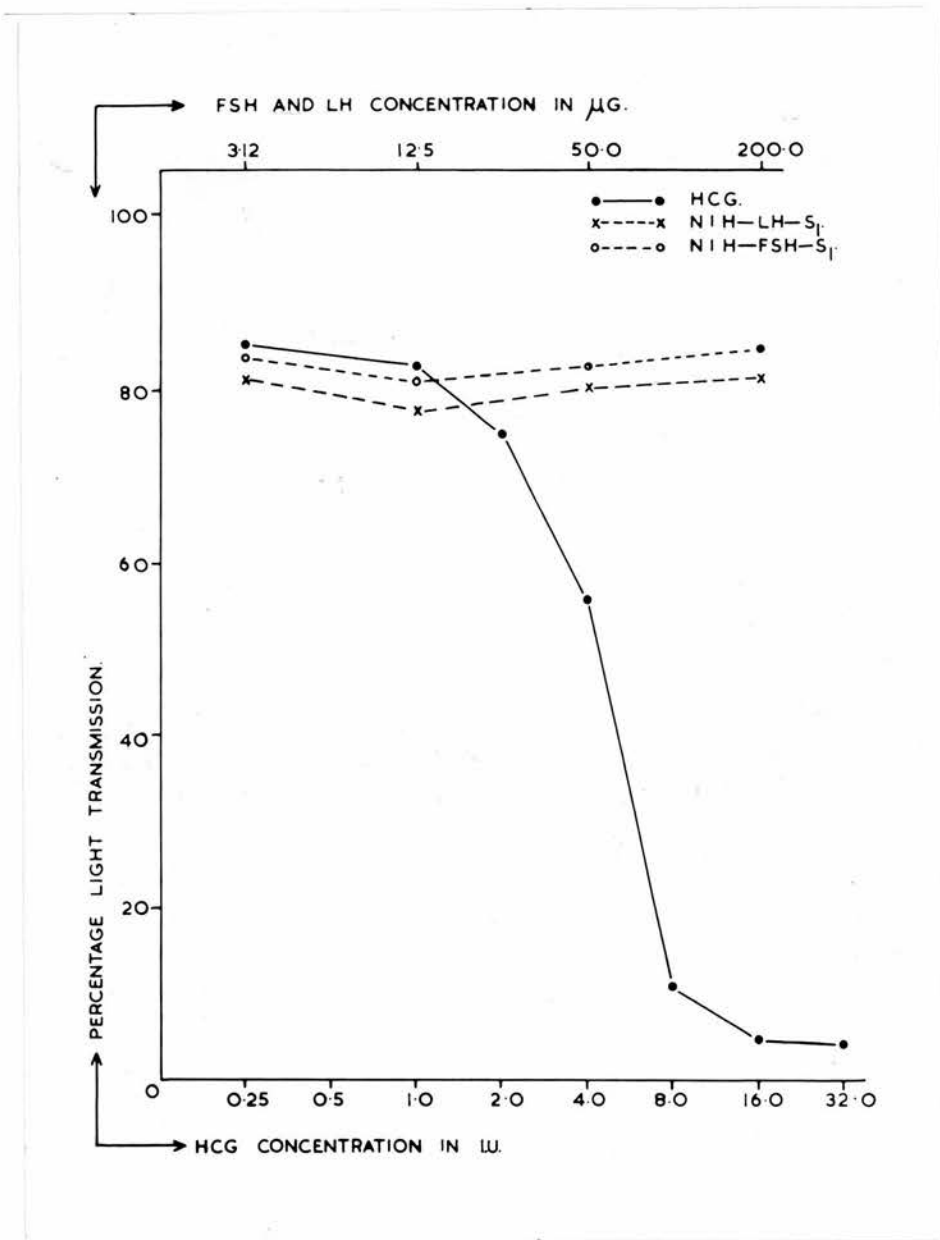


Fig.8.

TABLE 25

Results of a typical Growth Hormone Immuno-assay -
Haemagglutination-Inhibition Method.

Preparation	Concentration Growth Hormone/ Tube in ug.	No. of Tubes Agglutinated	Total No. of Tubes/ Dose Level	Probit
Armour BGH (standard)	4.0	8	10	6.28
	5.0	1	10	3.72
NIH - GH - B2 (unknown)	10.0	9	10	5.84
	12.6	1	10	3.72

log. dose interval 0.1

Relative Potency 1.02, 95% Confidence Limits 92 - 114%,
4-point assay design.

λ 0.043.

Deviation from parallelism is not significant.

TABLE 26

Replicate Estimations of NIH - GH - B2 in
terms of Armour BGH - H. - I. Method.

Assay Design	Relative Potency	Fiducial Limits (0.95)	λ		Activity in i.u./mg and fiducial limits in i.u.
2 + 2	1.02	92-114	0.043	Valid	0.546(0.50 - 0.62)
2 + 2	1.15	108-128	0.036	Valid	0.616(0.58 - 0.69)
2 + 2	1.00	90-111	0.045	Valid	0.536(0.48 - 0.59)

The mean relative potency is 1.06.

Calculated mean activity of NIH - GH - B2 is
0.57 i.u./mg. (Fid.Lim. 0.48 - 0.69 i.u./mg.)

TABLE 27

Duplicate Estimations of HGH - Raben 12 in terms
of HGH - Raben 6 - H.I. Method

Assay Design	Relative Potency	Fiducial Limits	λ		Activity in i.u./mg. and Fiducial Limits in i.u.
2 + 2	1.135	94 - 134	0.086	Valid	0.177 (0.146 - 0.209)
2 + 2	1.000	82 - 122	0.097	Valid	0.195 (0.160 - 0.238)

TABLE 28

Estimations of the Growth Hormone Activity of
Fractions Separated from a Crude Pituitary Extract
by Passage through a Decy cellulose Column

Preparation	Assay Design	Relative Potency	Fiducial Limits	λ		Activity in i.u./ mg. and fiducial limits in i.u.
Fraction 1	2 + 2	1.00	89 - 112	0.046	Valid	1.85 (1.65 - 2.07)
Fraction 2	2 + 2	0.98	81 - 118	0.072	Valid	9.05 (7.48 - 10.90)
Fraction 2	2 + 2	1.00	89 - 112	0.076	Valid	9.25 (8.23 - 10.36)
Fraction 3	2 + 2	1.01	89 - 116	0.050	Valid	0.93 (0.82 - 1.07)
Fraction 3	2 + 2	1.00	89 - 112	0.046	Valid	0.92 (0.82 - 1.03)
Fraction 4	2 + 2	1.04	88 - 122	0.054	Valid	0.038 (0.032 - 0.046)
Fraction 4	2 + 2	0.92	77 - 111	0.063	Valid	0.034 (0.028 - 0.041)

(see Table 26). The values obtained were respectively 0.546, 0.616 and 0.536 i. u. /mg. which gives a mean estimate of 0.57 i. u. /mg. This value, although somewhat lower than the estimate of potency made by the tibial test (0.745 i. u. /mg.) is well within the 95% confidence limits of the tibial estimation.

Using an antiserum to HGH, estimations of the potency of a growth hormone preparation Raben 12 were made on two occasions. (See Table 27). The values obtained were respectively 0.177 and 0.195 i. u. /mg. which gives a mean estimate of 0.186 i. u. /mg. This value, although slightly higher than the estimate of potency made by the tibial test (0.146 i. u. /mg.) is well within the fiducial limits of the tibial estimation.

Using an antiserum to HGH, estimations of the potency of four samples obtained by fractionating a crude pituitary extract on a deoxycellulose column were made (see Table 28). Where estimations of potency have been made more than once, agreement between the values on each occasion was good. In no case was sufficient material available to confirm the results obtained by re-estimating the potency using the tibial test.

C. Growth Hormone in Human Serum

No estimates of growth hormone could be made in fresh, untreated human serum from 9 normal subjects because of non-specific agglutination in control tubes.

All estimates of growth hormone made in sera (from 24 normal subjects) which had been incubated at 56°C. (to destroy

complement) and absorbed with fresh red cells (to remove non-specific agglutinins) showed that the level of growth hormone was below the sensitivity of the method i. e. less than 20 - 50 $\mu\text{g.}/\text{ml.}$ serum. A brief comparison of the growth hormone levels obtained herein and by other workers is shown in Table 29.

ii) Latex Particle Agglutination-Inhibition Method

D. Typical Human Chorionic Gonadotrophin Standard Curve

Experiments were carried out to establish the form of the HCG standard curve in a latex agglutination-inhibition reaction. The results of a typical experiment are recorded in table 30 and shown in graph form in fig 9. It will be seen that maximum agglutination of the particles, which occurs when little or no antigen is present, is accompanied by maximal light transmission, while minimum agglutination, which occurs when an excess of antigen is present, is accompanied by a high degree of turbidity and minimal light transmission. The steepest part of the curve appears to lie in the region of 1 - 10 i. u. HCG and for normal assay work the dose levels of the standards were chosen from this region.

E. Assay of HCG Standards

The results of a typical 6-point quantitative assay are shown in table 31. The slope of the dose response curve is reasonably steep and a log. dose interval of 0.301 was used. The λ figure is very low indicating a high degree of precision and the fiducial limits are relatively narrow (90 - 114%). There was no significant deviation from parallelism between the dose response curves

TABLE 29

Comparison of Human Serum Growth Hormone Levels Obtained by Different Workers

Worker	Normals*	Acromegalics**	Hypopituitary Dwarfs**	Hypophysectomised Subjects**
<u>Haemagglutination-Inhibition Method</u>				
Present Investigation	<10 - <50* (25)	Within normal range	90	
Boucher & Mason (1961)	100 - 480	" (3)	158 ± 41 (7)	0 - 480 (20)
Dominguez & Pearson (1962)	80 ± 400 (14)	" (4)	<20*	<20*
	<20*	35 - 300*		20 - 290 (10)
Ehrlich & Randle (1961a)	80 - 300 (24)	220 - 470		
(1961b)	100 - 250			45 - 76 (4/7)
Hartog & Russell Fraser (1961)	88 - 256 (18)	320 - 462 (7/10)		
Hayashida (1961)	15 - 45 (6)	30 - 240 (7)		
Kaplan & Grumbach (1962)	<20* (6)	120 - 240* (5)	<20* (1)	
Read (1960)	98 - 360 (7)	534 - 1141 (8)	31 - 87 (7)	
Read & Bryan (1960)	90 - 400 (9)	>500 (9)	50 (9)	
<u>Radio-Immuncassay</u>				
Glick et al. (1963)	0 - 15.0(64)	2 - 250 (30)		undetectable (21)
Hunter & Greenwood (1962)	2.4 - 5.6 (7)	25.2- 67.4 (7)		
Utiger et al. (1962)	0 - 50 (30)	15 - 260 (22)	0 - 30 (5)	

* Serum incubated at 56°C. to destroy complement and absorbed with fresh R.B.C.

* Extracted serum

** Range in m ug/ml. serum (no. of cases).

TABLE 30

Typical HCG dose response curve.

Concentration of HCG in i.u.	Percentage Light Transmission at 630 m μ .
40	3.50
20	4.25
10	5.05
5	31.25
2.5	65.60
1.25	71.00
0.625	76.35
0.312	80.80

Fig. 9:- THE LATEX PARTICLE AGGLUTINATION-INHIBITION ASSAY FOR HUMAN CHORIONIC GONADOTROPHIN. TYPICAL DOSE RESPONSE CURVE.

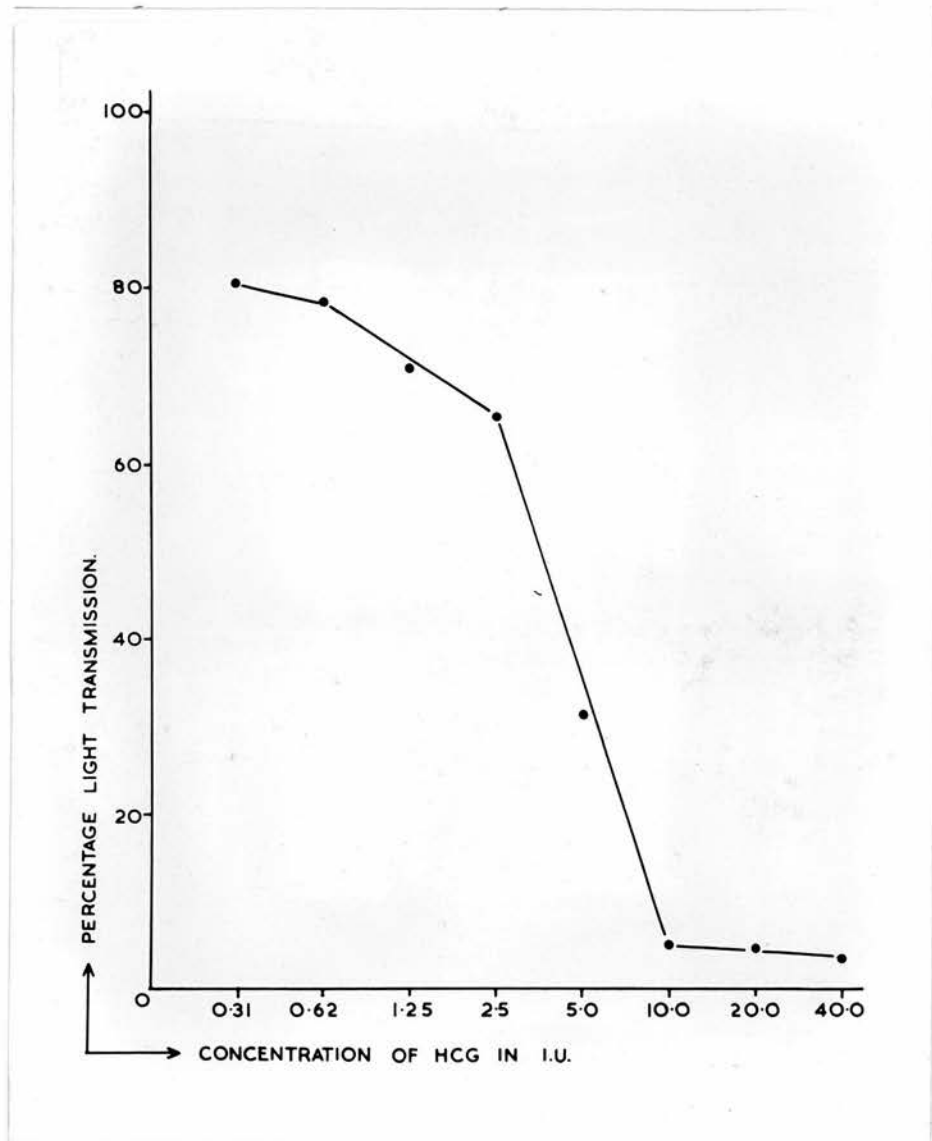


Fig. 9.

TABLE 31

Results of a Typical HCG Assay by Immuno-assay -
Latex Particle Agglutination - Inhibition Method.

Preparation	Concentration in i.u.	Mean Percentage Light Trans- mission at 650m μ	Range in Spectro- photometer Readings	Number of Obser- vations
International Standard	6.0	65.8	1.8	3
	3.0	36.2	1.4	3
	1.5	14.3	19.9	3
Unknown 'Pregnyl'	6.0	57.6	2.0	3
	3.0	41.1	6.6	3
	1.5	14.7	3.4	3

3 + 3 Assay design;
Relative Potency 1.028

Index of Precision λ 0.044
95% Confidence Limits 90.6 -
114.0%.

Validity :- No significant deviation from parallelism
(P > 0.05)

No significant curvature of dose response
curve standard (P > 0.1)

No significant curvature of dose response
curve unknown (P > 0.05)

of the standard and the unknown, nor did the two curves show any significant curvature of the dose response curve.

The results of replicate estimations of the potency of a commercial HCG preparation "Pregnyl" are shown in table 32. There is good agreement between the relative potencies, (1.028 and 1.020 respectively,) obtained, which makes the commercial preparation virtually equipotent with the International Standard, i. e. the results obtained here confirm the potency claimed for the preparation by the manufacturers.

F. Assay of HCG in Urine

None of the estimates of the HCG content of pregnant urine were statistically valid. All the assays showed highly significant (P 0.005) deviation from parallelism between the dose response curves of the standard and the unknown.

The results of a number of recovery experiments are shown in table 33 and fig 10. In no case was the dose response curve of the HCG in urine parallel with the dose response curve of the HCG in water.

It would appear, therefore, that this assay method cannot be applied to the direct estimation of HCG in urine, although apparently satisfactory for the estimation of purified HCG preparations.

DISCUSSION

The application of immunological techniques has given considerable impetus to research in endocrinology. In theory, immunological methods are highly specific, since a given antigen

TABLE 32

Estimations of HCG activity in 'Pregnyl' in terms
of the International Standard

Assay Design	Relative Potency	λ	Fiducial Limits	
3 + 3	1.028	0.044	93 - 114%	Valid *
3 + 3	1.020	0.041	90 - 113%	Valid *

* Valid - No significant deviation from parallelism
and no significant curvature of the dose response
curves.

TABLE 33

Estimates of the Percentage Light Transmission following the Addition of Various Concentrations of HCG to a Number of Urine Samples - Latex Agglutination - Inhibition Method.

Concentration of Added HCG in i.u./tube	Mean Percentage Light Transmission at 650 m μ				
	Water	3 month old nonpregnant + urine	Fresh Nonpregnant + urine	Pregnancy Urine	Male urine over 65 years
8	10.7	3.1	10.0	55.2	21.25
4	55.6	21.7	44.4	72.4	62.75
2	74.4	46.1	53.1	81.3	69.30
1	82.6	55.7			

Fig. 10:- THE LATEX PARTICLE AGGLUTINATION-INHIBITION ASSAY FOR HUMAN CHORIONIC GONADOTROPHIN. RECOVERY OF HCG ADDED TO A NUMBER OF URINE SAMPLES.

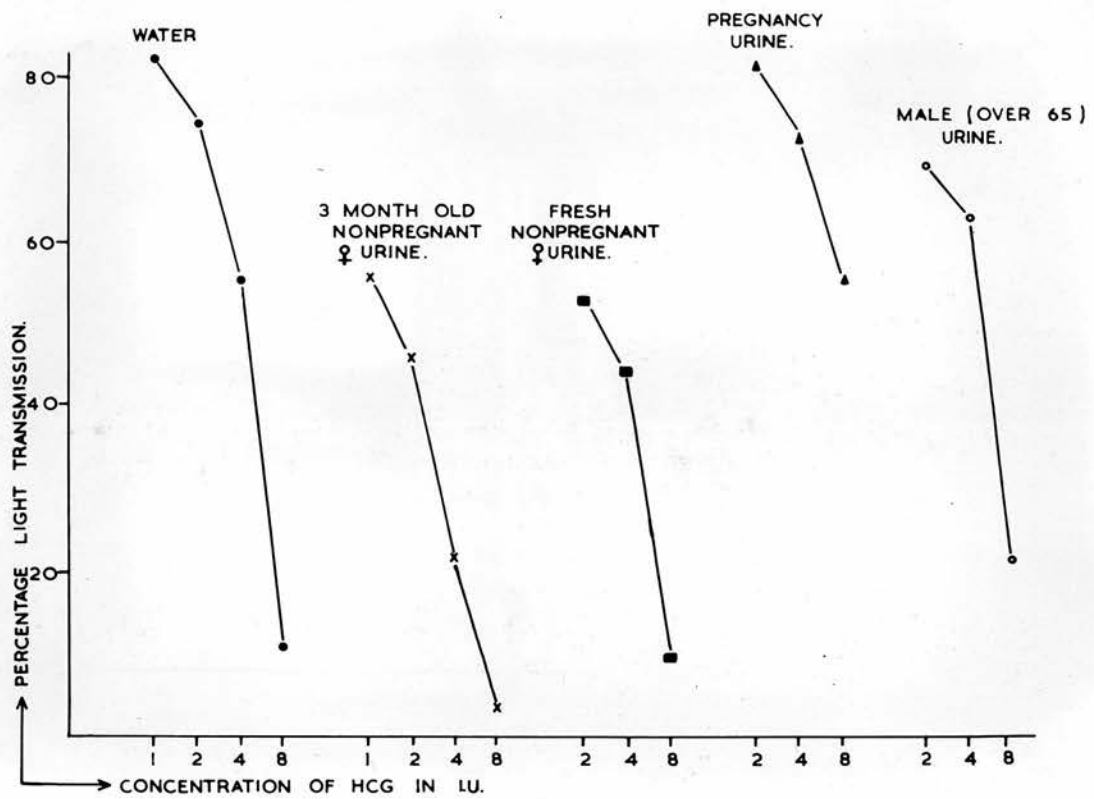


Fig. 10.

will only react with its appropriate antibody and nothing else. In practice, however, this does not always hold because it is often extremely difficult to obtain a system in which only a single antigen and antibody exists. Immunological techniques have been applied in two main fields. They have been used firstly, to provide information about the potency of purified hormone preparations and secondly, in the development of specific and sensitive methods of estimating hormone levels in blood and urine.

The protein hormones are in themselves only weakly antigenic, and so, to obtain a high titre antiserum, have to be injected in a suitable adjuvant. That an adjuvant is necessary has been shown by various workers including Elberg and Li (1950) and Emmerson and Emmerson (1960) who were unable to demonstrate the presence of antibodies to growth hormone following the repeated administration (to rats) of bovine growth hormone given without adjuvant. Although, Read (1958, 1960 ab) claimed that a satisfactory growth hormone antibody titre could be obtained using the light mineral oil mixture known as Freund's Incomplete Adjuvant, only two other groups namely, Irie and Barrett (1962) and Hayashida and Li (1958 abc) have used this method of raising antibodies to growth hormone. Most workers (including Brody and Carlstrom, 1960; Hunter and Greenwood, 1962; Lunenfield et al., 1962; McGarry and Beck, 1963) prefer to use the stronger adjuvant known as Freund's Complete Adjuvant to raise antibodies to hormone preparations. The results obtained in the present chapter appear

to confirm this preference since the presence of antibodies could not be detected following treatment with F.I.A., but high titres were obtained when the hormones were administered in F.C.A.

The use of an adjuvant increases the danger of selective fixation of antibody i. e. the production of antibodies in greater concentrations to the contaminants rather than to the main antigen present. It is, therefore, essential to purify the antiserum as much as possible before use. In the present chapter the cleanliness and specificity of all the antisera prepared have been checked by immuno-diffusion and cross-haemagglutination reactions.

Hayashida and Li (1958 b) noted that prior to absorption an antiserum to BGH showed cross-precipitation with ovine prolactin and γ -globulin, but that following purification no reaction to prolactin, TSH, ACTH, LH, FSH and γ -globulin could be detected. While the appearance of precipitin lines between an antiserum and an antigen indicates the presence of an antigen-antibody system, their absence does not necessarily mean that such a system does not exist. The bands may not appear because one or both of the components of the system may be so weak that the precipitin complex may not be visible; or one of the components may be present in such an excess that the antigen-antibody complex precipitate may have dissolved in the component which is in excess. A more sensitive index of the presence of impurities can be the haemagglutination reaction itself. Cross-reaction in haemagglutination systems may be demonstrated in one of two ways, 1) antiserum will

agglutinate cells which have been tagged with 'foreign' antigens or
2) the agglutination reaction may be inhibited by a 'foreign' antigen.
In this context the term 'foreign' antigen is applied to any antigen
other than the one to which it was intended to raise antibodies.

Using the criterion of absence of lines of identity, it was
shown that following preliminary purification both the antisera to
BGH and HGH appeared to be reasonably free of non-specific
antibodies (see p. 89). No cross reactions with human pituitary
growth hormone, ovine prolactin, ovine FSH and LH, bovine TSH
and a commercial preparation of ACTH have been observed against
the BGH antiserum. Unlike Hayashida and Li (1959) lines of
identity between ovine growth hormone and the BGH antiserum were
not seen. There was apparent confirmation of the above results,
in that, no inhibition of the agglutination reaction was observed,
when any of these hormones (in amounts of up to 200 μ g./tube)
was added to tubes containing BGH antiserum prior to the addition
of BGH-coated red cells, some haemolysis of the cells was noted
when ACTH was added to the system, this was probably due to the
presence of a non-specific haemolytic substance in the commercial
preparation used. When HGH-coated cells were added to the anti-
BGH serum no agglutination of the cells occurred. It would appear
therefore that a reasonably pure antigen-antibody system to BGH
had been obtained and that this was species specific.

A similar apparently pure species specific antigen-antibody
system was also obtained for HGH. In spite of the apparently

identical electrophoretic patterns of ovine prolactin and human growth hormone (observed by Ferguson and Wallace, 1961) precipitin lines - indicating a common immuno-identity - between sheep prolactin and the anti-HGH serum were not observed; a similar finding has been reported by Hayashida and Grunbaum, 1962. As no human prolactin was available it was not possible to confirm the observation of Hayashida (1962) of a continuous band between HGH, human prolactin and an anti-HGH serum. It would seem probable that this indicates a common immuno-chemical property of the two hormones rather than a non-specific human protein as there are no reports of the presence of a line of immuno-identity between normal human serum and the anti-HGH serum.

Although there had been several reports in the literature (Bourdell, 1961; Gold et al., 1960; Moudgal and Li, 1961 ab and Segal et al., 1962) of the successful production of antibodies to ovine FSH and LH, the single attempt reported herein was unsuccessful. As by this time it was obvious that all the endocrine antigen-antibody systems were species specific and the aim of this work was to set up methods for the assay of human FSH and LH, it did not appear worthwhile to undertake further attempts to obtain antibodies to the ovine hormone.

The HCG antiserum obtained was apparently free of antibodies to ovine FSH and LH, and to bovine GH and TSH. Like Carlström and Brody (1962) no cross-reaction with PMSG was noted. As no human LH (HLH) was available it was not possible to investi-

gate the claim of Wide, Roos and Gemzell, (1961) and Goss and Taymor (1962) of a cross-reaction between HCG and HLH. In fact Taymor, Goss and Buytendorp, (1962) ascribe this cross-reaction to the presence of a major antibody component which is common to both HCG and HLH. More recently, Goss and Lewis (1964) have shown that while HCG and HLH possess a common antigenic group, each has specific antigenic components which permit their immunologic differentiation, but do not affect their biological activity.

Goss and Taymor (1962) claimed that it was possible to adapt the Ortho Pregnancy Diagnosis Kit to the assay of HLH, by measuring the percentage light transmission of the supernatant. They found that there was a proportional decrease in the light transmitted when HLH was added to the tube, the effective straight line dosage was 15 - 50 μ g. HLH/tube.

More recently Taymor, Buytendorp and Goss (1962) claim to have measured the levels of HLH in human urine using this method and that the results obtained agree well with those obtained using the comparative bioassay - the ventral prostate test. Their immunological assays have been performed not on untreated urine, but on urine which has been extracted according to the method of Albert (1955).

In the experiments reported here attempts were made to convert the Ortho Pregnancy Diagnosis Kit into a quantitative assay for HCG. As will be seen from the results obtained it proved to be a very precise method of estimating the potency of reasonably

pure preparations with a sensitivity comparable to that found by the bioassay method depending on the increase in weight of the prostate in intact immature rats. However, attempts to apply the method to the measurement of HCG in urine failed because of lack of parallelism between the standard and the unknown. Even in recovery experiments, when known amounts of HCG were added to urine from male and non-pregnant female subjects, the dose response curves of HCG in water and of the same concentrations of HCG in urine were not parallel. It would appear therefore that the addition of urine to the reaction mixture interferes in some non-specific manner with the agglutination and subsequent precipitation of the latex particles. It is not clear whether this interference is a pH effect or linked in some way with the ionic concentration and the specific gravity of the urine. Singer and Plotz (1956) noted that spontaneous agglutination of coated particles occurred between pH 5.5 and 8.0, while a total inhibition of reaction occurred between pH 10 and 12.6. To eliminate this pH effect, the pH of all urines examined in this study was adjusted to between pH 8.0 and 8.5 before preliminary centrifugation, although, Goldin (1962) claims that urine pH is not critical. It would appear that pH is not critical for uncoated particles, as, in some preliminary experiments, for a particular particle concentration there was very little difference in the percentage light transmitted (see table 22), whether the particles were suspended in distilled water or in pH adjusted urine.

Although it has not been found possible to use this method for the quantitative estimation of HCG in urine (Goss and Taymor, 1965 use it in a semi-quantitative form to give rapid estimates of HCG levels in pregnancy) this does not invalidate its use a) for the quantitative estimation of potency of purified preparations of HCG and b) for the diagnosis of pregnancy. As a pregnancy diagnosis kit the conditions of the assay have been so weighted that it only very rarely gives a false positive result. Approximately 2% false negative results are obtained from either testing urine too early in pregnancy, or from the use of a urine sample with a low specific gravity. Correct results generally range from about 95 - 100% (Ortho Research Foundation, Goldin, 1962; Henry and Little, 1962; Raj et al., 1963 amongst others and compare favourably with the results obtained by the more conventional pregnancy tests.

It was hoped that the use of latex particles would eliminate some of the difficulties encountered in the standard haemagglutination reaction. In the latex particle system the amount of agglutination occurring can be measured quantitatively and accurately by estimating the residuary turbidity of the solution while, direct methods for the accurate estimation of red cell agglutination do not exist.

Although coated latex particles are unfortunately sensitive to alterations in salt concentrations and the specific gravity of the fluid in which they are suspended, they are inert and therefore should not be affected by the non-specific antibodies which fre-

quently upset haemagglutination reactions. Following the finding that Boyden type cells, both control and antigen-coated, were agglutinated when added to either normal human serum or plasma, two courses were open. Either the serum or the plasma must be purified in some way to remove the interfering agglutinins or another type of coated (indicator) cell, which was not affected in this way, must be used in this system.

From the literature it appeared that treatment of red cells with formalin had the effect of not only strengthening the cell and making it more resistant to non-specific agglutinins but also of preserving the cell, i. e. coated cells could be prepared in advance and used for upwards of 3 months. A number of different methods, all claimed to give good results, were tried. In one instance (Csizmas) the cells were so crenallated following formalinisation as to be totally unusable, in spite of the author's claim that no crenellation occurred. Cells treated according to the methods of Fulthorpe and Weinbach were dark, sticky and showed a totally haphazard agglutination reaction in both diluent and buffer. The best results were obtained with those prepared according to the method of Fauconnier, in which the cells were gassed with carbon monoxide before formalinisation. Cells prepared in this way were of good colour and were not crenellated, but even these showed frequent spontaneous agglutination in diluent which made them useless as indicator cells in the haemagglutination reaction.

Butt et al. (1961) claimed good results in an HCG system

using cells prepared according to the method of Ling. In this method pyruvic aldehyde was substituted for formalin. Cells prepared according to this method were not crenellated and did not show spontaneous agglutination in buffer and diluent. Unfortunately all attempts to tag these cells with growth hormone failed. As the various attempts to prepare a better cell for use in the system had failed, the only alternative left was to attempt to clear the sample of unwanted antigens.

The simplest method of clearing the serum sample was firstly to destroy the complement content by heating, and secondly, to absorb the serum with the red cells to be used in the assay. Fresh cells had to be used, because uncoated tanned cells would remove all the protein including the growth hormone from the sample. Following this procedure the non-specific agglutinants appeared to have been removed. It now, however, appeared that the growth hormone level of normal serum was below the level of sensitivity of the assay i. e. less than 10 - 50 $\mu\text{g.}/\text{ml.}$ serum. This very low (below the sensitivity of the method) result was very puzzling in the light of the values for normal serum (range 80 - 480 $\mu\text{g.}/\text{ml.}$ serum) reported by other workers at this time (see Table 29), although Kaplan and Grumbach (1962) using similar heat treated and absorbed serum reported the growth hormone levels in normal and hypopituitary subjects to be less than 20 $\mu\text{g.}/\text{ml.}$ i. e. below the sensitivity of the method. It was some time before it was accepted that these apparently

reasonable results (normals < 500 $\mu\text{g.}/\text{ml}$; acromegalics > 500 $\mu\text{g.}/\text{ml}$. serum) were in fact over estimates of growth hormone activity. These results became suspect because of the high levels reported in clinical conditions such as pituitary dwarfs and hypophysectomised subjects (range $100 - 200$ $\mu\text{g.}/\text{ml}$. and $20 - 290$ $\mu\text{g.}/\text{ml}$. respectively), when low or undetectable levels would be expected. It was thought that these false high values were due to the presence of non-specific inhibitors in the test sera; a view apparently confirmed by the findings of Dominguez and Pearson (1962) who used unextracted serum and showed values in the same range from normal subjects, hypophysectomised subjects, hypopituitary dwarfs and acromegalics. When the serum samples were extracted, values below the sensitivity of the method were obtained in the case of the first three groups.

It would appear therefore, that the failure to apply this method to clinical problems lies in its lack of sensitivity, not in its performance. This method gave good, reproducible and precise results when used for assaying the hormone content of pituitary extracts and where the potency of a pituitary preparation has been estimated by both the haemagglutination-inhibition method and the tibial assay, reasonable agreement exists between the results obtained by the two methods.

More recently, radio-immunological assay methods have been developed. The estimated potency of some pituitary extracts (ICRF 6, ICRF 6 - peak I and ICRF 6 - peak II) as measured by the

Hunter radio-immuno-electrophoretic method agreed with the results obtained by the tibial test (see Pages 50-51). The values for human plasma (range 0 - 50 $\mu\text{g.}/\text{ml.}$) published by Utiger et al. (1962) using a modified precipitin test are less than most of the published values for the haemagglutination-inhibition method, but greater than the values obtained by Hunter and Greenwood, 1962 (range 2.4 - 5.6 $\mu\text{g.}/\text{ml.}$ plasma) and Glick et al., 1963 (range 0 - 15 $\mu\text{g.}/\text{ml.}$ plasma) who have used a modified electrophoretic technique. It is interesting to see that the more sensitive radio-immunological assay methods have confirmed the normal growth hormone level in blood to be less than 10 $\mu\text{g.}/\text{ml.}$ which is the lower limit of sensitivity reached in the experiments reported here. It is probable that the radio-immuno-electrophoretic techniques which are precise and not too laborious will become the methods of choice in the clinical field, despite the fact that they are time consuming.

SUMMARY

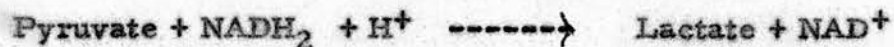
1. Specific antisera have been raised to the following hormones :-
bovine and human growth hormone and human chorionic gonadotrophin.
2. The haemagglutination - inhibition assay method for growth hormone has been established and some of the conditions of assay examined.
3. The potency of two pituitary growth hormone preparations and four pituitary fractions has been estimated by this method.

4. The haemagglutination-inhibition method was used to estimate the growth hormone content of human sera from 24 normal subjects.
5. The Ortho pregnancy diagnosis kit was modified to give a quantitative method of estimating HCG, and the specificity of the method examined.
6. The potency of 'Pregnyl' was determined using this method.
7. The method was applied to the quantitative estimation of HCG in urine.

Methods of estimating mevalonic acid are complex, time consuming and not suitable for incorporation into bioassays, and for this reason no attempt has yet been made to study the endogenous levels of this substance. Attempts to measure acetate as an index of the acetylcoenzyme A level in the ovary failed because the techniques suitable for extensive application were not sensitive enough to detect the presence of acetate even in ovaries pre-treated with PMSG and HCG.

Pyruvic acid readily forms a coloured hydrazone with 2, 4-dinitrophenylhydrazine, and methods of estimating pyruvate using this property of the compound are reasonably sensitive and easy to perform. The method of Katsuki et al. (1961) was finally chosen and the results obtained with this procedure are described herein.

A very sensitive enzymic method which depends on the reaction



is also available, and the data presented here was obtained with the method of Bucher et al. (1963).

In the present section, the results of an investigation undertaken to study the endogenous pyruvic acid level in the ovaries and testes of immature rats, is reported. The effect of gonadotrophic stimulation on the pyruvate levels of the ovary is also described.

MATERIALS AND CHEMICAL METHODS OF ESTIMATION

A. Dinitrophenylhydrazine

Reagents:-

1. 0.1% dinitrophenylhydrazine solution - 100 mg. DNPH (BDH - reagent grade) are dissolved in 100 ml. 2N HCl with the aid of gentle heating.
2. 0.1N sodium carbonate - 5.3 g. anhydrous sodium carbonate (BDH - AR grade) and 0.84 g. sodium bicarbonate (BDH - AR grade) are dissolved in distilled water and the volume made up to 1 litre.
3. Ethyl acetate - ethyl acetate (M & B - reagent grade) was redistilled and the fraction collected from boiling point 74 - 77°C was used.
4. Petroleum ether - petroleum ether (BDH - AR grade) was redistilled and the fraction collected from boiling point 60 - 80°C. was used.

Method (Katsuki et al., 1961):-

1. 4.0 ml. deproteinised tissue preparation and 1.0 ml. DNPH solution are placed in a 10.0 ml. stoppered, graduated, conical centrifuge.
2. The tube was shaken and incubated for 10 minutes in a 25 - 30°C water bath.
3. 5.0 ml. ethyl acetate was then added, the tube again shaken and nitrogen bubbled through the mixture for 2 minutes.
4. On standing, two layers became apparent and when completely separated the lower aqueous layer was discarded.

5. The ethyl acetate layer was washed twice with 2 - 3.0 ml. distilled water.
6. 5.0 ml. petroleum ether and 2.0 ml. carbonate solution were then added, the tube was again shaken and nitrogen bubbled through the mixture.
7. After standing, the upper ethyl acetate layer was discarded .
8. The carbonate layer was then washed-three times with 4.0 - 5.0 ml. of a 1 : 1 mixture of ethyl acetate and petroleum ether.
9. Nitrogen was then bubbled vigourously through the carbonate solution until the odour of ethyl acetate could no longer be detected.
10. The carbonate extract was made up to 10.0 ml. with more carbonate solution and the absorbancy measured at 355 m μ using a DNPH blank. Silica cells with a 1.0 cm. light path were used.
11. 5.0 ml. 2N NaOH were added to 5.0 ml. of the carbonate extract and the absorbancy measured at 417 m μ .
12. The value of the pyruvic acid content of the sample was then calculated from standard curves.
13. Blanks were prepared in the same way, 4.0 ml. of deproteinising agent being substituted for the 4.0 ml. deproteinised sample.

Eight samples could be carried through the method at one time.

Modifications to the Method

- 1). Deproteinising Agent.

The type of deproteinising agent used in this procedure is of

importance. Katsuki et al. (1961) recommended the use of metaphosphoric acid for this purpose. They found that trichloroacetic acid gave unsatisfactory results because it interfered with the final extraction of pyruvate dinitrophenylhydrazone. On the other hand Neish (1953, 1957) found that a freshly prepared tungstic acid solution was the best tissue extractant and deproteinising agent for pyruvate.

Pyruvic acid standards were made up in both 10% metaphosphoric acid and in freshly prepared tungstic acid (8 ml. N/12 H₂SO₄ : 1 ml. 10% Na tungstate) and taken through the procedure for the extraction and estimation of pyruvate. The results obtained (read against the appropriate DNPH blank) are shown in table 34 and figure 10.

From these it can be seen that the two curves follow each other closely and when the central part of the two curves was analysed on the basis of a 6-point Gaddum assay (see Appendix), they were found to be parallel and to show no significant curvature.

In all the experiments reported using the DNPH method tungstic acid was used as the deproteinising agent.

2). Absorbancy measurements.

The pyruvic dinitrophenylhydrazone has a faint yellow colour in dilute carbonate solution with a maximum absorbance at 355 m μ . If sodium hydroxide is then added there is a colour change to red and the point of maximum absorbance is altered to 417 m μ . Katsuki and his colleagues suggest that it is better to measure the absorbance

TABLE 34.

Standard Curves - Comparison of the optical density of pyruvic acid standards made up in metaphosphoric acid and in tungstic acid at 355 m μ and 417 m μ . Dinitrophenylhydrazine Determinations.

Pyruvic Acid in $\mu\text{g.}/\text{sample}$	Metaphosphoric Acid		Tungstic Acid	
	O.D. 355 m μ .	O.D. 417 m μ .	O.D. 355 m μ .	O.D. 417 m μ .
0.125	0.094	0.044	0.088	0.042
0.25	0.201	0.090	0.155	0.067
0.50	0.519	0.210	0.353	0.149
1.00	0.713	0.300	0.687	0.280
2.00	1.368	0.560	1.344	0.550

**Fig.11:- COMPARISON OF THE OPTICAL DENSITY OF PYRUVIC ACID
STANDARDS MADE UP IN METAPHOSPHORIC ACID AND IN
TUNGSTIC ACID AT 355 MU AND 417MU WAVELENGTH.**

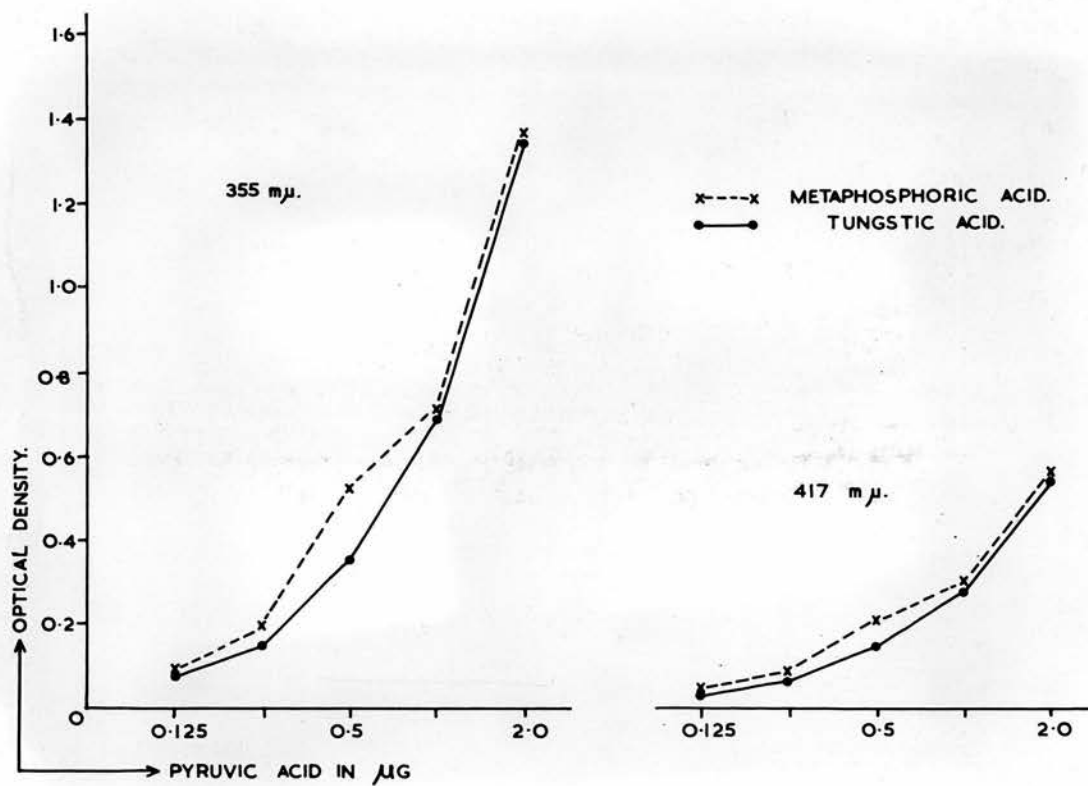


Fig. II.

of solutions at 417 $m\mu$, as interference by coloured impurities in the sample was then largely avoided. The red colour is not however very stable and samples must be read within 10 - 20 minutes of the addition of the sodium hydroxide.

Pyruvic acid standards were therefore made up in both metaphosphoric acid and freshly prepared tungstic acid and taken through the procedure. The absorbancy of the solutions was measured at both 355 $m\mu$ and following hydroxide addition at 417 $m\mu$. The results obtained are summarised in table 34 and in figure 11.

At both 355 $m\mu$ and 417 $m\mu$ the curves relating the optical densities of the pyruvic acid standards in both metaphosphoric acid and in tungstic acid follow each other closely, and the central parts were found to be parallel and to show no significant curvature. However when the absorbancy curves at 355 $m\mu$ and 417 $m\mu$ are compared it can be seen that while they follow the same pattern of increased optical density with increasing concentrations of pyruvic acid, the curves are no longer parallel. The steeper dose response curve is obtained when the absorbancy is measured at 355 $m\mu$.

3). Cuvettes and Spectrophotometer.

Fused silica quartz cuvettes were used throughout. Initially the carbonate extracts were diluted to 10 ml. with more carbonate solution and the absorbance measured using 1 cm light path cuvettes with a 4 ml. capacity. It was thought that it would be possible to make the assay more sensitive if microcuvettes of less than 1 ml. capacity were used. Pyruvic acid standards were made up in

tungstic acid in concentrations ranging between 0.125 and 16.0 $\mu\text{g}/$ sample. The absorbancy of these samples was measured in either the diluted form in the large cuvettes or undiluted in the micro-cuvettes. The results are summarised in table 35 and in figure 12.

It can be seen that the two curves are of identical form and parallel. The use of microcuvettes increases the sensitivity of the method by a factor of about eight.

All measurements of absorbancy were made in a Pye spectrophotometer model Sp. 500.

4). Modifications to the method adopted.

- a. Tungstic acid as deproteinising agent.
- b. Microcuvettes.
- c. Undiluted carbonate extract.

B. Enzymic Method

Reagents:-

- 1). 5 M potassium carbonate - about 69g. K_2CO_3 (BDH - AR grade) are dissolved in distilled water and made up to 100 ml.
- 2). Methyl orange indicator (0.05% w/v) - 50 mg. methyl orange are dissolved in distilled water and made up to 100 ml.
- 3). Perchloric acid (6% w/v) - 13 ml 70% HClO_4 (BDH - AR grade) are diluted to 250 ml. with distilled water.
- 4). 0.4 M triethanolamine buffer, pH 7.6 - 18.6g. triethanolamine hydrochloride (BDH - reagent grade) are dissolved in 200 ml. distilled water, 18 ml. 2N NaOH (BDH - volumetric solution) and 3.7g. of the disodium salt of ethylene-diamine-tetra-

TABLE 35.

Standard Curves - Comparison of the optical density of pyruvic acid standards in standard and microcuvettes at 355 m μ . Dinitrophenylhydrazine Determinations.

Pyruvic Acid in $\mu\text{g.}/\text{sample}$	O.D. 355 m μ .	
	4.0 ml. cuvettes	micro- cuvettes
0.125	-	0.088
0.25	-	0.155
0.50	-	0.353
1.00	0.164	0.687
2.00	0.230	1.344
4.00	0.420	-
8.00	0.740	-
16.00	1.300	-

**Fig.12:- COMPARISON OF THE OPTICAL DENSITY OF PYRUVIC ACID
STANDARDS IN STANDARD AND MICROCUVETTES AT 355 MU WAVELENGTH.**

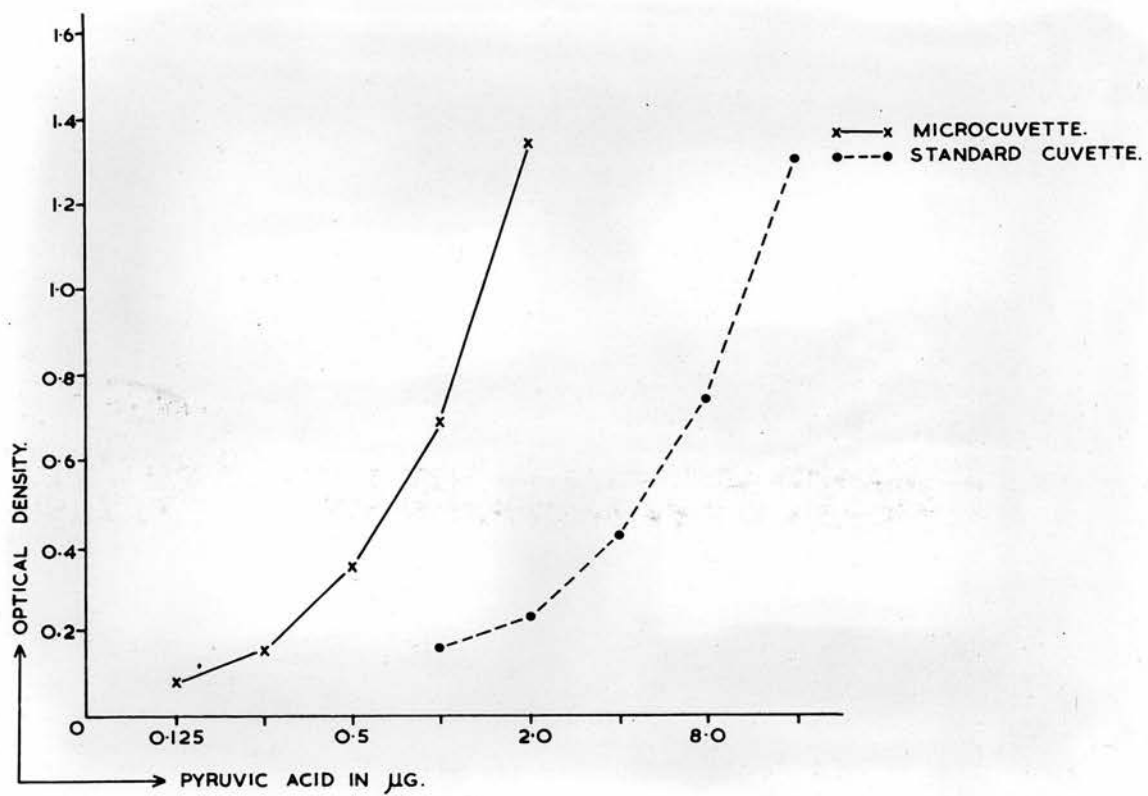


Fig.12.

acetic acid (BDH - reagent grade) are then added and the volume made up to 250 ml.

- 5). Reduced diphosphopyridine nucleotide (NADH) - 7 mg. of the disodium salt (BDH) are dissolved in 1.5 ml. distilled water to give an approximately 5×10^{-3} M β NADH₂ solution.
- 6). Lactic dehydrogenase (LDH) - a 10 mg/ml protein solution with an enzymatic activity of 75U/mg. (supplied by Koch-Light Laboratories) is diluted 1 : 10 with 2.1 M ammonium sulphate. (BDH - AR grade)

All solutions were stored in a refrigerator at +4°C., the NADH₂ solution was freshly prepared each week, the others were kept as long as no bacterial contamination occurred.

Method:-

To a Silica quartz cuvette with a 1.0 m. light path a 3.0 - 4.0 ml. capacity was added.

1. 2.0 ml. deproteinised sample.
2. 1.0 ml. 0.4 M triethanolamine buffer.
3. 0.04 ml. NADH₂ solution.

The contents of the cuvette were well mixed and of this solution two measurements of the optical density against a blank containing 3.0 ml. buffer + 0.03 ml. indicator were made at 340 m μ at three minute intervals. Then 0.01 ml. of enzyme solution were pipetted onto a small glass spatula and stirred into the solution and the optical density again measured 3 and 6 min. after the addition of the enzyme. The amount of pyruvate present can then be calculated.

Calculation:-

The concentration of pyruvate in the sample is estimated from the equations:-

$$\frac{\Delta E \times V}{\xi \times d} = \mu\text{moles pyruvate/assay mixture}$$

$$\text{or } \frac{\Delta E \times V}{\xi \times d \times A} = \mu\text{moles pyruvate/gram tissue}$$

where ΔE = decrease in optical density (O.D.) following the addition of lactic dehydrogenase

V = volume of assay mixture.

ξ = extinction coefficient of DPNH which is 6.3 $\text{cm.}^2/\mu$ mole at 340 $m\mu$ for a light path of 1cm.

d = the light path.

and A = weight of tissue in the assay sample, which
= $\text{g. tissue} \times \frac{\text{ml. extract taken for assay}}{\text{total ml. extract}}$

('total ml. extract' means the volume of extractant used plus the correction for the volume of water contained in the tissue. For this purpose it is assumed that the water content of tissues is 75%. In the experiment reported here the correction for the water content of the tissues was neglected, as a 5.0 ml. extractant volume was used on tissue weights ranging from 20 - 200 mg. the error introduced by neglecting this factor was not more than 3%.)

Modifications to the Method:-

The only modification to the enzymatic method which has been introduced, is the approximately twenty-fold increase in the volume of tissue extractant used. This is discussed in the section on tissue preparation (see p. 122 - 124)

METHODS OF TISSUE PREPARATION

A. Tissue Freezing

It is generally accepted that pyruvic acid is rapidly destroyed in tissues by autolytic procedures and Neish (1953) has recommended that the animal should be anaesthetised and the appropriate tissue be frozen in situ, before removal to a container precooled in a mixture of acetone and dry ice. In the present investigation it was found difficult to free the ovary from the surrounding fat, capsule and fallopian tube following quenching in situ. In view of the fact that an ovary could usually be dissected out, cleaned and quenched within one minute of removal from the rat, it was decided, therefore to check if freezing of the tissue was obligatory.

Twelve rats which had been treated with 50 i.u. PMSG prior to the experiment were anaesthetised, one ovary from each animal was quenched and frozen, the other ovary was not frozen, but put into the deproteinising solution as rapidly as possible. Material from the rats was pooled and the results are shown in table 36.

The difference of 3.4 μ moles noted between the pyruvate level in frozen and in unfrozen tissue is less than the standard deviation (± 16.5) of the mean of the five separate observations

TABLE 36

Estimation of pyruvic acid concentration in ovarian material which has been frozen and not frozen before extraction with tungstic acid. Dinitrophenylhydrazine Determinations.

Overian Material	Result mu moles pyruvate/ 100 mg.	Design	Rel. Pot.	Fid. Limits	λ	Validity
Frozen	39.8	2 + 2	1.05	78 - 139%	0.073	Yes
Not frozen	43.2	2 + 1	0.76	67 - 86%	0.046	

made of the pyruvate level three days after treatment with 50 i. u. PMSG (see p. 127). It therefore seemed unlikely that any marked deterioration in pyruvic acid levels had occurred and for this reason freezing of the ovarian tissue was discontinued.

B. Volume of Protein Extractant.

1) In the dinitrophenylhydrazine method.

Although it had been shown that there was no difference in the recovery of standards made up in metaphosphoric acid and in tungstic acid, it seemed relevant to repeat the experiment using tissue pyruvic acid rather than the pure compound. The ovaries from 7 rats which had been pretreated with 50 i. u. PMSG at age 21 days and with 25 i. u. HCG on the third day after PMSG, were removed 11 days after the administration of the HCG. One ovary from each rat was extracted by metaphosphoric acid and the other extracted by tungstic acid. The results obtained with each extractant are shown in Table 37.

It can be seen that the results 12.7 μ moles/100 mg. ovarian tissue extracted by metaphosphoric acid and 14.1 μ moles/100 mg. ovarian tissue extracted by tungstic acid were not significantly different. It was, therefore, decided to use tungstic acid as the protein extractant of choice.

Neish recommended the use of 9.0 ml. tungstic acid to 1.0 g. tissue as the optimal tissue to protein extractant ratio. As ovarian material was somewhat scarce, it was decided to estimate the

TABLE 37

Estimation of pyruvic acid concentration in ovarian material which has been extracted with metaphosphoric acid and with tungstic acid. Dinitrophenylhydrazine Determinations.

Tissue Extractant	Result μ moles pyruvate acid/100 mg.	Design	Rel. Pot.	Fid. Limits	λ
Metaphos- phoric acid	12.68	2 + 1	0.96	82 - 113%	0.078
Tungstic acid	14.12	2 + 1	0.99	82 - 120%	0.095

pyruvate level in ovarian material which had been extracted at the following ratios:-

- a). 0.1 g. tissue : 8.0 ml. tungstic acid.
- b). 0.1 g. tissue : 1.0 ml. tungstic acid.

Pooled ovarian material from rats which had been treated with 50 i.u. PMSG two days before was used for this experiment. The results are summarised in table 38.

It can be seen that there was little difference in the results obtained when different volumes of tissue extractant were used. It would appear therefore that increasing the volume of tissue extractant used does not adversely affect the extraction of pyruvate from the material, but does reduce the lower limit of sensitivity of the procedure and was therefore used in future experiments.

2). In the enzymic method.

Bucher et al. (1963) recommends that 2.0 g. tissue should be extracted with 6.5 ml. 6% perchloric acid. It is apparent from the text that this ratio has been chosen in order to simplify the final calculation. It was assumed that to increase the volume of perchloric acid used per gram of tissue would not materially interfere with the extraction of pyruvate from the tissue.

A 24 day old rat was killed and the liver dissected free. The liver was then homogenised in 6% perchloric acid in the following ratios:-

- 1). 0.1 g. tissue : 6.5 ml. perchloric acid.
- 2). 0.2 g. tissue : 6.5 ml. perchloric acid.

TABLE 38

Estimation of pyruvic acid concentration in ovarian material which has been extracted with different volumes of tungstic acid. Dinitrophenylhydrazine Determinations.

Tissue: Acid Ratio g.:ml.	Result μm. moles pyruvic acid /100 mg.	Design	Rel. Pot.	Fid. Limits	λ
0.1:8.0	84.8	2 + 1	0.75	65 - 88%	0.079
0.1:1.0	85.9	2 + 1	0.76	61 - 91%	0.098

3). 2.0 g. tissue : 6.5 ml. perchloric acid.

The results are summarised in table 39.

It can be seen that there is little difference in the estimated level of pyruvic acid when liver tissue is extracted in ten or twenty times the recommended volume of extractant. It was decided, therefore, to use a constant (5.0 ml.) volume of perchloric acid for the extraction process, and to correct for tissue weight in the final calculation.

C. Method of Tissue Preparation Adopted.

1) In the dinitrophenylhydrazine method.

Immature female rats were anaesthetised, and the ovaries rapidly dissected free and cleaned. The ovaries were weighed to the nearest mg. and then homogenised in freshly prepared tungstic acid. The volume of acid used was adjusted to the weight of the available material (1.0 ml. : 0.1 g.). The homogenised solution was then filtered through Whatman no. 50 paper. The clear filtrate obtained represents the 'deproteinised solution' on which the pyruvate estimations were made.

2). In the enzymic method.

Mortars containing a little sand and 5.0 ml. of an approximately 6% (w/v) perchloric acid solution were prepared. Immature female rats were killed by cervical dislocation. The ovaries were rapidly removed, cleaned, blotted and weighed in pairs on a torsion balance to the nearest mg. Paired ovaries were then ground in a

TABLE 39.

Estimation of pyruvic acid concentration in liver tissue which has been extracted with different volumes of 10% metaphosphoric acid. Enzymic Determinations.

Tissue: Extractant Ratio g:ml.	Optical Density at 340 mμ	Calculated Pyruvic acid Level mμ moles/ 100 mg.
0.1:6.5	0.011	21.3
0.2:6.5	0.019	18.4
2.0:6.5	0.207	20.1

mortar and left to stand for approximately 10 min. before the contents were transferred to a conical centrifuge tube. The centrifuge tube was then spun in the cold for 5 - 7 min. at approximately 2,500 rcf. The clear supernatant was decanted and 4.0 ml. were transferred to a 10 ml. conical flask, 0.01 ml. methyl orange indicator (0.05% w/v) was then added and the solution neutralised with an approximately 5M potassium carbonate solution. Following neutralisation the conical flasks were left in a tilted position in the refrigerator for at least 10 min. Most of the supernatant was then poured off carefully. This was the extract or 'deproteinised solution' on which the enzymic estimations were made.

Experimental Design.

1. Rats

Animals from a closed colony were used throughout these experiments. The rats were from Wistar strain and usually varied in age from 21 - 24 days, in some experiments where large numbers of animals were required rats of up to 30 days in age were used. The body weight at the commencement of an experiment was usually within the weight range 25 - 35 g.

2. Pretreatment

The hormones used in pretreatment were:

- a). Pregnant mare serum gonadotrophin (PMSG) - 'Gestyl'
 - b). Human chorionic gonadotrophin (HCG) - 'Pregnyl'
- and were supplied by the courtesy of Organon.

The hormones were dissolved in distilled water and adminis-

tered subcutaneously, the injection volume being kept constant at 0.5 ml.

Rats receiving PMSG alone were given either a single injection of 25, 50 or 100 i.u. or two injections each of 50 i.u. The second injection was administered 1, 2 or 3 days after the first.

Rats receiving both PMSG and HCG were given 50 i.u. PMSG followed 3 days later by 25 i.u. HCG.

3. Sampling.

Experiments were usually longitudinal in design, i. e. estimations were made at daily intervals following hormone administration. When estimations were made by the DNPH method pooled material from groups of 3 - 6 rats was usually used; although for the estimations on untreated rat ovaries, the material from approximately 50 rats had to be pooled. When the enzymic method was used, estimations could usually be made on the combined material from a single pair of ovaries.; however for the estimations on ovarian material from untreated animals, the ovaries from 2 - 7 animals were pooled.

4. Calculation of Results

In the DNPH method results were calculated from standard curves. The statistical evaluation of the results was carried out according to the simplified three - point, four - point and six - point assay procedure described by Gaddum (1953 b).

In the enzymic method results were obtained directly from the appropriate formula (see page 119) and the standard deviation for each group calculated.

The results obtained by the two methods were then compared by the student 't' test. (see Appendix).

RESULTS

A. Dinitrophenylhydrazine method.

Experiment 1. To establish the pyruvic acid concentration in the seminal vesicles and the ventral lobe of the prostate in immature male rats.

The accessory gland material from approximately 50 rats aged between 21 and 30 days was pooled for this experiment. The results are shown in table 40. The estimated value of 2.84 μ moles/100 mg. tissue for the seminal vesicle tissue cannot be accepted as a correct estimation as the assay was statistically invalid. This level does however appear to be of the same order as that obtained for the prostate tissue (3.45 μ moles/100 mg.) this assay being valid.

Experiment 2. To establish the pyruvic acid concentration in the ovaries of untreated immature female rats.

The pooled ovarian material from approximately 50 rats aged between 21 and 30 days was used. The results of this experiment are shown in table 40. The assay was statistically valid and the estimated level 3.75 μ moles/100 mg. is of the same order as

TABLE 40.

Estimation of pyruvic acid content in gonadal and accessory gland material from untreated immature rats. Dinitrophenylhydrazine Determinations.

Tissue	mu moles pyruvic acid/100 mg.tissue	Assay Design	Relative Potency	Fiducial Limits %	λ	Validity
Seminal Vesicle	2.84	2 + 2	0.81		0.035	Not
Ventral Lobe Prostrate	3.45	2 + 2	0.97	93 - 102	0.100	Yes
Ovarian	3.75	2 + 2	1.06	93 - 121	0.370	Yes

that found in the immature male gonadal accessory gland material.

Experiment 3. To ascertain the effect of different concentrations of PMSG on the ovarian pyruvic acid level.

A group of 36 rats was divided into 3 groups at weaning and each group was then injected with one of the following:- 25, 50 or 100 i. u. PMSG/rat. The results of this experiment are contained in tables 41 - 43 and summarised in table 44.

It can be seen that except on day 5, the pyruvic acid levels in the ovarian material was much the same for all doses of PMSG. The very high level 40.9 μ moles/100 mg. for the material from rats injected with 25 i. u. PMSG, 5 days previously is probably an artifact due to the use of a three-point assay for the statistical analysis of the results. If the pyruvic acid level is estimated by directly reading from the standard curve, a value of 31.1 μ moles/100 mg. is obtained. While this figure is still markedly higher than the comparable values for rats treated with 50 and 100 i. u. PMSG, i. e. 23.1 and 25.9 μ moles/100 mg. respectively, the difference can be ascribed to the error inherent in the use of single estimations in bioassay.

It would appear that, while, stimulation of ovarian growth by PMSG is accompanied by a marked rise in the level of pyruvic acid in the ovary, increasing the concentration of PMSG between 25 and 100 i. u. does not further increase the pyruvic acid concentration in the ovary.

Experiment 4. To establish the ovarian pyruvic acid level

TABLE 41.

Estimation of pyruvic acid concentration in ovarian tissue following a single injection of 25 i.u. FMSG. Dinitrophenylhydrazine Determinations.

Days after Injection	mu moles pyruvic acid/100 mg. tissue	Assay Design	Relative Potency	Fiducial Limits	λ	Validity
3	33.84	2 + 2	1.193	103 - 138%	0.063	Yes
4	17.73	2 + 1	0.626	20 - 99%	0.248	
5	40.91	2 + 1	1.152	82 - 162%	0.159	
6	28.18	2 + 1	0.792	64 - 98%	0.089	

TABLE 42.

Estimation of pyruvic acid concentration in ovarian tissue following a single injection of 50 i.u. PMSG. Dinitrophenylhydrazine Determinations.

Days after Investigation	mu moles pyruvic acid/100 mg. tissue	Assay Design	Relative Potency	Fiducial Limits	λ	Validity
3	29.54	2 + 2	1.040	92 - 117%	0.066	Yes
4	20.34	2 + 2	0.718	55 - 84%	0.091	Yes
5	23.18	2 + 2	1.303	155 - 180%	0.233	Yes
6	31.14	2 + 2	0.878	76 - 103%	0.087	Yes

TABLE 43

Estimation of pyruvic acid concentration in ovarian tissue following a single injection of 100 i.u. PMSG. Dinitrophenylhydrazine Determinations.

Days after Injection	μ moles pyruvic acid/100 mg. tissue	Assay Design	Relative Potency	Fiducial Limits	λ	Validity
3	27.95	2 + 2	0.985	82 - 117%	0.096	Yes
4	23.50	2 + 2	0.827	69 - 99%	0.066	Yes
5	25.91	2 + 1	0.914	68 - 117%	0.186	
6	28.98	2 + 2	0.816	67 - 100%	0.102	Yes

TABLE 44

Comparison of the pyruvic acid concentration in ovarian tissue following treatment with various concentrations of PMSG.

Dinitrophenylhydrazine Determinations.

Days after Injection	mu moles pyruvic acid/100 mg. ovarian tissue		
	25 i.u. PMSG	50 i.u. PMSG	100 i.u. PMSG
3	33.84	29.54	27.95
4	17.73	20.34	23.50
5	40.91	23.18	25.91
6	28.18	31.14	28.98

in immature rats consequent to the administration of a single injection of 50 i. u. PMSG.

This experiment was repeated a number of times and the results are contained in table 45 and summarised in table 48 and figure 13. It will be seen that following an injection of 50 i. u. PMSG, there is a marked rise in the pyruvic acid content by the second day after the injection, which then proceeds to fall and appears to plateau at about 20 μ moles/100 mg. ovarian tissue by the fifth to sixth day after the injection.

Experiment 5. To establish the ovarian pyruvic acid level in immature rats which have twice been treated with 50 i. u. PMSG.

In this experiment the second injection of PMSG was administered three days after the first. Rats were killed on the first and third days after the second injection. The results of this experiment are contained in table 46 and summarised in table 48 and figure 13.

It will be seen that immediately following a second injection of 50 i. u. PMSG there appears to be no further increase in the pyruvic acid level of ovarian material. One day after a second injection of PMSG the mean pyruvic acid level was 28.4 ± 11.2 μ moles/100 mg. tissue which is not significantly different from the mean level 24.4 ± 5.1 μ moles/100 mg. for tissue from rats which had been treated with a single dose of PMSG four days before. It is, however, possible that following a second injection of PMSG there is a reduction in the pyruvic acid content of ovarian material

TABLE 45.

Estimation of pyruvic acid concentrations in ovarian tissue following a single injection of 50 i.u. FMSG. Dinitrophenylhydrazine Determinations.

Days after 50 i.u. FMS	μm moles pyruvic acid/100 mg.	Assay Design	Relative Potency	Fiducial Limits	λ	Validity
2	84.8	2 + 1	0.75	65 - 88	0.079	
	65.9	2 + 1	1.16	95 - 142	0.095	
	70.6	2 + 1	1.24	101 - 153	0.095	
	85.9	2 + 1	0.76	61 - 91	0.098	
3	29.3	2 + 2	0.77	57 - 105	0.073	Yes
	43.1	2 + 1	0.76	67 - 87	0.046	
	23.5	2 + 2	0.83	73 - 94	0.066	Yes
	20.4	2 + 2	0.72		0.127	Not
	29.5	2 + 2	1.04	92 - 117	0.066	Yes
	39.8	2 + 2	1.05	78 - 139	0.073	Yes
	43.2	2 + 1	0.76	67 - 86	0.046	
4	16.2	3 + 3	1.15	99 - 133	0.127	Yes
	25.9	2 + 1	0.91	80 - 103	0.074	
	28.2	2 + 1	0.99	86 - 115	0.089	
	29.3	2 + 2	1.03	83 - 129	0.122	Yes
	26.6	2 + 2	0.94	25 - 351	0.072	Yes
	20.3	2 + 2	0.72	55 - 84	0.091	Yes
5	14.8	3 + 3	1.04	92 - 105	0.058	Yes
	23.2	2 + 2	1.30	155 - 180	0.233	Yes
6	20.0	2 + 2	1.41	94 - 215	0.180	Yes
	24.4	2 + 2	1.72	156 - 193	0.243	Yes
	22.8	2 + 2	1.6	37 - 188	0.172	Yes
	31.1	2 + 2	0.88	76 - 103	0.087	Yes

TABLE 46.

Estimation of pyruvic acid concentration in ovarian tissue following a second injection of 50 i.u. PMSG, 72 hours after the first. Dinitrophenylhydrazine Determinations.

Days after Second Injection	mu moles pyruvic acid/ 100 mg. tissue.	Assay Design	Relative Potency	Fiducial Limits %	λ	Validity
1	44.8	2 + 1	0.79	71 - 87	0.051	
	26.4	2 + 1	0.93	82 - 105	0.074	
	19.7	2 + 2	0.69	54 - 84	0.107	Yes
	22.8	2 + 2	0.83	69 - 97	0.093	Yes
3	11.3	3 + 3	0.79	64 - 99	0.139	Yes
	14.2	3 + 3	1.00	79 - 125	0.151	Yes

as three days after a second injection the mean level had fallen to 12.8 ± 2.0 μ moles/100 mg. which is significantly lower than the mean level 24.6 ± 4.7 μ moles/100 mg. for the comparable day (the sixth) after a single injection of PMSG.

Experiment 6. To establish the ovarian pyruvic acid level in immature rats which have been treated with both PMSG and HCG.

The HCG (25 i.u./rat) was administered three days after the injection of 50 i.u. PMSG. This experiment was repeated a number of times and the results are contained in table 47 and summarised in table 48 and figure 13.

It will be seen that the administration of both hormones does not appear to greatly alter the pyruvic acid level in ovarian material. One day after the injection of HCG the mean pyruvic acid level was 24.8 ± 6.9 μ moles/100 mg. tissue which is virtually the same as the mean level 24.4 ± 5.1 μ moles/100 mg. tissue from rats which had been treated with a single dose of PMSG four days before. Two to three days after the HCG injection the ovarian pyruvic acid level appears to level off at about 10 - 15 μ moles/100 mg. tissue, which is significantly lower than the comparable levels obtained for rats treated with PMSG alone.

B. Enzymic Method.

Experiment 7. To establish the pyruvic acid concentration of the testis of immature male rats.

Separate estimations were made on each testis. The

TABLE 47.

Estimation of pyruvic acid concentration in ovarian tissue following an injection of 25 i.u. HCG, 72 hours after the administration of 50 i.u. PMSG. Dinitrophenylhydrazine Determinations.

Days after 25 i.u. HCG	μ moles pyruvic acid/100 mg. tissue.	Assay Design	Relative Potency	Fiducial Limits %	λ	Validity
1	23.3	3 + 3	0.82	74 - 91	0.054	Yes
	34.9	2 + 1	1.23	105 - 143	0.089	
	29.5	2 + 2	1.04	95 - 115	0.051	Yes
	20.8	2 + 2	0.73	55 - 92	0.127	Yes
	25.5	2 + 2	0.89	78 - 103	0.079	Yes
	15.1	2 + 1	1.08	59 - 229	0.270	
2	8.6	3 + 3	1.22	97 - 152	0.074	
	18.7	3 + 3	1.32	104 - 171	0.151	Yes
3	11.8	2 + 2	0.83	49 - 130	0.217	Yes
	11.9	2 + 2	0.84	37 - 188	0.267	Yes
	22.9	3 + 3	1.60	125 - 219	0.162	Yes
4	11.0	3 + 3	0.77	47 - 125	0.164	Yes
5	8.5	2 + 2	1.19	74 - 243	0.212	Yes
6	17.4	3 + 3	1.22	95 - 153	0.137	Yes
7	14.5	2 + 2	1.02	55 - 198	0.233	Yes
8	7.4	2 + 2	1.04	76 - 152	0.231	Yes
9	12.7	2 + 2	0.89	74 - 108	0.103	Yes
10	9.0	2 + 2	1.28	75 - 311	0.224	Yes
11	11.7	2 + 2	0.83	71 - 101	0.096	Yes
	12.7	2 + 1	0.96	82 - 113	0.078	
	14.1	2 + 1	0.99	82 - 120	0.095	

TABLE 48.

IMMATURE RATS - PYRUVIC ACID LEVEL IN
OVARIES FOLLOWING TREATMENT WITH 50 i.u. FMSG AND 25 i.u. HCG
ESTIMATIONS BY DINITROPHENYLHYDRAZINE METHOD

Untreated rats - 3.75 μ moles/100 mg. ovarian tissue (n = 1)

Days after first injection	First Injection 50 i.u. FMSG		Second Injection 50 i.u. FMSG 72 hrs. after first injection		Second Injection 25 i.u. HCG 72 hrs. after first injection	
	n	μ moles/ 100 mg. tissue Mean - S.D.	n	μ moles/ 100 mg. tissue Mean - S.D.	n	μ moles/ 100 mg. tissue Mean - S.D.
2	4	76.8 \pm 10.1				
3	7	32.7 \pm 16.5				
4	6	24.4 \pm 5.1	4	28.4 \pm 11.2	6	24.8 \pm 6.9
5	2	19.0 \pm 5.9			2	13.6 \pm 7.1
6	4	24.6 \pm 4.7	2	12.8 \pm 2.0	3	15.5 \pm 6.4
7					1	11.0
8					1	8.5
9	1	13.2			1	17.4
10					1	14.5
11					1	7.4
12					1	12.7
13					1	9.0
14					3	12.8 \pm 1.2

Fig.13:- PYRUVIC ACID LEVELS IN THE OVARIES OF IMMATURE RATS
PRIOR TO AND FOLLOWING TREATMENT WITH PREGNANT MARE
SERUM GONADOTROPHIN (PMSG) AND HUMAN CHORIONIC
GONADOTROPHIN (HCG). ESTIMATIONS BY THE DINITROPHENYL-
HYDRAZINE METHOD.

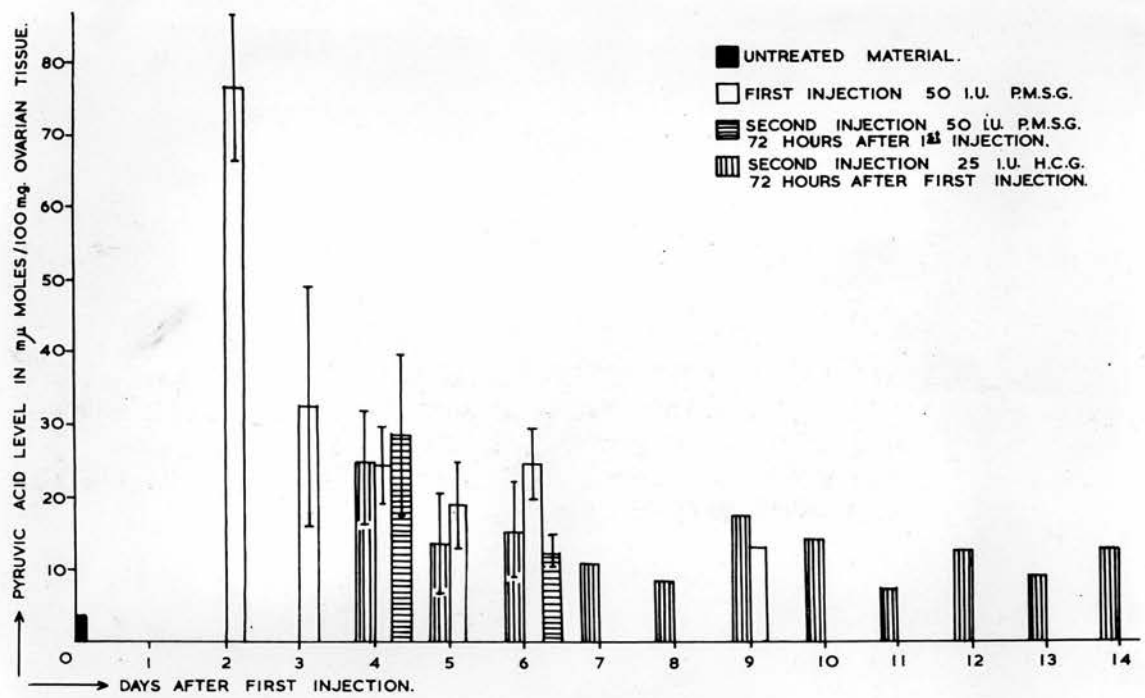


Fig.13.

estimated value 9.4 ± 3.4 μ moles/100 mg. tissue appears reasonable when compared with the values obtained for other gonadal tissues.

Experiment 8. To establish the pyruvic acid concentration in the ovaries of untreated immature female rats.

In this experiment estimations were made on pooled material. The results are shown in table 49. An estimate of the pyruvic acid content per pair of ovaries could not be made directly. The mean weight of a pair of immature ovaries was 5.3 mg. and the estimated pyruvic acid content per 100 mg. was 18.3 ± 7.3 μ moles. From these figures it can be deduced that the pyruvic acid content of a single pair of ovaries is about 1 μ mole.

Experiment 9 To establish the ovarian pyruvic acid level in immature rats consequent to the administration of a single injection of 50 i.u. PMSG.

This experiment was repeated a number of times; three observations were usually made on each occasion. The results are shown in table 50 and summarised in tables 57 and 59 and figure 14. It can be seen that following a single injection of 50 i.u. PMSG there is firstly a very marked rise in ovarian weight during the first three days, a plateau period from day 4 to day 6, and thereafter a steady falling off in weight. This pattern is reflected in the concentration of pyruvic acid per pair of ovaries but not in the concentration of pyruvic acid per 100 mg. ovarian tissue. The

TABLE 49.

Estimation of pyruvic acid concentration in gonadal tissue of untreated immature male and female rats. Enzymic determinations.

	Mean Weight of paired ovaries	mu moles/ paired ovaries	mu moles/ 100 mg. tissue mean - S.D.
Immature Testis			9.4 [†] ± 3.4 [†]
Immature Ovaries	5.3 mg. ⁺	1.0 [°]	18.3 [†] ± 7.3 [*]

† mean of 12 observations on separate testes from 6 animals

+ mean weight of pairs of ovaries from 50 rats

° calculated value

* mean of 7 observations on pooled material.

TABLE 50

Estimation of pyruvic acid concentration in ovarian tissue following a single injection of 50 i.u. PMSG. Enzymic determinations.

Days after Injection	No. of Observations	Wt. of paired ovaries in mg. Mean \pm S.D.	μ moles pyruvate pair ovaries Mean \pm S.D.	μ moles pyruvate/100 mg. Ovarian tissue Mean \pm S.D.
1	7	16.0 \pm 2.9	12.3 \pm 3.4	72.9 \pm 27.4
2	9	36.6 \pm 10.3	18.4 \pm 5.8	49.6 \pm 5.9
3	18	73.2 \pm 20.2	20.4 \pm 6.8	28.6 \pm 8.1
4	11	82.5 \pm 25.5	16.5 \pm 2.8	22.5 \pm 5.8
5	11	84.7 \pm 28.4	12.2 \pm 6.0	14.7 \pm 5.3
6	12	79.8 \pm 27.1	10.6 \pm 2.5	14.9 \pm 5.9
7	12	57.5 \pm 27.0	9.6 \pm 4.5	17.3 \pm 7.6
8	6	50.6 \pm 15.6	10.9 \pm 3.5	18.4 \pm 5.1
9	3	36.7 \pm 14.3	8.1 \pm 3.0	22.1 \pm 0.9
10	3	20.7 \pm 5.5	7.3 \pm 4.6	35.1 \pm 4.5
11	3	25.0 \pm 2.6	5.6 \pm 3.5	21.8 \pm 11.1

concentration per 100 mg. shows a four-fold rise in the first 24 hours after the injection followed by a steady fall in concentration which levels off at about the initial concentration by the fourth day after administration.

Experiment 10. To establish the ovarian pyruvic acid level in immature rats which have twice been treated with 50 i. u. PMS.

In this experiment the second injection of PMSG was administered one, two or three days after the first. Rats were sampled daily after the second injection. The results of this experiment are shown in tables 51 - 54 and summarised in tables 57 - 59 and figure 14.

It can be seen that a second injection of PMSG causes a very marked increase in the ovarian growth, which is reflected in the marked rise in the pyruvic acid content per pair of ovaries, whether the PMSG is administered one, two or three days after the first injection. A similar increase in the pyruvic acid content when expressed per 100 mg. tissue was not noted. When the second PMSG injection was given either one or two days after the initial injection there was little difference between the pyruvic acid content per 100 mg. of comparable ovaries from rats which had been treated with one or two doses of PMSG. However, when the second dose of PMSG was administered three days after the first injection there was a marked depression in the pyruvic acid content per 100 mg. in the ovarian material from rats which had had two doses of

TABLE 51

Estimation of pyruvic acid concentration in ovarian tissue following a second injection of 50 i.u. PMSG, 24 hours after the first. Enzymic determination.

Days after Injection	No. of Observations	Wt. of paired ovaries in mg. Mean \pm S.D.	μ moles pyruvic acid pair ovaries Mean \pm S.D.	μ moles pyruvic acid/100 mg. Ovarian tissue Mean \pm S.D.
1	6	56.7 \pm 5.5	18.7 \pm 3.2	33.1 \pm 4.6
2	6	122.1 \pm 27.1	29.3 \pm 2.3	24.0 \pm 5.7
3	6	174.3 \pm 35.2	39.9 \pm 6.7	17.8 \pm 3.2
4	6	182.7 \pm 23.8	31.2 \pm 10.7	16.9 \pm 4.9

TABLE 52

Estimation of pyruvic acid concentration in ovarian tissue following a second injection of 50 i.u. PMSG, 48 hours after the first. Enzymic determination.

Days after injection	No. of Observations	Wt. of paired ovaries in mg. Mean \pm S.D.	µm moles pyruvic acid pair ovaries Mean \pm S.D.	µm moles pyruvic acid/100 mg. Ovarian tissue Mean \pm S.D.
1	6	110.5 \pm 25.1	28.8 \pm 10.3	25.7 \pm 4.7
2	6	153.2 \pm 16.5	29.4 \pm 3.0	19.2 \pm 1.5
3	6	242.5 \pm 26.8	32.7 \pm 7.7	13.4 \pm 2.7

TABLE 53

Estimation of pyruvic acid concentration in ovarian tissue following a second injection of 50 i.u. PMSG, 72 hours after the first. Enzymic determination.

Days after 2nd injection	No. of Observations	Wt. of paired ovaries in mg. Mean \pm S.D.	mu moles pyruvic acid pair ovaries Mean \pm S.D.	mu moles pyruvic acid/100 mg. Ovarian tissue Mean \pm S.D.
1	8	124.0 \pm 24.9	19.7 \pm 5.6	16.6 \pm 7.2
2	9	201.0 \pm 18.0	26.9 \pm 12.1	13.3 \pm 6.2
3	5	247.6 \pm 36.5	22.5 \pm 10.2	9.2 \pm 1.1
4	9	257.7 \pm 63.9	14.4 \pm 3.9	5.6 \pm 1.3
5	4	214.3 \pm 46.0	17.2 \pm 1.5	8.3 \pm 1.5
6	3	177.0 \pm 38.3	25.8 \pm 5.7	14.9 \pm 4.0
7	3	171.3 \pm 66.5	21.4 \pm 7.8	13.1 \pm 3.8
8	3	200.0 \pm 7.0	12.0 \pm 3.2	6.1 \pm 1.7

TABLE 34

Comparison of the ovarian pyruvic acid content following a single and a double injection of 50 i.u. PMSG. Enzymic determinations.

	Days after 2nd	Pyruvic acid concentration mμ moles/100 mg. tissue		Degrees of Freedom	t	P	Difference
		Single PMSG	Double PMSG				
Injection interval 24 hours	1	49.6	33.1	13	5.77	< 0.001	Significant
	2	28.6	24.0	21	1.18	0.2 - 0.3	Not significant
	3	22.5	17.8	15	1.81	0.05 - 0.1	Not significant
	4	14.7	16.9	15	0.83	0.4 - 0.5	Not significant
Injection interval 48 hours	1	28.6	25.7	22	0.83	0.4 - 0.5	Not significant
	2	22.5	19.2	15	1.34	0.2 - 0.3	Not significant
	3	14.7	13.4	15	0.55	0.5 - 0.6	Not significant
Injection interval 72 hours	1	22.5	16.6	17	1.96	0.05 - 0.1	Not significant
	2	14.7	13.3	18	0.54	0.5 - 0.6	Not significant
	3	14.9	9.2	15	2.11	0.05 - 0.1	Not significant
	4	17.3	5.6	19	4.53	< 0.001	Significant
	5	18.4	8.3	8	3.77	0.001-0.01	Significant
	6	22.1	14.9	4	7.27	0.001-0.01	Significant
	7	35.1	13.1	4	6.44	0.001-0.01	Significant
	8	21.8	6.1	4	2.43	0.05 - 0.1	Not significant

PMSG when compared with the appropriate single dose material.

Experiment 11. To establish the ovarian pyruvic acid level in immature rats which have been treated with both PMSG and HCG.

The HCG (25 i.u./rat) was administered three days after the injection of 50 i.u. PMSG. This experiment was repeated a number of times, three observations were usually made on each occasion. The results are contained in tables 55 and 56 and summarised in tables 57 - 59 and figure 14.

It will be seen that in the first 24 hours following the administration of 25 i.u. HCG, the ovarian weight doubles and then almost immediately thereafter is maintained at about that level. The pyruvic acid content per pair of ovaries following the administration of HCG shows a somewhat erratic pattern but on the whole is considerably higher than the level of comparable ovaries from rats which have been treated with a single dose of PMSG, this is however simply a reflection of the higher ovarian weights in rats which have had combined PMSG and HCG treatment rather than PMSG alone. When the pyruvic acid content is expressed as μ moles per 100 mg. ovarian tissue there is a marked depression in pyruvic acid immediately following the administration of HCG, which plateaus in two to three days to a level considerably lower than that seen in animals treated with PMSG alone.

C. Comparison of results obtained by the two methods.

TABLE 55

Estimation of pyruvic acid concentrations in ovarian tissue following the administration of both PMSG and HCG.
Enzymic determinations.

Days after second injection	No. of Observations	Wt. of paired ovaries Mean \pm S.D.	μ moles pyruvic acid/ paired ovaries Mean \pm S.D.	μ moles pyruvic acid/ 100 mg. tissue MEAN \pm S.D.
1	5	160.5 \pm 61.5	22.51 \pm 12.2	14.3 \pm 4.7
2	6	161.0 \pm 48.4	15.15 \pm 4.2	9.5 \pm 1.5
3	5	196.4 \pm 26.1	15.25 \pm 10.6	7.8 \pm 6.0
4	6	178.8 \pm 40.2	17.14 \pm 2.7	8.4 \pm 1.3
5	4	192.7 \pm 32.1	18.15 \pm 1.0	10.4 \pm 1.6
6				
7	6	180.5 \pm 26.2	28.64 \pm 15.3	16.9 \pm 11.7
8	3	153.7 \pm 25.1	20.57 \pm 6.4	13.4 \pm 3.3
9	3	157.3 \pm 19.9	15.73 \pm 1.2	10.0 \pm 1.2
10	4	157.7 \pm 15.7	25.71 \pm 8.8	18.2 \pm 8.4
11	3	173.0 \pm 15.6	17.34 \pm 1.4	10.0 \pm 0.8

TABLE 56

Comparison of the ovarian pyruvic acid content following a single injection of 50 i.u. FMSG and after treatment with both 50 i.u. FMSG and 25 i.u. HCG. Enzymic determinations.

Days after HCG	Pyruvic acid concentration mM moles/100 mg. tissue		Degrees of Freedom	t	P	Difference
	Single PMS	PMS + HCG				
1	22.5	14.3	14	2.74	0.01 - 0.02	Significant
2	14.7	9.5	15	2.29	0.02 - 0.05	Significant
3	14.9	7.8	15	2.25	0.02 - 0.05	Significant
4	17.3	8.4	16	2.80	0.01 - 0.02	Significant
5	18.4	10.4	8	2.97	0.01 - 0.02	Significant
6						
7	35.1	16.9	7	2.53	0.02 - 0.05	Significant
8	21.8	13.4	4	1.26	0.2 - 0.3	Not significant

TABLE 57

IMMATURE RATS - WEIGHT OF PAIRED OVARIES
FOLLOWING TREATMENT WITH 50 I.U. PMSG AND 25 I.U. HCG

Estimations by the enzymic method.
 Untreated rats - Mean weight 5.3 mg. (n = 50)

Days after first injection	First injection 50 I.U. PMSG		Second injection 50 I.U. PMSG 24 hours after first injection		Second injection 50 I.U. PMSG 48 hours after first injection		Second injection 50 I.U. PMSG 72 hours after first injection		Second injection 25 I.U. HCG 72 hours after first injection	
	n	Wt. in mg. mean ± S.D.	n	Wt. in mg. mean ± S.D.	n	Wt. in mg. mean ± S.D.	n	Wt. in mg. mean ± S.D.	n	Wt. in mg. mean ± S.D.
1	7	16.0 ± 2.9								
2	9	36.6 ± 10.3	6	56.7 ± 5.5						
3	18	73.2 ± 20.2	6	122.1 ± 27.1	6	110.5 ± 25.1				
4	11	82.5 ± 25.5	6	174.3 ± 35.2	6	153.2 ± 16.5	8	124.0 ± 24.9	5	160.5 ± 61.5
5	11	84.7 ± 28.4	6	182.7 ± 23.8	6	242.5 ± 26.8	5	247.6 ± 36.5	6	161.0 ± 48.4
6	12	79.8 ± 27.1					9	201.0 ± 18.0	5	196.4 ± 26.1
7	12	57.5 ± 27.0					9	257.7 ± 63.9	6	178.8 ± 40.2
8	5	50.6 ± 15.6					4	214.3 ± 46.0	4	192.7 ± 32.1
9	3	36.7 ± 14.3					3	177.0 ± 38.3	-	
10	3	20.7 ± 5.5					3	171.3 ± 66.5	6	180.5 ± 26.2
11	3	25.0 ± 2.6					3	200.0 ± 7.0	3	153.7 ± 25.1
12									3	157.3 ± 19.9
13									4	157.7 ± 15.7
14									3	173.0 ± 15.6

**Fig.14:- PYRUVIC ACID LEVELS IN THE OVARIES OF IMMATURE RATS
PRIOR TO AND FOLLOWING TREATMENT WITH PREGNANT MARE
SERUM GONADOTROPHIN (PMSG) AND HUMAN CHORIONIC
GONADOTROPHIN (HCG). ESTIMATIONS BY THE ENZYMIC
METHOD.**

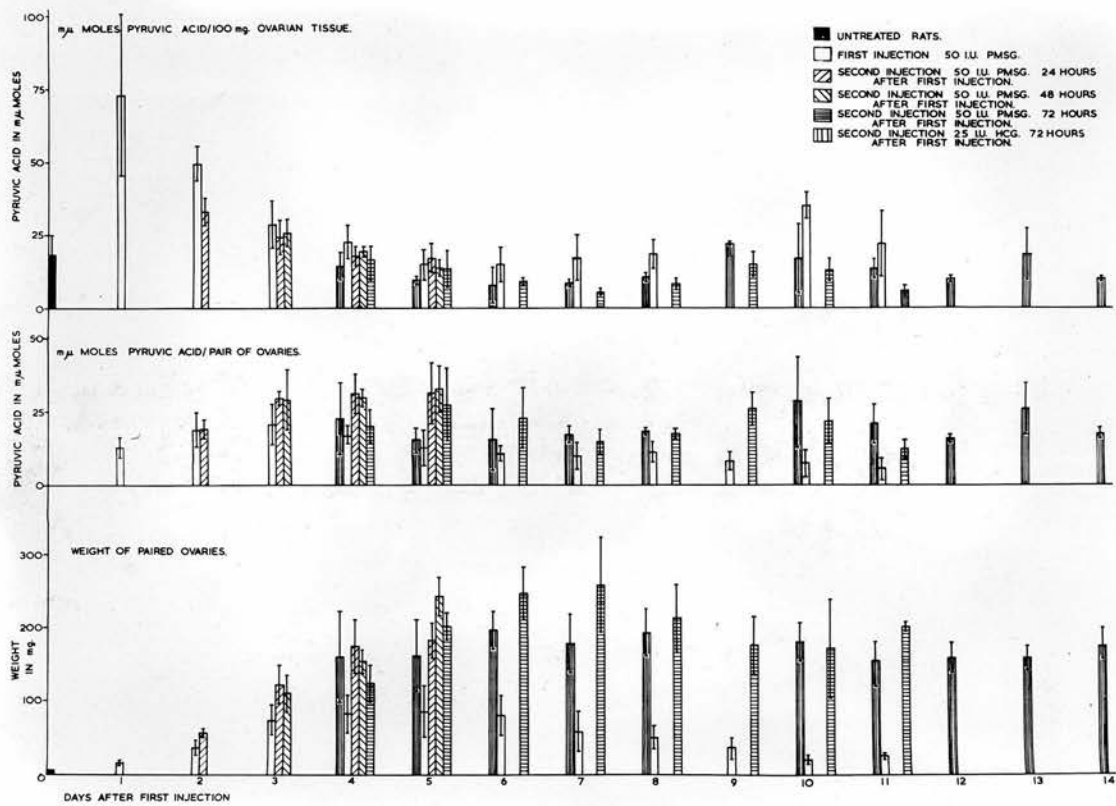


Fig.14.

Experiments 2 and 8

The results are compared in table 60. A significant 5 - fold difference in the pyruvic acid level in untreated immature ovaries was observed. It is probable that this difference in values can be discounted. For a discussion on this point see page 135.

Experiments 4 and 9.

The results of these experiments are compared in table 61. It will be seen that on the second and sixth days after the administration of PMSG, the difference in the results obtained by the two methods was significant, but on the third, fourth and fifth days thereafter the difference was quite clearly not significant. While the difference in values for the second and sixth days is statistically significant, the concentrations obtained by the DNPH method are less than twice the values obtained by the enzymic method and can probably be discounted for the following reasons :-

- a). a smaller number of estimations were made by the DNPH method, and
- b). seasonal animal differences due to the estimations by the two methods being made at different periods of the year.

Experiments 5 and 10

Only two comparisons could be made in this category, and these are shown in table 62. It will be seen that one shows a significant difference between the results obtained by the two methods, while the other does not. When the results for the third

TABLE 60

Comparison of estimations of pyruvic acid content
in the ovaries of untreated immature rats
obtained by the DNPH and enzymic methods.

Pyruvic acid concentration mu moles/ 100 mg.tissue		Degrees of Freedom	t	P	Difference
DNPH method	Enzymic method				
3.75	18.30	6	5.31	0.01 - 0.001	Significant

TABLE 61

Comparison of estimations of pyruvic acid concentration in ovarian material following the administration of 50 i.u. PMSG, obtained by the DNPH and enzymic method.

Days after PMS	Pyruvate concentration mu moles/ 100 mg. tissue		Degrees of	t	P	Difference
	DNPH method	enzymic method				
2	76.8	49.6	11	6.18	< 0.001	Significant
3	32.7	28.6	25	0.76	0.4 - 0.5	Not significant
4	24.4	22.5	15	0.66	0.5 - 0.6	Not significant
5	19.0	14.7	13	1.34	0.2 - 0.3	Not significant
6	24.6	15.0	14	2.93	0.01- 0.02	Significant

TABLE 62

Comparison of ovarian pyruvic acid content following the administration of two injections of 50 i.u. PMSG, 3 days apart, obtained by the DNPH and enzymic methods.

Days after double PMS (3 day interval)	Pyruvate concentration μ moles/100 mg. tissue		Degrees of Freedom	t	P	Difference
	DNPH method	enzymic method				
1	28.4	16.6	10	2.22	0.05 - 0.1	Not significant
3	12.8	9.2	5	3.16	0.02 - 0.05	Significant

day are considered the actual difference between the two means is seen to be quite small, but as the standard deviations obtained in this experiment were exceptionally low, the difference between the two means becomes statistically significant.

Experiments 6 and 11.

Following the administration of HCG, results were obtained on days 1, 2, 3 and 11 by the two methods. These are compared in table 63, where it can be seen that on the first and eleventh days after HCG, the results obtained by the two methods show significant differences. As in the previous experiments, it is probable that a major factor in the causation of apparently statistically significant differences between the two methods, is the low standard deviation shown by the results obtained by the enzymic method.

Comment.

It would be reasonable to say that the results obtained by the two methods are comparable. While 50% of the comparisons apparently show a significant difference in the results obtained by the two methods, at least half of these have quite low actual differences which would probably not be significant, if the group means had shown higher standard deviations. Excepting the values obtained for untreated immature ovaries, the higher value is never as much as twice the lower result. Other contributory causes are probably animal differences and the low number of replicate estimations by the DNPH method.

TABLE 63

Comparison of estimations of ovarian pyruvic acid content, following the administration of 50 i.u. PMSG and 25 i.u. HCG, obtained by the DNPH and enzymic methods.

Days after HCG	Pyruvate concentration μ moles/ 100 mg. tissue		Degrees of Freedom	t	P	Difference
	DNPH method	enzymic method				
1	24.8	14.3	9	2.83	0.02 - 0.05	Significant
2	13.6	9.5	6	1.54	0.1 - 0.2	Not significant
3	15.5	7.8	6	1.60	0.1 - 0.2	Not significant
11	12.8	10.0	4	3.73	0.02 - 0.05	Significant

DISCUSSION

The difference in the results for the pyruvic acid concentration per 100 mg. ovarian tissue obtained by the dinitrophenylhydrazine method when compared with those obtained by the enzymic method is not significant in most cases. As the two methods involved are totally different in concept and performance, this implies that the values obtained are reasonably comparable and therefore probably represent a true assessment of the pyruvic acid level.

The greatest discrepancy in the results appears in the value for immature untreated ovarian material for which the values obtained are 3.75 and 18.3 μ moles/100 mg. for the dinitrophenylhydrazine and the enzymic methods respectively. This 5-fold difference is somewhat disquieting but can probably be discounted for the following reasons:-

- 1) Only one assay was carried out on this material by the dinitrophenylhydrazine method due to the large number of animals required per assay.
- 2) The range in values 9.3 - 27.6 μ moles by the enzymic method is very wide.
- 3) Seasonal variation - estimations by the DNPH method were made in the spring and early summer, by the enzymic method in the autumn and winter months.

Similar reasons probably account for the discrepancy noted

two days after PMSG stimulation. The difference here is, however, considerably less and may also in part be accounted for by differences in ovarian weight.

Despite these differences in the values for the immature ovaries it is obvious that, initially, prior to hormonal stimulus the pyruvic acid level is relatively low. It is therefore interesting to note that, in the first 24 hours after PMSG stimulation, there is an approximately 5-fold increase in pyruvic acid concentration. This high value then rapidly falls off and about four days after PMSG administration has fallen to a level which is of the same order as the initial concentration. It is postulated that this initial rise is connected with the marked increase in ovarian growth which is caused by the administration of PMSG. However the increase in ovarian growth continues for about three days when it reaches a plateau and then progressively decreases from the seventh day onwards, while a continued rise in pyruvic acid was not seen. It was then thought that perhaps the rise in pyruvic acid concentration only occurred during the initial phase of a growth spurt and so attempts were made to increase the pyruvic acid concentration by further increases in ovarian weight obtained by either the administration of a second injection of PMSG or of HCG.

It was, however, found that in the first few days following the administration of a second dose of PMSG, whether the second injection was given one, two or three days after the first, there

was no significant difference in the ovarian pyruvic acid concentration between animals which had received one or two doses of PMSG. It was noted that following the administration of HCG that there was a further significant depletion of ovarian pyruvic acid. It would appear therefore that renewing the ovarian growth is not a cause of ovarian pyruvate build up, but, rather of pyruvate depletion.

One finding noted in the laboratory, which may have some bearing on this problem is that the ovary and uterus of untreated immature rats are usually deeply imbedded in fat. Following the administration of PMSG and during the consequent ovarian growth period, this fat disappears and by the third to fourth day after PMSG the ovary is completely free of surrounding fat. It is known that during periods of fasting free fatty acids form the main metabolic fuel of the heart (Olson, 1962), and Garland, Newsholme and Randle (1962) have shown in in vitro experiments that the addition of free fatty acids to the medium diminishes the uptake and oxidation of pyruvate by rat heart and diaphragm muscle. It is therefore reasonable to suggest that the administration of PMSG with consequent high ovarian activity stimulates in some way the mobilisation of the surrounding fat depot and that, the pyruvate build up noted is in fact the consequence of pyruvate sparing, due to the use of the surrounding fat as the preferred metabolic fuel. The plateaued level noted three to four days after PMSG administration thus reflects a basic turnover level reached when the surrounding fat

depots have been exhausted. The even lower level noted following the administration of HCG probably reflects an even higher turnover rate in pyruvate consequent to the increased metabolic rate which occurs during the ovarian luteinising process following HCG.

SUMMARY

1. Two methods for the estimation of pyruvic acid have been established. One is dependant on the formation of a coloured hydrazone, the other on the enzymic conversion of pyruvic acid to lactic acid.
2. Both methods have been used to estimate the pyruvic acid content of ovarian material from immature rats, both prior to and following treatment with PMSG and HCG.
3. The results obtained by the two methods have been compared.

CHAPTER V

DISCUSSION

It is over thirty years since Evans and Simpson (1931) reported the first quantitative method for the assay of pituitary growth hormone. In the succeeding decades many workers have tried to develop more sensitive methods of assay and as purer hormone preparations became available, the specificity of the methods has been studied. Over the last ten years, sensitive and specific immunological techniques have been applied to the assay of growth hormone. Initially, it was not known whether the biologically active growth promoting principle was the same as the immunologically active material, and few investigators have reported the results of comparative assays. The growth hormone content of serum could not be measured by both the tibial test and by immunological methods, because of the relative lack of sensitivity of the former, however, comparative estimates of potency of standard growth hormone preparations have been made by the two methods. In the present study the results of such a comparison are reported (see Chapters 2 and 3).

In the tibial section (Chapter 2), the potencies of a large number of growth hormone preparations of pituitary origin were measured by this method, and in Chapter 3, the potencies of one bovine and one human growth hormone preparation were again estimated, but this time by the haemagglutination-inhibition assay. It was shown that there was reasonable agreement between the two

estimates of potency for these preparations, the indices of discrimination were 1.32 and 0.82 for the BGH and the HGH preparations respectively. It is interesting to note the differences between the two methods. The response in the tibial test is a graded one and is characterised by having a relatively low slope and high standard deviation, while the response of the haemagglutination-inhibition assay is of an all-or-none type and consequently has a very steep dose response curve. These differences are highlighted by statistical analysis, which indicates that the tibial test is a relatively imprecise method having high lambda figures and very wide fiducial limits, while the immunological technique is very precise with low lambda figures and narrow fiducial limits.

In this study, it has been shown that the index of discrimination for the two assay methods is approximately unity. Therefore, it would appear that the biological and immunological activity of growth hormone as measured by the tibial test and the haemagglutination-inhibition were respectively contained in different chemical groupings of the same molecule and that the two methods were measuring the same substance. This study has also shown that from a statistical viewpoint, the haemagglutination - inhibition method is a more precise assay system for the quantitative assay of purified growth hormone.

Recently, there has been considerable controversy as to whether the haemagglutination-inhibition method for growth hormone

determination as described by Read (1960 ab), is in fact a suitable method for the measurement of the hormone in body fluids. The present investigation has shown that the procedure meets the requirements of practicability as suggested by Borth (1957) in that it is cheap, rapid and requires little skill of the operator. There is also extremely good agreement between replicate estimations (see Chapter 3). Providing that the necessary steps to remove interfering substances are taken, the method appears to be specific and other anterior pituitary hormones do not interfere with the results obtained (see Chapter 3). The high degree of specificity shown by the immunological method is in marked contrast to the lack of specificity shown by the tibial test and the method depending on the increase in total body weight in rats (see Chapter 2).

The immunological technique has other advantages over the bioassay methods in its greater sensitivity and its extensive dose response curve. While, its ultimate limit of sensitivity appears to be about 10 $\mu\text{g.}/\text{tube}$, preliminary scanning experiments can cover a range of up to 1 $\text{mg.}/\text{tube}$. The method is therefore much more sensitive than the tibial test for which the dose response curve extends over the range 5 - 250 $\mu\text{g.}/\text{rat}$. The two-fold form of the haemagglutination-inhibition assay (see Chapter 3, table 24), with its extensive preliminary scanning experiment followed by the narrow range accurate assay made this a very useful tool for estimating the growth hormone content of a purified preparation whose approximate potency was completely unknown. Such preparations

are extremely difficult to measure by the tibial test as valid estimates can only be made when approximately the same effective doses of the standard and the unknown preparations are administered i. e. ideally the final relative potency of the assay should be approximately unity, the amount of the unknown used being adjusted to this end.

As was to be expected, the agreement between replicates using the immunological assay was excellent (see Chapter 3, tables 26 - 28), and considering the inherent inaccuracies of the tibial test agreement between replicates here was also good (see Chapter 2, tables 11 and 12). Despite the apparent advantages of both these methods it is therefore most unfortunate that neither of these assays is sufficiently sensitive to be used for the estimation of growth hormone in human body fluids. Provided that the haemagglutination-inhibition assay is correctly set up, it has been shown in Chapter 3 that the growth hormone content of human serum cannot be measured and is therefore less than 10 $\mu\text{g./ml.}$ an estimate which is in agreement with the more recent findings of Glick et al. (1963) and Hunter and Greenwood (1964) who have used radio-immunological assay techniques. However, it is worthy of note that even with the more sensitive method, growth hormone levels are frequently below the limit of sensitivity in normal subjects.

Attempts to modify the Ortho Pregnancy Diagnosis Kit for quantitative clinical use were also unsuccessful. Several workers

(for references see Chapter 1) have shown it to be an excellent method for the diagnosis of pregnancy. It is probable that this is due to the fact that as a qualitative test it has been weighted to give a positive reaction only when very high concentrations of HCG are present, and so interference by non-specific agents is relatively unimportant. However, when attempts have been made to quantitate this method, the presence of these non-specific substances becomes increasingly important. It is still a matter of controversy as to what is the precise form of these non-specific agents. It has been suggested that as the endpoint measured was the percentage of light transmitted through the test sample, that this interference is due to the presence of some non-specific light absorbant substance. Since the test samples were urines (which naturally contain substantial amounts of chromagens and non-specific absorbants), this appeared to be a reasonable hypothesis. If, however, this is the explanation, then the use of the same urine as the blank should compensate for, and thus neutralise any such absorbant effects in the test sample. Even though this was the procedure used here, interference was still noted. The work presented here, has thus shown that the presence of chromagens in the test samples was not the major cause of interference, although it may yet prove to be a minor contributory factor. Although, all samples were brought to a pH of 8.0 - 8.5 (the near optimal pH figure quoted by Singer and Plotz, 1956) before use, interference still occurred, and so the work presented here (see Chapter 3.) has shown that pH was not a

major cause of interference, a finding which confirms Goldin's observation (1962) that urine pH was not a critical factor during pregnancy diagnosis tests. It is probable that the specific gravity of the sample plays a relatively important part; some workers (Goldin, 1962; Henry and Little, 1962) have observed either inconclusive or false results when urines with a specific gravity of less than 1.015 were tested. If the specific gravity had proved to be the most important cause of interference, this could have been overcome by concentrating the sample to some extent. However, it now seems certain that the most important factor is the ionic concentration of the sample. Singer and Plotz (1956) noted that there was marked spontaneous agglutination of latex particles when the electrolyte concentration was less than 0.3% and greater than 1.25% sodium chloride, and advised suspending particles in a buffer containing 3.85% NaCl. It would, however, be a somewhat complex procedure to stabilise the electrolyte concentration of urine at the optimum level, and for this reason attempts to develop a quantitative direct assay for HCG in urine using a modified form of the latex pregnancy diagnosis test do not seem to be worthwhile. It is interesting to note that Goss and Taymor (1962) claimed satisfactory results using urine which was first extracted by the kaolin-acetone method of Albert (1955) prior to resuspension of the extract in the buffered saline.

Although the two immunological methods examined here have proved to be unsuitable for clinical use, they are both of value

and complementary to the bioassay methods for growth hormone and HCG, when estimates of the potency of standards or pituitary extracts are required. Read's haemagglutination-inhibition method for the assay of growth hormone could easily be used instead of the tibial test in laboratories which require only occasional estimates of the potency of purified growth hormone preparations, either for the purpose of checking the potency of standards or for the standardisation of pituitary extracts. The only special requirements for this assay are a high titre antiserum (which can be readily prepared and stored until required at - 20°C.) and a source of sheep or human red cells (both of which are readily available.) The method is much cheaper and more practicable than is the tibial test in which large numbers of hypophysectomised weanling rats are required. It is also easier to perform replicate estimations of potency by the immunological method. As hypophysectomised animals are not required for the bioassay of HCG and the sensitivity of the two methods is approximately the same, there is no great advantage in using the immunological methods for quantitative work except in laboratories which have no animal house and only require occasional estimations. It can, however, be used in a supplementary form to confirm the potency of new standards in places which habitually use bioassay methods.

Although, the relatively simple immunological assays of the haemagglutination-inhibition and latex particle agglutination-inhibition types had initially shown great promise, the data presented

(Chapter 3) has shown that they are not suitable for clinical work. With this in mind, and following the successful development of the ovarian cholesterol depletion assay for LH (Bell et al., 1964), attention was turned towards the measurement of ovarian pyruvic acid. (See Chapter 4.)

Little is known about the level of pyruvic acid in the ovary. Neish (1953) has shown the presence of fairly high concentrations of pyruvic acid in rat blood and brain tissue. He also measured the pyruvic acid concentration in tissue from the lungs, liver, spleen and kidney, but did not investigate the gonads. Pyruvate is known to be an important general metabolic precursor and it has been shown to be a precursor of mevalonic acid and hence of cholesterol (Cornforth, 1960). Methods of estimation were known to be reasonably sensitive, specific and practicable. However, as was pointed out by Neish (1957) although pyruvic acid is the most stable of the α -keto acids found in animal tissues, it is advisable to carry out estimations as rapidly as possible to avoid post-mortem autolysis.

The results presented here (Chapter 4) proved to be very interesting. As was to be expected, the level in immature untreated ovarian material was very low. Estimates were made by two methods and the values 3.75 μ moles/100 mg. (DNPH method) and 18.3 μ moles/100 mg. (enzymic method) which are equivalent to 0.33 and 1.61 mg/100 g. respectively are of the same order as the values quoted by Neish (1953) e.g. 0.16 - 1.21 mg./100 g. for lung

tissue. Following the gonadotrophic stimulation consequent to the administration of PMSG there was a marked rise in pyruvic acid concentration which then fell off rapidly. There was no further increase in pyruvic acid concentration when ovarian growth was further stimulated by the administration of a second dose of PMSG or HCG; in fact a relative depletion of pyruvic acid was observed. It seems that the most likely explanation of this curious pattern of pyruvic acid response to gonadotrophic stimulation lies outwith the ovary itself.

It was observed, during the course of these experiments and by other workers in the laboratory in Edinburgh, that initially the immature ovary was surrounded by a mass of fat, which was difficult to remove, but following the administration of PMSG and HCG, as the ovary grew, this fat depot was used up and had disappeared 2 - 3 days after the administration of HCG, incidentally making the ovary much easier to clean. It is suggested therefore, that the pyruvic acid build up occurs in the ovary during the initial growth period following a single injection of PMSG, because the surrounding fat was being preferentially metabolised for energy requirements and that when this was exhausted, pyruvic acid was then used as a precursor thus causing the falling off in pyruvic acid concentration noted.

It seems unlikely that the initial rise in pyruvic acid concentration observed here was due to either a simple growth effect or to the development of follicles in the ovary. If this was the

explanation one would expect the increase to be either further enhanced or at least maintained following the administration of a second dose of PMSG. The marked depletion which does in fact occur can however, be explained by the fat mobilisation theory. Ovarian growth is very rapid following the administration of either HCG to the PMSG primed ovary or of a second dose of PMSG. Thus the fat depot would be exhausted even more rapidly and the pyruvic acid called on as a precursor earlier, thus depleting the reserves.

An alternative explanation of the marked depletion of pyruvic acid which occurs may lie in the observations of Szarka, Mayer and Evans (1933). Pyruvic acid is known to be metabolised to lactic acid and these workers noted that in their in vitro experiments ovarian material from rats which had been treated with PMSG at least 4 days before, converted 1 molecule of oxygen to 2.55 molecules of lactic acid, while untreated animals showed a conversion rate of only 1 : 1.36, although the total oxygen consumption by material from both groups, was virtually the same. It is thus possible that the marked fall in pyruvic acid levels (from the third day onwards after PMSG administration) observed in the experiments presented herein (Chapter 4) reflects an increased rate of conversion of pyruvic acid to lactic acid.

Even the approximately six-fold rise in pyruvic acid (to about 6.5 $\mu\text{g}/100 \text{ mg.}$), which followed the administration of a single injection of PMSG, did not raise the ovarian pyruvate concentration to the level of cholesterol (up to 1000 $\mu\text{g}/100 \text{ mg.}$, Bell et al., 1964)

seen in the ovary. This implies that while pyruvic acid is a precursor of cholesterol, its turnover rate is so rapid that none is stored by the ovary. The basic ovarian pyruvic acid levels are so low and difficult to measure that it would not be possible to detect any further gonadotrophic effect and for this reason no attempt was made to develop the findings obtained as a possible assay method for either FSH or LH. However, the data presented here provides further information about the effect of the gonadotrophic hormones on intermediary metabolism in the ovary.

Although bioassays are intended to give quantitative information about the substances they are measuring, they can sometimes be usefully adapted to supply qualitative information. The data presented here (see Chapter 2) has shown that the tibial test can be used in this way. It has been shown that in the hypophysectomised rat, the alteration in the width of the proximal epiphysis of the tibia known as the tibial test is simply a relatively sensitive but non-specific index of the growth of the animal. Using this qualitative form of the tibial test, the growth promoting or inhibiting properties of new compounds have been investigated. The dithiocarbamoylhydrazine derivative studied herecin has been shown to have a marked growth inhibitory action, while this may well be a phenomenon of the dosage used, it could be a reflection of the recently proved general toxicity of the compound (Brown, 1963). Similarly the lack of effect of the ACTH-like synthetic polypeptide could be a dosage effect, although it might reflect a real loss of

biological activity. A clinical problem (muscular atrophy) was also investigated using the tibial test in this way. The work presented here has shown that surgically induced muscular atrophy of the gastrocnemius muscle does not affect cartilage growth and that treatment of these pituitary intact animals with substantial doses of the anterior pituitary hormones did not modify cartilage growth although it had some effect on the atrophied muscle. It would appear, therefore, that lameness due to muscular disease is not accompanied by a shortening of the bone. The lack of effect of administered anterior pituitary hormones on bone growth in these animals was surprising, but growth effects may have been masked by the use of pituitary intact rats rather than the hypophysectomised animal of the standard growth hormone tibial assay.

Thus the work presented here has shown that even though new assays have been developed and old ones modified, there is still a need for more sensitive procedures which can be applied to clinical problems and that attempts to study the physiology of the hormones involved remain an important field for future investigation.

SUMMARY

1. The Tibial Test for growth hormone has been established; its conditions of assay and the specificity of the method have been investigated.
2. The tibial test has been applied to the estimation of the potency of a number of pituitary growth hormone preparations from different animal species and to an assessment of the effects on cartilage growth of two synthetic compounds and of artificially induced muscular atrophy.
3. Antisera have been raised to bovine and human growth hormone and to human chorionic gonadotrophin, and the specificity of the antisera examined.
4. The Haemagglutination-Inhibition method for the determination of both bovine and human growth hormone was established; the conditions of assay and the specificity of the method have been examined.
5. The haemagglutination-inhibition method has been applied to the estimation of the potency of a bovine and a human pituitary growth hormone preparation and of four human pituitary fractions. It has also been applied to the estimation of the growth hormone content of sera from normal human subjects.
6. The Ortho Pregnancy Diagnosis Kit has been modified to provide a quantitative method for the estimation of purified HCG preparations.
7. This latex particle agglutination-inhibition method has been

- applied to the quantitative estimation of HCG in urine.
8. Two methods for the estimation of pyruvic acid, one dependant on the formation of a coloured hydrazone, the other on the enzymic conversion of pyruvic acid to lactic acid, have been established.
 9. Both these methods have been applied to the determination of the pyruvic acid content of ovarian material from immature rats, both prior to and following the administration of PMSG and HCG.
 10. The results obtained have been discussed.

APPENDIX

STATISTICAL METHODS

All the assay procedures which depended on a graded or a measured effect (i. e. tibial assays, latex agglutination-inhibition assays and the estimations of pyruvate by the dinitrophenylhydrazine method) were of a three-point, four-point or six-point design as described by Gaddum (1953a). The assay procedures dependant on an all - or - none or quantal response (i. e. haemagglutination-inhibition assays) were of a four-point or six-point design as described by Gaddum (1933, 1953a). The calculations of the relative potency, the fiducial limits of error and the index of precision for both types of assay have been carried out according to the simplified formulae of Gaddum (1953b).

One of the most commonly used methods for estimating the precision of bioassay procedures is the calculation of the index of precision (λ), which provides an estimate of the standard deviation of the logarithms of the individual effective doses. For assays dependant on measured effects this is calculated from the formula:-

$$\lambda = \frac{s}{b}$$

where s is the mean standard deviation of the responses, and b is the slope of the dose response curve.

In quantal assays where the response is submitted to probit transformation before calculation, and b , while, still the slope of the dose response curve, has a somewhat different meaning, the

index of precision is calculated from the formula :-

$$\lambda = \frac{1}{b}.$$

The precision of a given assay is considered to be high when s is small and b is large, i. e. the lower the figure for λ the more precise the assay. In practice, an assay is considered to be very precise when the index of precision is less than 0.2, while an assay with a λ figure greater than 0.2 is described as having a low precision.

The validity of the four-point and six-point assays was also tested according to the methods of Gaddum (1953b). An assay was considered to be valid when no significant difference between the slopes of the log dose-effect curves of the standard and unknown preparations could be demonstrated. While, an absence of parallelism between the slopes of the standard and the unknown can be taken as a positive indication of lack of identity between the standard and test preparations, the presence of parallelism between the two slopes cannot be taken as irrefutable evidence that the two preparations are identical.

The statistical method of Gaddum (1953b) was also used to determine the linearity of the log dose response curves of the standard and the unknown preparations in six-point assays. Ideally the response observed for the middle dose should lie midway between the responses observed for the high and low doses. A significant deviation from the straight line indicates that the response measured

is not being altered in a regular manner and invalidates the results obtained.

The fiducial limits or 95% confidence limits as they are sometimes called, are the limits within which the potency may be expected to lie in 95% of assays. Where there is a good overlap between the fiducial limits of separate assays using the same standard, it is reasonable to assume a common identity for the unknown preparations. Where there is no overlap in the confidence limits, it can be considered that there is a qualitative dissimilarity between the preparations. Fiducial limits can also be used to compare different assay methods. Where comparable relative potencies with overlapping confidence limits are obtained from different assay methods for the same preparation, it can be argued that the methods are measuring the same substance.

In some experiments (tibial tests and pyruvate estimations) the standard deviation of a response was calculated according to the method of Gaddum (1953b). For the tibial test where the standard deviations are large, it has been the convention to express the results as the mean \pm the standard error of the mean. The standard error of the mean is in fact the square root of the variance (or standard deviation squared) divided by the number of observations; this was also calculated according to the method of Gaddum (1953b).

When comparisons of results were required, the student 't' test as described by Gaddum (1953b) was used. This test indicates

whether the discrepancy between two sets of results can be accounted for by the errors of the test, and is used to ascertain the significance of the difference between the means. It is an estimate of the variance of the difference of the means; the significance of this value is determined by consulting a table of 't' (Fisher and Yates, 1957).

The Index of Discrimination was used to compare the results obtained by the tibial test and the haemagglutination-inhibition assay for growth hormone. It is the ratio of the results obtained by the two methods and was calculated according to the method of Gaddum (1955) i. e.

$$\text{Index of Discrimination} = \frac{\text{Relative potency by tibial test}}{\text{Relative potency by immuno-assay}}$$

When the index of discrimination is unity, it is assumed that the active principle measured by the two tests is identical. If, however, the value is widely divergent from unity then a qualitative difference between the two assays is thought to exist.

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