ABSTRACT OF THESIS

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Title of Thesis EARLY EMBRYOLOGY OF THE MOUSE AS STUDIED BY TRANSPLANTATION OF OVA.

Hormonal induction of oestrus and ovulation (superovulation) in the sexually immature mouse was studied with the principal purpose of determining the viability and developmental capacity of the superovulated eggs. The observation of responses (and factors influencing them) of prepuberal female mice to the gonadotropic treatment employed supplemented the main investigation.

Response to the gonadotropic treatment. A relationship between prepuberal age at treatment and the number of Graafian follicles ruptured was demonstrated (and confirmed in later work) in some of the stocks of mice tested. Mice three weeks old at the time of initial treatment yielded the highest numbers of ova (maximum obtained in this study from one mouse, 98 ova). However, genetic, nutritional and other environmental factors appear to influence the relationship between age and number of ova. The proportion of three- to four-week old sexually immature females which mated was observed in one outbred stock of mice to be positively correlated with age at treatment. Time of onset of follicular rupture (which was shown to be independent of diurnal regulation) was found to be so uniform among mice of the hybrid stock used that both the rate and the duration of superovulation could be estimated.

Viability and developmental capacity of the eggs. The percentage of superovulated eggs (from prepuberal mice) which underwent normal maturation, penetration by spermatozoa and formation of pronuclei was found to be as high as that among eggs from mature, untreated mice. Both the viability and stage of development attained by superovulated eggs at 3 1/2 days post coitum (p.c.), i.e., shortly before uterine implantation, indicated that the treatment of immature mice with gonadotropins had resulted in ovulation of qualitatively normal ova. However, an inverse

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relationship existed between the number of eggs superovulated and their subsequent rate of survival as well as rate of cleavage up to 3 1/2 days p.c. Despite this reciprocal relationship, the absolute numbers of normally developing blastocysts increased proportionally to the yield of eggs (no increase when fertilization was experimentally delayed by more than 10 hours). Transplantation of eggs to mature hosts enabled the demonstration that normally cleaved 3 1/2-day eggs from mature and treated immature mice were comparable in viability and in capacity for normal embryonic growth up to 18 1/2 days of gestation (i.e., one day before birth). The efficacy of transplantation of eggs in the mouse has been discussed in the light of results obtained following use of the technique.

It is concluded from the findings presented that superovulation of the sexually immature mouse can be highly recommended as an experimental approach to a wide variety of biological research problems.

EARLY EMBRYOLOGY OF THE MOUSE AS STUDIED BY TRANSPLANTATION OF OVA

By

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Submitted to the
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To

my uncle

PROF. WILLIAM H. GATES, SC.D.

PREFACE

Not so very many years ago a geneticist might have felt secure in his belief that the egg came first -- the organism later. In the present generation, however, researches in physiology of reproduction are able to shed new light on the age-old question by demonstrating some of the roles which the maternal soma plays in determining ontogenetic and even phylogenetic development.

The studies reported here deal with the ovum of the sexually immature mouse. Inasmuch as ovulation has been induced experimentally, the investigation, of necessity, touches on aspects of the physiology of the prepuberal mouse which regulate her response to the hormonal treatments used. The developmental fitness of artificially ovulated eggs is demonstrated by several procedures, including transplantation to mature hosts. As a consequence of the findings, new experimental approaches are made available to biological research.

The author's sincere gratitude is expressed to Professor C. H. Waddington for his supervision of this investigation and for having made available the research facilities of the Institute of Animal Genetics, University of Edinburgh. For financial support during the initial period of study, many thanks go to the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and to Dr. C. C. Little for his efforts in procuring the necessary fellowship funds. Financial assistance for one year of research was gratefully received in the

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Dr. R. A. Beatty's supervision, encouragement and very helpful advice throughout this investigation has been deeply appreciated. The author is also indebted to Drs. M. N. Runner, A. W. H. Braden and R. G. Edwards for their constructive criticism of the work of this thesis. For statistical advice, thanks are due to Drs. B. Woolf, A. K. Robertson and C. K. Chai. The foundation stocks and all hybrid mice used in these studies were kindly supplied by Drs. D. S. Falconer and T. C. Carter, respectively.

Appreciation for technical assistance is expressed to the following: J. Isaacson and his staff, for care of the mice used; D. Roberts and R. Soper, for art work; D. Pinkney, his assistants, and G. McKay, for photography; J. Armstrong, for histology; and Marjorie Higgins, for typing of the thesis.

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CHAPTER I

GENERAL INTRODUCTION

Hormonal Induction of Ovulation and Oestrus in Laboratory Animals

The induction of ovulation and/or oestrus by hormone treatment, although first applied in the mouse more than 30 years ago, has scarcely begun to be exploited in laboratory animals despite its potential usefulness as a research tool. Early use of the technique in the laboratory, primarily on rabbits, was devoted to the study of fundamental problems in application of the method to agricultural breeding programs. Now, hormonal control over reproductive processes is widely and successfully employed in farm animals in conjunction with artificial insemination, in pioneer work on transplantation of eggs, and in therapeutic treatment of non-breeders (these uses having been reviewed for work on the cow, by Lamming and Rowson, 1952 and by Willett, 1953; the sheep, by Robinson, 1951; and the sow, by Spalding et al., 1955). However, the application to laboratory animals of hormonally induced oestrus as a tool in biological research has received little attention relative to the prospective value of such a procedure.

Artificially induced breeding in laboratory animals is applicable to a variety of experimental uses. Under the appropriate regimen of gonadotropin injections, the laboratory animal may be induced to

superovulate[1], i.e., to ovulate more ova than would normally be shed in the absence of treatment. Superovulation is an excellent source of ova for much-needed descriptive studies (histochemical, cytological and physiological) on preimplantation stages of mammalian development. Similarly, superovulation can provide large numbers of ova for experimental treatments such as are used in the induction of heteroploidy and parthenogenesis (Beatty, 1957). When applied to the production of animals in the laboratory, induction of oestrus permits the implantation and possibly the birth of excessive numbers of young. Also, by hormonal treatment and mating of sexually immature animals, the generation interval can be shortened (e.g., to as little as 34-39 days in the mouse, as shown by Adams, 1954). The ability to increase litter size and to shorten the generation interval could be of considerable importance in genetic testing programs where increased production would mean more rapid accumulation of genetic ratios. Treatment with gonadotropins has already been successfully applied to large-scale production of female animals for assays (Gates, unpublished), large litters from superovulated mice having been reduced to normal size by the removal of males at birth. For studies on embryology and physiology of reproduction, treatment with gonadotropins affords a means of making large numbers of animals in oestrus available at any

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[1]The verb "to superovulate", in addition to its original connotation, "to ovulate large numbers of ova, following hormone treatment", has come through common usage to mean also "to induce superovulation". Accordingly phrases like "mice were superovulated" and "the technique of superovulation" are used throughout this text.

given time. By this means, problems involved in ovulation, egg transport, fertilization and fertility of females may be studied under controlled conditions. Thus, at least three important uses of hormonally controlled oestrus in the laboratory animal are: (1) an abundant source of mammalian ova for experimental studies, (2) a technique applicable to increased production of animals and (3) a means of studying basic problems in embryology and physiology of reproduction.

Among the laboratory animals, the mouse is well-suited to hormonally induced oestrus for the uses cited above. Since there is relatively little variation in diameter between the ova of eutherian mammals (Parkes, 1952), the mouse ovum, in terms of size, is suitable for descriptive studies of the mammalian egg. In view of the wealth of knowledge about its genetic constitution, the mouse is especially useful for studies on the genetic effects of heteroploidy after its experimental induction in superovulated ova. Recent successes in the culture of mouse ova (Whitten, 1956) now make practicable the treatment of mouse ova <u>in vitro</u>. After culture and treatment, mouse ova may be transplanted to mature hosts for gestation; resistance of the mouse to infection is high, favouring the relatively easy method of transplantation of eggs by means of laparotomy. In addition to these factors, economy and rapid breeding make the mouse an excellent experimental animal for the application of hormonally controlled oestrus to the study of problems in mammalian embryology or reproduction.

The sexually immature mouse offers several unique advantages over the adult in the induction of oestrus. Higher egg yields after superovulation are usually obtained from immature mice (Fowler and Edwards, 1957, for the mature mouse; and Gates and Runner, 1957, for the immature mouse). Since the prepuberal mouse does not secrete appreciable amounts of gonadotropin, there is little competition between administered and intrinsic hormones as could be the case with adult animals (Gates and Edwards, in preparation). When it is desired to have the combined advantages of supernumerary ovulations and pregnancy, progesterone therapy permits gestation in the immature mouse (Smithberg and Runner, 1956). Finally, the greater availability of immature mice is of considerable advantage over the use of mature females in the application of superovulation to research problems.

Aims of the Investigation

In view of the apparent advantages of the prospective use of superovulation in the sexually immature mouse for biological research, it was considered worthwhile to study those aspects of the technique about which present knowledge is inadequate. The main phases of the work are covered in Chapters II through V, references to pertinent literature being presented in the introduction to each chapter. In Chapter II, consideration is given to some of the factors influencing the numbers of eggs superovulated and the percentage of treated females which mate. The ability to induce ovulation of excessive

numbers of eggs with regularity is of considerable advantage when superovulation is used as a source of fertilized or unfertilized ova for experimentation. Chapter III is a study of the accuracy with which induced ovulations may be timed -- knowledge which is of value in experiments requiring study, treatment, or fertilization of eggs at a known time before or after ovulation. Data are presented in Chapter IV which indicate the rate of survival and the development reached by eggs from superovulated immature mice during early stages of fertilization and just prior to implantation. The viability and the capacity of superovulated eggs for normal growth during embryonic and foetal development is assessed in Chapter V by the method of transplantation to a mature host. The information presented in Chapters IV and V should be of value in determining the suitability of the technique for use as a source of normally developing ova, embryos or live young. As a consequence of the experimental results presented in this investigation, it has been possible to evaluate (in Chapter VI) the efficacy of utilizing superovulation in the immature mouse as a technique in approaching specific research problems.

History of the Induction of Oestrus in Immature Rodents

The development of refined methods for induction of oestrus and superovulation in the immature rodent began with the publication of important works by Zondek and Ascheim (1927) and Smith and Engle (1927). Both groups of workers demonstrated that sexual precociousness could be induced in prepuberal mice by treatment with anterior

hypophyseal tissue. Smith and Engle further reported that superovulation invariably occurred in mice treated while they were still sexually immature. Leonard and Smith (1933) induced superovulation in hypophysectomized rats with a 5-day series of injections of postmenopausal urine, containing a follicle-stimulating hormone (FSH) principle, followed by Chorionic Gonadotropin (CG). Superovulation, mating, pregnancy and parturition were obtained in prepuberal rats with a single injection of an FSH-containing hormone by Cole (1936, 1937, and 1940), using Pregnant Mares' Serum (PMS), and by Evans and Simpson (1940), using sheep pituitary extract. Rowlands (1944), employing Leonard and Smith's procedure of a priming hormone followed by an ovulatory hormone, was able to induce superovulation in 100% of immature rats by treatment with PMS, followed 56 hours later by CG. Rowlands' refined procedure (but with a 48-hour interval) was also found by Runner and Gates (1954a) to induce ovulation accompanied by mating in about 90% of 5- to 6-week old mice and is basically the method used throughout the following studies for superovulation of the sexually immature mouse.

CHAPTER II

OPTIMAL CONDITIONS FOR SUPEROVULATION AND THE INDUCTION OF OESTRUS IN THE IMMATURE MOUSE

INTRODUCTION

The first account of superovulation in the mouse by Smith and Engle (1927) states that as many as 48 eggs were found in a single Fallopian tube of an immature mouse. However, subsequent reports have indicated that similar high yields of eggs have not been obtainable consistently from the immature mouse or rat.

Relatively little is known about the factors which determine the number of Graafian follicles matured and ruptured. Rowlands (1944) found that in the immature rat treated with Pregnant Mares' Serum (PMS) followed by Chorionic Gonadotropin, the number of eggs ovulated could be varied by the PMS dose. However, the optimal dose of PMS resulted in a mean number of only 25 eggs superovulated. After the work presented in this chapter had been completed, Bodemer and Rumery (1957) reported in an abstract that their work on the immature golden hamster had provided evidence indicating that both age and the amount of PMS injected influenced the numbers of eggs ovulated.

The methods of superovulation developed for the immature rat and mouse by Rowlands (1944) and by Runner and Gates (1954a), respectively, while capable of evoking ovulation in nearly all treated females, have yielded variable results with regard to both the numbers

of eggs superovulated and the percentage of females showing an oestrus response. Austin (1950) reported that among treated immature female rats which had ovulated a high number of eggs (mean 37.2), the percentage of matings was low (34%). In contrast, Runner and Gates (1954a) treated 5- to 6-week old mice with the result that a high proportion had mated (88%) but had ovulated very few eggs (mean, 5.8). For the practical application of the technique of superovulation in the laboratory rodent, it is desirable that any factors which influence either (or both) the egg yield and the proportion of females mating, be known and controllable.

The objective of the work in this chapter is to analyze the influence of age, weight and precocity on the responsiveness of prepuberal mice to gonadotropic hormones, in terms of numbers of eggs superovulated and receptivity to a male.

MATERIALS AND METHODS

Female mice used for the work in this, and the succeeding chapters, were drawn partly from the P, Q, and R stocks, maintained by Dr. T. C. Carter and referred to generally in the text as the "hybrid" stocks. The majority of females used were of the R stock, a cross between PCT females[1] and F_1 (C_3H X strain 101) males. The

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[1]The PCT stock is derived from a colony bred stock named "T" sent to T. C. Carter in 1953 by W. L. Russell. The stock is homozygous for the 7 recessive genes, aa, bb, $c^{ch}c^{ch}$, dd, sese, ss, and pp.

P stock is derived from PCX females[2] crossed to CBA strain males. Stock Q results from matings of PCX females and KL strain males, the latter an inbred line maintained by Dr. D. S. Falconer.

The remaining females used in the work of the thesis were from an outbred stock. These mice originated from a colony designated as the J stock and maintained by Dr. D. S. Falconer. The stock contains the recessive genes a, b, c, m and si; a few J stock mice bear the genes se and d. When matings were required, fertile males of the inbred A strain were utilized.

The gonadotropic hormones used were the Organon products[3] "Gestyl", or Pregnant Mares' Serum (PMS), and "Pregnyl", or Chorionic Gonadotropin (CG). Both hormones were dissolved in physiological saline and injected intraperitoneally in a volume of 0.5 ml. per mouse.

Preliminary experiments were conducted to establish the optimum conditions of hormone treatment for the induction of oestrus and superovulation in the stocks of mice used throughout these studies. The PMS-CG interval of 56 hours reported by Rowlands (1944) to be optimal for superovulation in the rat was found to be excessive and resulted in sporadic ovulation, some of which occurred independently of the CG injection. However, a single dose of two international units (i.u.) of PMS, followed 36 hours later by 2 i.u.

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[2]The PCX stock is a sextuply recessive, colony bred stock developed by T. C. Carter, M. F. Lyon and R. J. S. Phillips.

[3]Acknowledgement is due to Organon Laboratories Ltd., London, for donating initial supplies of the hormones used.

CG, was found to result in the ovulation of high numbers of eggs, a reasonable percentage of matings and accurate control of the time of ovulation (see Chapter III).

Unless otherwise stated, estimates of the numbers of eggs superovulated were based on the numbers of eggs recovered from the Fallopian tubes (in physiological saline, 0.85%). Up to 12 hours after ovulation, the ova are located in the enlarged first loop, or ampulla of the Fallopian tubes. At this stage, the eggs are attached to one another by their surrounding cumulus cells, thus facilitating their recovery en masse.

In experiments requiring matings, females were allowed to mate, one or two per fertile male, between 9 and 14 hours after CG.

DESCRIPTION AND ANALYSIS OF RESULTS

Number of Eggs Superovulated with Respect to Age

Hybrid stocks. The possibility of a relationship between number of eggs shed and initial age and weight was studied in 117 superovulated P, Q, and R stock females. Only females with imperforate vaginas before injection were included, this being proof of sexual immaturity. Seventy-six of the females were killed immediately following the expected time of completion of ovulation, i.e., from 14 to 18 hours after CG injection (see Chapter III), and eggs were recovered from their Fallopian tubes. Forty-one females were killed at 3½ days post coitum (p.c.), and eggs were recovered from each of their uterine horns. The completeness of egg recovery at 3½ days p.c. is discussed in Chapter IV.

Table II.1 gives the mean numbers of eggs recovered from hybrid females at the ages of 19 to 29 days, at the time of PMS injection. It is apparent from these data that the number of eggs superovulated tended to be negatively correlated with age over the range of ages tested. However, age and body weight were found to be highly correlated in these animals ($r=0.644$, $P<0.01$). Thus, it was necessary when testing for the effect of age on number of eggs to also consider any influence due to body weight.

A multiple regression revealed the degree to which age and body weight influenced the number of eggs superovulated. The mean square for the multiple regression of number of eggs on both age and weight was significantly greater ($F=21.411$, $P<0.001$) than the mean square for variance not attributable to regression. The regression coefficient expressing the relationship of age to number of eggs ovulated, independent of the effect of body weight, was significantly different from zero ($b_1 = -4.91$, S.E. = 0.95). However, there was an insignificant regression of number of eggs on weight, independent of age ($b_2 = 0.285$, S.E. = 1.45). Thus, in the hybrid stocks used, it was found that over the age range tested, age at the beginning of hormone treatment, but not body weight, exerted a strong influence on the number of eggs a given mouse could superovulate.

In view of the non-significant effect of body weight, a simple regression of number of eggs on age was calculated. The mean square for regression was highly significant in comparison with the mean square for variance not due to regression ($F=43.90$, $P<0.001$). The

Table II.1

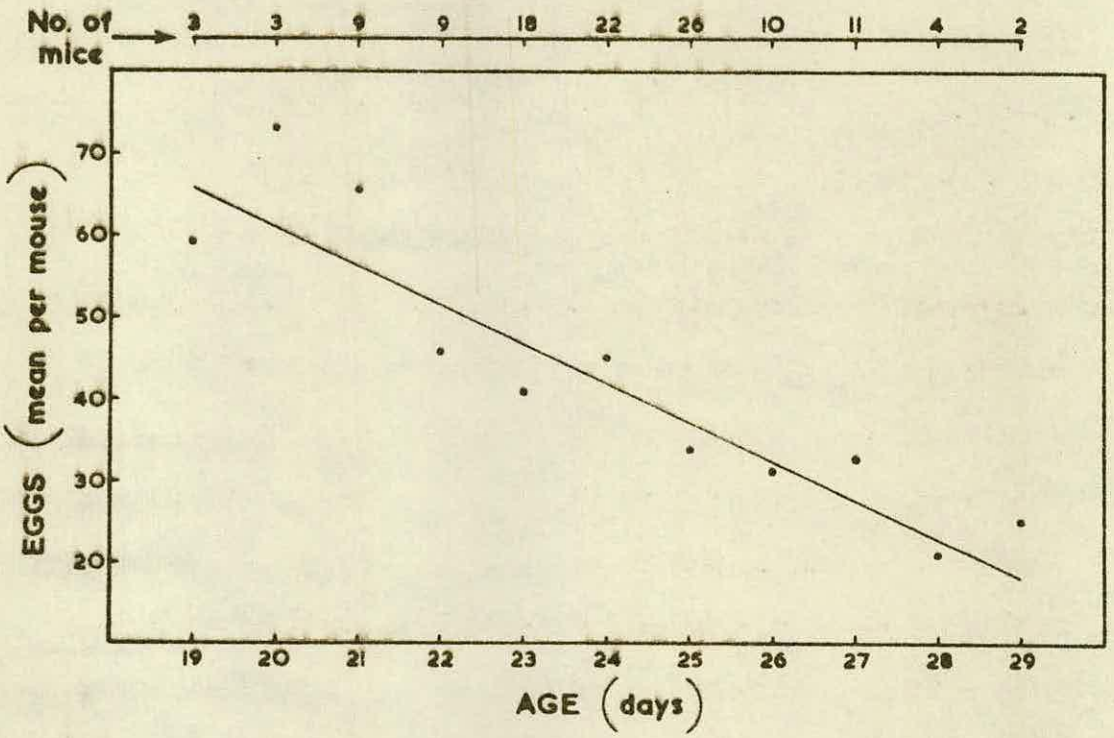
RELATIONSHIP IN HYBRID MICE BETWEEN AGE AT BEGINNING OF GONADOTROPIC HORMONE TREATMENT AND NUMBER OF EGGS SUPEROVULATED

Data Presented graphically in Figure 1.

Age of ♀ (days)	Mean No. of eggs per ♀ + std. error (or range)	No. of ♀♀
19	59.3 (72, 53, 53)	3
20	73.3 (98, 91, 31)	3
21	65.8 ± 4.9	9
22	45.9 ± 8.3	9
23	40.9 ± 3.7	18
24	45.0 ± 3.4	22
25	34.0 ± 3.2	26
26	31.1 ± 3.4	10
27	32.6 ± 4.4	11
28	21.0 ± (9 - 30)	4
29	25.0 ± (16 & 34)	2
19-29	41.2 ± 1.8	117

regression coefficient of -4.79 ± 0.72 differed significantly from zero. For the hybrid stocks of mice used, the negative correlation between number of eggs superovulated and initial age of the females in days can be expressed by the regression equation $\hat{Y} = 156.73 - 4.79\ X$, where $\hat{Y}$ is the mean number of eggs expected at a given age, X. Thus, with each increment in age of one day, between the ages of 19 and 29 days, there were, on the average, 4.79 fewer eggs superovulated. The inverse relationship between age and number of eggs may be seen in Figure 1, in which a regression line based on the above equation is superimposed over points representing the mean number of eggs recovered from animals of each age (from Table II.1).

Outbred stocks. A similar investigation was made in an outbred stock of mice which superovulates relatively low numbers of eggs. This study is based on 273 J stock females which, following gonadotropin treatment at 21 to 32 days of age, were autopsied at 14 to 24 hours after injection of CG for the recovery of tubal eggs. In these outbred mice, the mean number of eggs superovulated, 15.26, was about one third that recovered from hybrid females (refer back to Table II.1) and was little more than the mean number spontaneously ovulated by mature females of the J stock, estimated from corpora lutea counts as 11.6 eggs. As can be seen in Table II.2, not only was there a large range in number of eggs recovered from females of each age group, but also, there was no demonstrable trend between number of eggs ovulated and increasing age of the outbred females from 3 to $4\frac{1}{2}$ weeks. These results indicate that a relationship of age to number



RELATIONSHIP BETWEEN FEMALE AGE AND NUMBER OF EGGS SUPEROVULATED BY HYBRID MICE. (Fitted regression line)

FIGURE 1

Table II.2

LACK OF CORRELATION IN OUTBRED J STOCK MICE BETWEEN AGE OF FEMALES AND NUMBER OF EGGS SUPEROVULATED

Age of ♀ (days)	Mean No. of eggs per ♀	(Range)	No. of ♀♀
21	12.5	(2 - 36)	11
22	10.0	(4 - 33)	21
23	25.2	(4 - 43)	18
24	9.7	(3 - 36)	20
25	15.1	(3 - 55)	29
26	15.3	(7 - 51)	32
27	12.8	(4 - 34)	26
28	16.6	(2 - 34)	38
29	17.8	(5 - 41)	41
30	18.0	(9 - 30)	10
31	13.3	(4 - 34)	21
32	16.3	(9 - 25)	6
21-32	15.3	(2 - 55)	273

of eggs superovulated, as obtained in the hybrid P, Q and R stocks, may not be demonstrable in all mouse stocks.

Percentage of Superovulated Females which Mate

Influence of age. A study was made in 316 mice of the outbred J stock to determine whether age had any influence on readiness to mate after superovulation. At the start of gonadotropin treatment, 268 of these females had closed vaginas, evidence that they had not as yet reached puberty. Table II.3 shows the percentage of these prepuberal females which mated, with respect to their ages at injection of PMS. Since the numbers of females used at 30 and 32 days of age were only 4 and 5, respectively, the data are combined for females 30 to 32 days old. With the exception of a low proportion of matings among 24-day females, the percentage of females mating can be seen to increase steadily with increase in age between 21 and 32 days. A significant χ^2 for total heterogeneity ($\chi^2 = 21.16$, $P<0.025$), indicated that the probability of mating was dependent upon the age-group sampled. That the relationship between age and percentage of females mating was statistically significant is shown by a high value for the corresponding χ^2 for trend [4] ($\chi^2 = 14.10$, $P<0.001$), as shown in Table II.3. If these results are generally applicable to other stocks of mice, there would seem to be an inverse relationship between the number of eggs ovulated and receptivity to a male, among females with closed vaginas treated between the ages of 3 and 4½ weeks of age.

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[4] Trend χ^2, described by Armitage (1955), was calculated by a convenient formula made available by Dr. B. Woolf.

Table II.3

RELATIONSHIP BETWEEN PREPUBERAL AGE OF MICE AND PROBABILITY OF MATING AFTER SUPEROVULATION

Outbred J stock females with imperforate vaginas at PMS injection

Age at PMS	Total ♀♀	No. ♀♀ mated	% ♀♀ mated
21	17	4	23.53
22	27	14	51.85
23	19	10	52.63
24	24	7	29.17
25	23	13	56.52
26	40	23	57.57
27	26	16	61.54
28	43	27	62.79
29	23	15	65.22
30 - 32	26	20	76.92
Totals	268	149	55.60 %

Heterogeneity χ^2 Analysis of Percentage of Females Mated and Trend with Age at Treatment with PMS

Source of Variation	D.F.	χ^2	P
Trend	1	14.11	<0.001
Remainder	8	7.05	>0.10
Total heterogeneity	9	21.16	<0.025

Influence of precocity. Finally, the data were analyzed to determine if the improving response to mating which occurs with advancing prepuberal age was primarily associated with the approach to sexual maturity. Such a study is possible if, in a given age group, the females with vaginas indicating recently established patency can be considered as advanced in sexual maturity over females with imperforate vaginas.

Table II.4 shows the percentage of females in two arbitrary age groups which mated according to whether or not their vaginas were open at the time the gonadotropin treatment was started. In the younger age group, 22 to 26 days old, the percentage of females with open vaginas which subsequently mated (78.26% of 23 females) was significantly greater than that among females with closed vaginas (50.38% of 133 females), with $\chi^2 = 6.15$ and $P<0.025$.

However, in comparison with those mice of the older (27-32 days) age group which had open vaginas (68% of 25 females mated), females that were approaching maturity precociously at 22-26 days of age mated in at least as high proportions. Therefore, the percentage of treated females which mate appears not only to be associated with age as shown previously (see also the 3rd and 4th rows of Table II.4) but in addition, it is greatest among females in which vaginal patency is established relatively early.

Table II.4

RELATIONSHIP BETWEEN PRECOCITY (ESTABLISHMENT OF VAGINAL PATENCY) AND PROBABILITY OF MATING AFTER INDUCED OVULATION

Outbred J stock mice of 2 age groups

State of vagina at PMS	Age group (days)	Total No. of ♀♀	Females which mated	
			No.	% of total
Open	22 - 26	23	18	78.26[a]
Open	27 - 32	25	17	68.00
Closed	22 - 26	133	67	50.38[a,b]
	27 - 32	118	78	66.10[b]

[a]Difference between these %'s has $\chi^2 = 6.15$, $P<0.025$.

[b]Difference between these %'s has $\chi^2 = 6.34$, $P<0.025$.

DISCUSSION

Number of Eggs Superovulated with Respect to Age

That a strong relationship exists in some stocks of mice between prepuberal age and the number of eggs that can be superovulated has been confirmed in subsequent work and published in brief (Gates and Runner, 1957). Unpublished data of interest in the present discussion is given in this, and the following paragraph. In two inbred strains, BALB/c and LG (selected for large body size), the earliest age at which hormone treatment led to subsequent ovulation was found to be 15 days as compared to 14 days for the F_1. Beginning with females two weeks old, there was a steady increase in mean number of eggs ovulated, reaching a maximum in females treated at 20 to 22 days of age: 65.8 eggs for BALB/c, 31.3 eggs for LG, and 51.7 eggs for the F_1. With further increase in age, from 3 to 4 weeks, there was a concomitant decrease in egg number in BALB/c and F_1 females, confirming the results obtained in this chapter with hybrid P, Q, and R females. On the other hand, in the LG strain females, the decrease between 3 and 4 weeks was slight (mean of 22.4 eggs ovulated at 4 weeks) and not significant. (The LG strain animals were not found to require greater doses of PMS for maximum superovulation.) In BALB/c X LG hybrid mice, the number of eggs remained constant among females treated between the ages of 4 and 8 weeks. Not only do these data extend the ages over which exists the relationship of age to number of eggs ovulated, but also the data reaffirm

that the relationship may be characteristic only of certain stocks of mice.

Also, unpublished work done subsequently to that reported in this chapter has revealed that the dose of PMS administered, along with age, is important in determining the numbers of eggs which can be superovulated. Thus, in each of three age groups (21, 24 and 29 days) of tri-hybrid (BALB/c X LG x strain 129) females, egg number increased with increase in PMS dose, from 1 to 4 international units (i.u.), then decreased with further increment in dosage to 8 i.u. In 76 tri-hybrid females treated at 21 days with an initial injection of 4 i.u. PMS, the mean number of eggs superovulated was 62.3 $\pm$ 2.21 (one female having ovulated 111 ova, another, 117 ova). It may be seen, then, that following gonadotropin treatment at an optimum age, and with the optimum PMS dose, some mouse stocks are capable of superovulating extremely high numbers of eggs.

In the untreated adult mouse, a relationship between body weight and the number of eggs ovulated (estimated from counts of corpora lutea) has been demonstrated by MacDowell et al. (1929). Also, in treated or untreated adult rabbits, the relationship of number of corpora lutea to age or weight has been recently reviewed and studied by Beatty (1958). However, there may be little basis for comparing findings in adult animals with those reported herein for the prepuberal mouse. This is especially so since, whereas in the adult the relationship between age or weight and number of eggs is continuously correlated, in the superovulated immature mouse the

relationship (with age) is a changing one, and only demonstrable between 2 and 4 weeks of age.

The few references in the literature to histology of the ovary of the sexually immature mouse and rat, with and without gonadotropin treatment, provide a possible explanation for a relationship of age to the number of eggs which can be superovulated. Antra were reported to first appear in Graafian follicles of the mouse ovary between the 12th and 14th day post partum (p.p.) by Brambell (1927) and by Engle (1931a), and have been first seen to be present at 15 days in the PMS-treated inbred mouse (Pfeiffer and Hooker, 1942). On about the 15th day, the diameters of some follicles show a marked increase in size, approaching that of follicles ruptured at the first oestrus (Engle, 1931a, for the mouse, and Hargitt, 1930, for the rat). These events correlate well with the earliest age at which gonadotropins could stimulate follicles for subsequent ovulation (Gates and Runner, 1957).

In the untreated mouse, Brambell (1927) reported that the follicles which were the first to develop antra reached their maximum development at 21 days p.p., at which time they constituted a large portion of the ovary. At this age, as many as 50 large follicles had been seen in a single section of an ovary. At 4 weeks of age, the majority of oocytes involved in this first wave of follicular growth had degenerated and only an occasional full-grown oocyte remained. Similar changes in oocyte size and number in the rat were reported by Arai (1920), who measured the diameter of oocytes in

animals at various ages. The first appearance of large numbers of oocytes 40 to 60μ in diameter occurred at 10 to 15 days. Rats 20 to 26 days old had the greatest numbers of oocytes measuring more than 60μ in diameter: an average of 138 for both ovaries. Oocytes of this size reached a relatively low frequency which persisted, beginning at 41 to 46 days of age and continuing into adulthood. Hargitt (1930) observed comparable changes in the rat ovary, with the first climax in follicular maturation reached at 26 days, and a paucity of large follicles (resulting from mass atresia) between 29 and 32 days of age.

Summarizing, in the untreated rodent, the 4 stages representing changes in the frequency of the largest oocytes--(1) first appearance at two weeks, (2) greatest number at three weeks, (3) subsequent decrease in number until about 4 weeks of age, and (4) maintenance of a relatively low number after 4 weeks--correspond well with the changing potentialities for egg production which have been demonstrated in superovulated prepuberal mice of some strains (see first paragraph of Discussion). It seems highly likely that the number of nearly mature non-atretic oocytes present in the ovaries of an immature mouse when gonadotropin treatment is begun may limit, if not actually represent, the number of eggs that can be superovulated.

Since polyovular follicles are not infrequent in the immature mammal (Hartman, 1926) the question might be raised as to whether their occurrence could be causally related to the large numbers of ova that can be superovulated in the sexually immature mouse. Hartman

states that most polyovular follicles undergo atresia, but that evidence of this is incomplete. Non-atretic polyovular follicles have been observed in the ovaries of the immature mouse by Chappellier (1909). However, occurrence of the phenomenon may be more frequent in some mouse strains than in others (Fekete, 1950). In order to determine whether the incidence of polyovular follicles varied appreciably with age in the gonadotropin-treated hybrid mice herein studied, serial sections of ovaries had been examined in 27 P, Q, and R stock females treated between 21 and 34 days of age in work not mentioned in this chapter. Since polyovular follicles did not occur in any of the ovaries of these hybrid females, the phenomenon can be discounted as a factor contributing to the numbers of ova superovulated by the sexually immature mouse.

In addition to age, general health of the mice chosen for hormone treatment may be an influencing factor in the number of eggs they will superovulate. Adams (1953, cited in Adams, 1954) was able to alter the age at which the formation of antra first occurred in the oocytes of the rabbit by regulating food intake. This, in turn, determined the age at which the ovaries could first respond to gonadotropin. Delay in formation of antra in the mouse could conceivably result from disease, such as infantile diarrhoea, indirectly affecting the intake and utilization of nutrition. The fact that animals appearing to be in ill health were deliberately culled from among those used in this work, may have reduced variability in the relationship of age to number of eggs superovulated.

Mating Response with Respect to Age and Precocity

The data have shown that in an outbred stock of mice the percentage of superovulated females which mated was proportional to prepuberal age of the females. In addition, females which were believed to be precocious for their age, on the basis of having recently opened vaginas, were correspondingly more receptive to a male after superovulation. Related to these findings are those of Wilson and Young (1941). These workers concluded that sensitivity to oestrogen in the guinea pig and rat, as detected by the copulatory response elicited by manual stroking of the back and perineal region, was correlated with age. The rat attains a high sensitivity at about the 30th day and maintains it through 10 months of age. Removal of the ovary and uterus did not alter the age at which a given degree of sensitivity was acquired. Furthermore, previous literature had indicated that the gonadotropins were not apparently involved in the relationship. Thus, although the ovary of the prepuberal mouse can produce oestrogen in response to gonadotropin treatment at as early as 11 days of age (Runner, unpublished), there may be a subsequent interval of several days before sensitivity to the intrinsic supplies of oestrogen is high enough to evoke receptivity to a male. Findings in this chapter with regard to the effect of precocity (early opening of the vagina) suggest that the percentage of superovulated females capable of mating is more closely associated with physiological age than with chronological age.

Summarizing in brief, the data presented in this chapter indicate that with increasing prepuberal age between 3 and 4 weeks the potential for high egg production following superovulation falls off (as demonstrated in hybrids) as probability of mating increases (as demonstrated in outbred mice).

CHAPTER III

TIME OF ONSET, RATE, AND DURATION OF SUPEROVULATION

INTRODUCTION

Accurate timing of ovulation may be a necessary requirement in embryological investigations such as those involving the study or treatment of mammalian eggs at a known time relative to their release from ovarian follicles. For such studies in polytocous species it is important to have knowledge of the time of onset of ovulation (when the first follicle ruptures), the rate at which ovulations occur, and the duration of ovulation (the interval between rupture of the first and last follicles). Those studies which necessitate timing the development of the egg from fertilization onwards require knowledge of the time at which ova enter the site of fertilization. The observations reported in this chapter provide an indication as to the extent to which these time relationships of ovulation can be established and controlled in the immature mouse treated with gonadotropins.

In the untreated laboratory animal there is generally much variability between females as to time of ovulation. Much use has been made of the rabbit for timing ovulations, which are generally stated as occurring about 10 hours after coitus. In actual fact, Walton and Hammond (1928) reported that the time of follicle rupture

as observed by them ranged from 9 3/4 to 13½ hours after coitus. In the mouse and rat, ovulation, which occurs independently of mating, is predictable at best only to within a few hours. Boling et al. (1939) found that rats of the Wistar strain ovulated usually about 9 hours after the onset of oestrus, but the range was more than 5 hours. In later work, Boling et al. (1941) reported that ovulation in their rat colony occurred between 6½ and 10 hours after onset of heat, as determined by the copulatory response. Ovulation timed in the mouse either from the beginning of oestrus or according to the hour of day during oestrus is at least as variable as that for the rat (see Snell, 1941, and recently, Braden, 1957a). Unfortunately, timing ovulation in the unmated mouse or rat can be a tedious procedure in that the day of oestrus must usually be established by taking daily vaginal smears. An alternative method is that of Runner and Ladman (1950) who timed ovulation in the mouse relative to the time of observed parturition; however, the variability was such that only 88% of mice ovulated within a predictable 6-hour period.

Artificial regulation of the periods of light and dark was first shown by Hemmingsen and Krarup (1937) to afford a means of superceding the diurnal control of ovulation, i.e., nocturnal ovulation in the laboratory rodent. With rats maintained under reversed day-night conditions, Austin and Braden (1954a) observed a spread in time of onset of ovulation of more than three hours which was similar to that of animals kept under normal colony conditions. Likewise, mice maintained under conditions of controlled illumination had a

long range in time of onset of ovulation of more than 5 hours (Braden and Austin, 1954a).

Can ovulation in hormonally treated immature rodents be induced to occur within more narrowly defined time limits than those of the untreated animal? The findings of Rowlands (1944) and Austin (1951) indicated that the times of onset of hormonally induced ovulations in immature rats varied by as much as 3-4 hours. Though Blandau (1955) was able to observe follicle rupture in the living animal by utilizing superovulation, he did not state how variable were his rats in the time of onset of ovulation. From the work of Runner and Palm (1953), in 4 inbred strains of mice the range in time of commencement of induced ovulation was about 2-4 hours. In contrast to the response of rats and inbred strains of mice to induced ovulation, the hybrid stocks of mice used in the present work were found to be unusually uniform in time of onset of superovulation. This regularity of response has made possible a discussion in this chapter of the rate at which ova were shed during superovulation and some of the factors associated with this rate.

MATERIALS AND METHODS

The mice used in this investigation, unless otherwise specified, were of the hybrid P, Q, and R stocks described in Chapter II. At the start of treatment with gonadotropins, their ages ranged from 18 to 29 days, and their body weights, from 9 to 15 grams. Their imperforate vaginas provided evidence that they had not yet reached puberty.

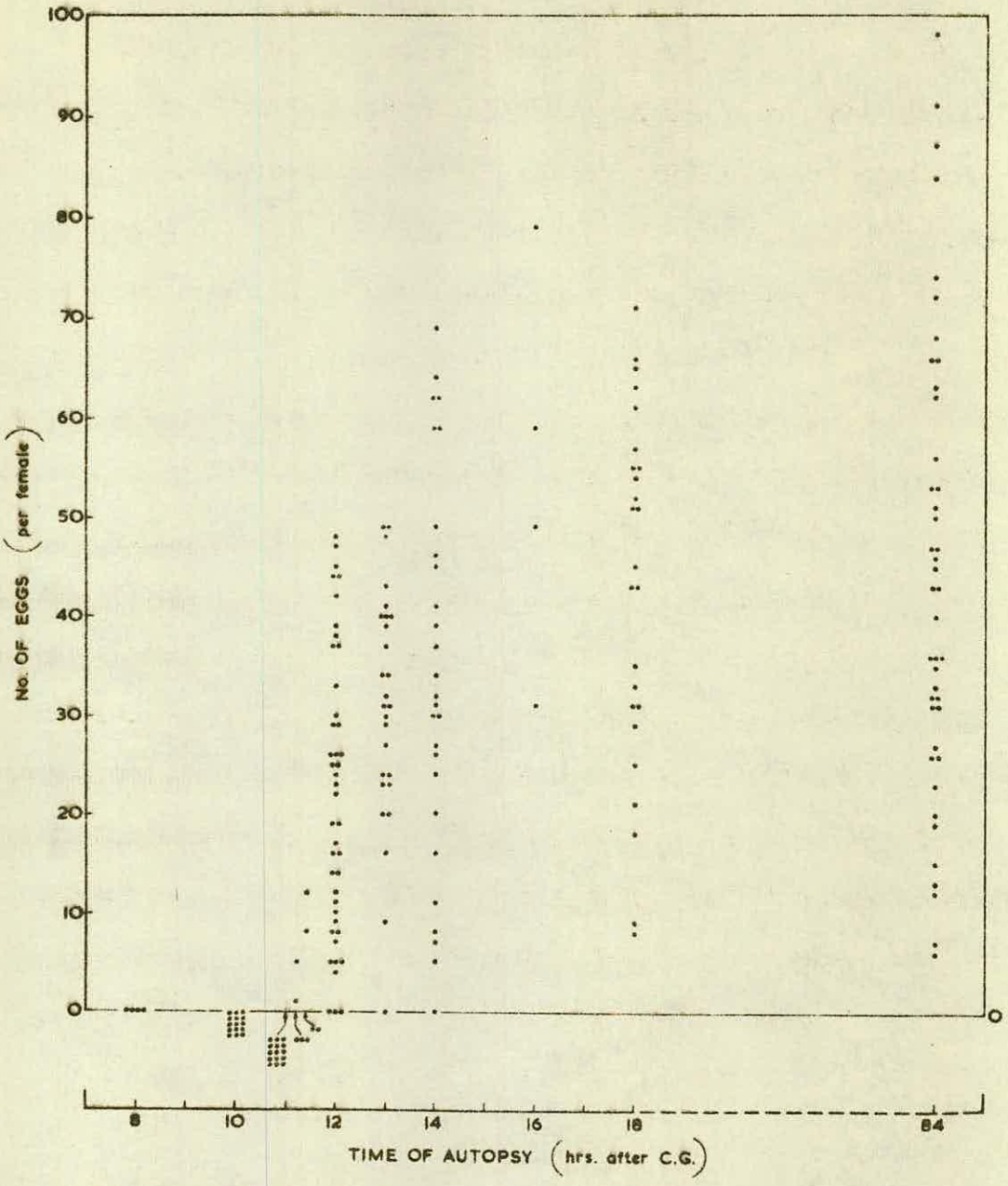
All mice received an injection of 2 i.u. Pregnant Mares' Serum (PMS) followed 36 hours later by 2 i.u. of Chorionic Gonadotropin (CG). This procedure for gonadotropin treatment which had proved to be optimal for induction of oestrus and ovulation (Chapter II) was also found to result in good control of time of ovulation, as will be shown in the results.

Determination of the time of onset, the rate and the duration of superovulation was based on the numbers of tubal ova recovered from 169 females at the following intervals after injection of CG: 8, 10, 11, 11 to 12, 12, 13, 14, 16, and 18 hours. Included also are results from the recovery of uterine eggs from 43 mice at $3\frac{1}{2}$ days p.c. These latter data are based on work in connection with the fourth chapter in which evidence is presented which indicates that the recovery of uterine eggs was thorough enough to enable a reasonable estimate of total eggs ovulated. The methods for recovery of eggs from Fallopian tubes and uteri are given in Chapters II and IV, respectively.

DESCRIPTION AND ANALYSIS OF RESULTS

Time of Onset of Superovulation

All of the data on recovery of tubal and uterine eggs at various intervals after CG are presented in Figure 2. As can be seen, superovulation began for most females over a remarkably short range of time. Tubal ova were not found in any of the 15 females



DISTRIBUTION OF FEMALES BY NUMBERS OF TUBAL OVA AT TIME OF AUTOPSY.

FIGURE 2

autopsied between 11 hours and 11 hours, 10 minutes after CG injection (nor in 19 females killed at 8 to 10 hours). However, by 12 hours after CG, ovulation had commenced in 40 out of 43 mice autopsied. Thus, it can be assumed that about 93% of the females in this hybrid stock began superovulating during the 50 minute interval prior to 12 hours after injection of CG.

It is notable that among 169 injected mice, only 5 had not ovulated when autopsied at 12 hours or later. Notes recorded in routine examination of the ovaries and reproductive tract during autopsy revealed the following information on the 5 mice without ova. The three females killed at 12 hours after CG and found not to have ovulated (representing 7% of 43 females autopsied at that time) had each responded to gonadotropin with good ovarian and uterine stimulation. Thus, there was no basis for assuming that any of these three females would not eventually have ovulated. On the other hand, of the two females without ova at 13-14 hours, the one which was killed at 13 hours, although showing normal uterine stimulation, did not have ovarian follicles showing the enlargement characteristic of a preovulatory phase. The other, killed at 14 hours, was atypical in that her uterus was still distended with fluid, a condition which does not normally persist for more than 12 hours after CG. Even if the latter two females mentioned were destined to ovulate eventually, despite evidence to the contrary, the percentage of prospective ovulators not having commenced ovulation by 13 hours would have been only 3.8% (2 out of 52). Thus, for the purposes of the following

discussion, virtually all superovulated females were considered to have begun ovulation by 13 hours after CG.

An indication of the times at which individual mice began superovulating was obtained from autopsies of 9 females between the 11th and 12th hours after CG. Among 5 females autopsied between 11 hours, 10 minutes and 11 hours, 15 minutes, three had no ova, one female had one ovum and another had 5 ova. In autopsies of 4 females between 11 hours, 20 minutes, and 11 hours, 25 minutes after CG injection, no ova were recovered from two females, one animal had 8 ova and another had 12 ova. In 3 of the 4 ovulated females mentioned, the numbers of unruptured follicles were greatly in excess of the numbers of ova recovered. The large numbers of unovulated follicles and low mean number of ova recovered (6.5 ova) from females autopsied between 11 hours, 10 minutes and 11 hours, 25 minutes, indicate that ovulation had begun recently and was still in progress in these early ovulators.

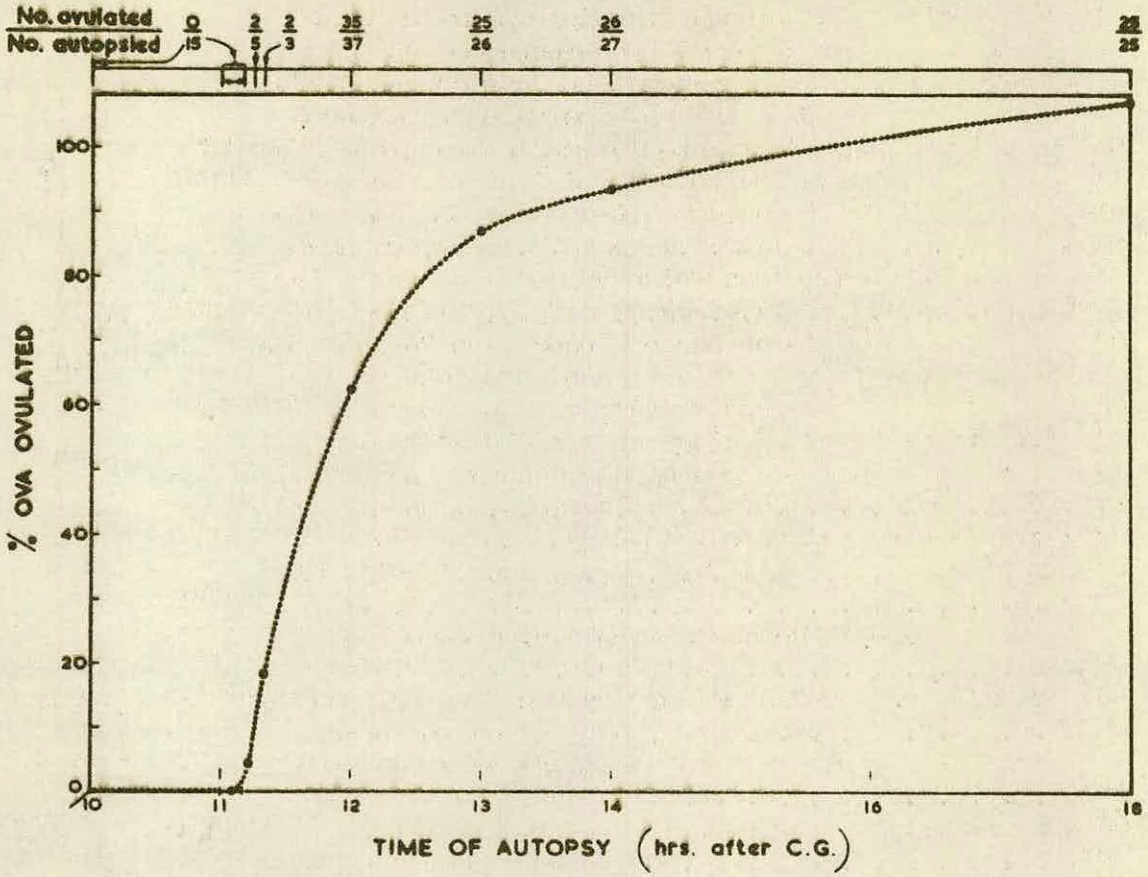
Ovum recoveries demonstrating the time of onset of ovulation in the hybrid stocks used herein were based on several separate experiments and were not confined to a limited season of the year. Including controls used for experiments in Chapter IV, series employing autopsies at both 11 and 12 hours after CG had been conducted during each of the months from November through February, and during May. In each of these series ova were never found in mice killed at 11 hours, whereas, the majority had begun superovulation when killed at 12 hours after CG. These results demonstrate that the occurrence

of onset of superovulation for a majority of females during the interval between 11 and 12 hours after injection of CG was repeatable throughout successive series in the hybrid stocks of mice used.

Rate and Duration of Superovulation

It can be seen in Figure 2 that there was marked variability in the number of eggs recovered from females at any given autopsy time. A large proportion of this variability may be attributable to range in age of the females used (see discussion of age-egg number relationship in Chapter II). For example, of the females that had ovulated more than 50 ova by 18 hours or more after CG, 26 of the 27 killed were between 19 and 25 days old at the beginning of hormone treatment. Conversely, among mice that had ovulated less than 30 ova during the same period, 15 out of 17 females were 24 to 28 days old. The effect of age on number of ova recovered at each autopsy interval can be further seen in Table III.1, to be discussed later.

An approximate estimation of the rate and duration of ovulation for females as a group may be obtained if it can be assumed that most of the variability in egg number is attributable to age of the females. In Figure 3, the total numbers of ova recovered from immature females at various intervals after CG are expressed as percentages of the total numbers of ova expected at completion of ovulation on the basis of age of the females used (from Table II.1). These expected totals were derived for mice at each CG-autopsy interval by summing the



RATE OF SUPEROVULATION FOR MICE AS A GROUP (Corrected for age)

FIGURE 3

products of (1) the number of mice of a given age, and (2) the mean number of eggs ovulated by mice of the corresponding age at completion of ovulation (read off from Table II.1). The percentage points plotted on Figure 3 are based on autopsies of females 21 to 29 days of age. Results from 18, 19, and 20 day old mice were excluded since Table II.1 contains insufficient data on mean numbers of eggs ovulated by animals less than 21 days of age. Also, it should be pointed out that the percentage of ova ovulated is based on all females, including unovulated ones. Exclusion of unovulated mice would increase the percentages of ova ovulated at 12, 13 and 14 hours to 65.8%, 90.2% and 97.2%, respectively. From the data presented in Figure 3, it would appear that approximately 90% of the ova to be superovulated by a given group of females arrive in the Fallopian tubes within the first two-hour period of ovulation, i.e., by 13 hours. The curve further suggests that from 3 to 5 hours may be required for completion of ovulation in all females.

Rate and Duration of Superovulation Relative to Potential Egg Yield

In Table III.1, the data are presented to illustrate the effect of age, and consequently, number of eggs eventually ovulated, upon rate and duration of ovulation. The mean numbers of tubal ova recovered at various intervals after CG is given both for females aged 18 to 23 days at PMS treatment (yielders of eggs in high numbers) and those 24 to 29 days (relatively poor egg yielders). In calculating mean numbers of ova recovered, the few non-ovulators, as well as

Table III.1

RELATIONSHIP BETWEEN AGE OF SUPEROVULATED MICE AND RATE OF ARRIVAL OF OVA IN THE TUBAL AMPULLAE

Based on mean number of tubal ova (± standard error) from females, including unovulated ones, autopsied at various intervals after CG. Immature P, Q and R stock hybrids. Data presented graphically in Figure 4.

Time of autopsy (hours after CG)	18- to 23-day old females			24- to 29-day old females		
	% ♀♀ ovulated	No. ♀♀ autopsied	Mean No. eggs ± std. error*	% ♀♀ ovulated	No. ♀♀ autopsied	Mean No. eggs ± std. error*
11	0	8	0.00	0	7	0.00
12	85.7	14	17.86 ± 3.11	96.6	29	23.72 ± 2.58
13	100	7	35.00 ± 3.47	95.0	20	29.40 ± 2.83
14	100	8	47.13 ± 7.69	93.8	16	30.06 ± 4.43
>14	100	24	56.83 ± 4.82	100	48	38.06 ± 2.41

*The following is a list of the pairs of means having significant "t" values, accompanied by statement of conclusion drawn from comparison.

Class	means compared	t	P	conclusion drawn
18-23 da.	12 & 13 hrs.	3.38	<0.005	1) Ovulation still in progress at 13 hrs. for both age groups.
18-23 da.	13 &>14 hrs.	2.37	=0.025	
24-29 da.	13 &>14 hrs.	2.08	<0.05	
14 hrs.	2 age groups	2.07	=0.05	2) Ovulation rate after 13 hrs. faster in younger age group.
>14 hrs.	2 age groups	3.89	<0.001	

females having ovulated are included. Graphic presentation of the data in Table III.1 is made in Figure 4.

Duration in females as a group. In each age group (Table III.1), the mean number of ova recovered at 12 and 13 hours after CG was significantly less than that found at autopsies later than 14 hours after CG. This suggests that regardless of the numbers of eggs to be eventually ovulated, the duration of ovulation for females as a group was at least as long as one hour, 50 minutes, (i.e., from 11 hours, 10 minutes to 13 hours after CG).

Duration in individuals. Evidence as to the variability in duration of ovulation among individual females at the time of autopsy is available from the data (Table III.2) recorded on the numbers of mature, unruptured follicles present in the ovaries of mice of the two age groups: 18-23 and 24-29 days old. Since ovulation is almost certainly complete in all females by 18 hours (see Fig. 3) it would appear from Table III.2 that 20% of females in both age groups killed at 16-18 hours (last row, sum of 6th and 7th columns) retained a few large follicles not destined to be ovulated. However, in both age groups, a larger percentage of females (44% in all) still had unruptured follicles when autopsied at 14 hours (8th row, 6th and 7th columns). This suggests that in some mice of each age group, ovulation was still in progress 14 hours after CG. By contrast, 19% of females (out of 21 in the 24- to 29-day age group) had no unruptured mature follicles at 12 hours, indicating that they had completed ovulation by then. It would seem, then, that there was considerable

TABLE III.2

RELATIONSHIP BETWEEN AGE OF SUPEROVULATED MICE AND RATE OF FOLLICLE RUPTURE

The percentage of females with varying numbers of mature unruptured follicles at increasing intervals after C G, as determined by gross examination of the ovaries. Immature P, Q and R stock hybrids.

Age of females (days)	Time of autopsy (Hrs. after C G)	Total ♀♀	Approximate No. mature unruptured follicles		
			None	1 to 5	>5
	12	10	0%	10%	90%
18 - 23	14	8	50%	38%	13%
	16 - 18	8	75%	13%	13%
	12	21	19%	29%	52%
24 - 29	14	15	60%	33%	7%
	16 - 18	13	85%	8%	8%
	12	31	13%	23%	65%
18 - 29	14	23	57%	35%	9%
	16 - 18	21	81%	10%	10%

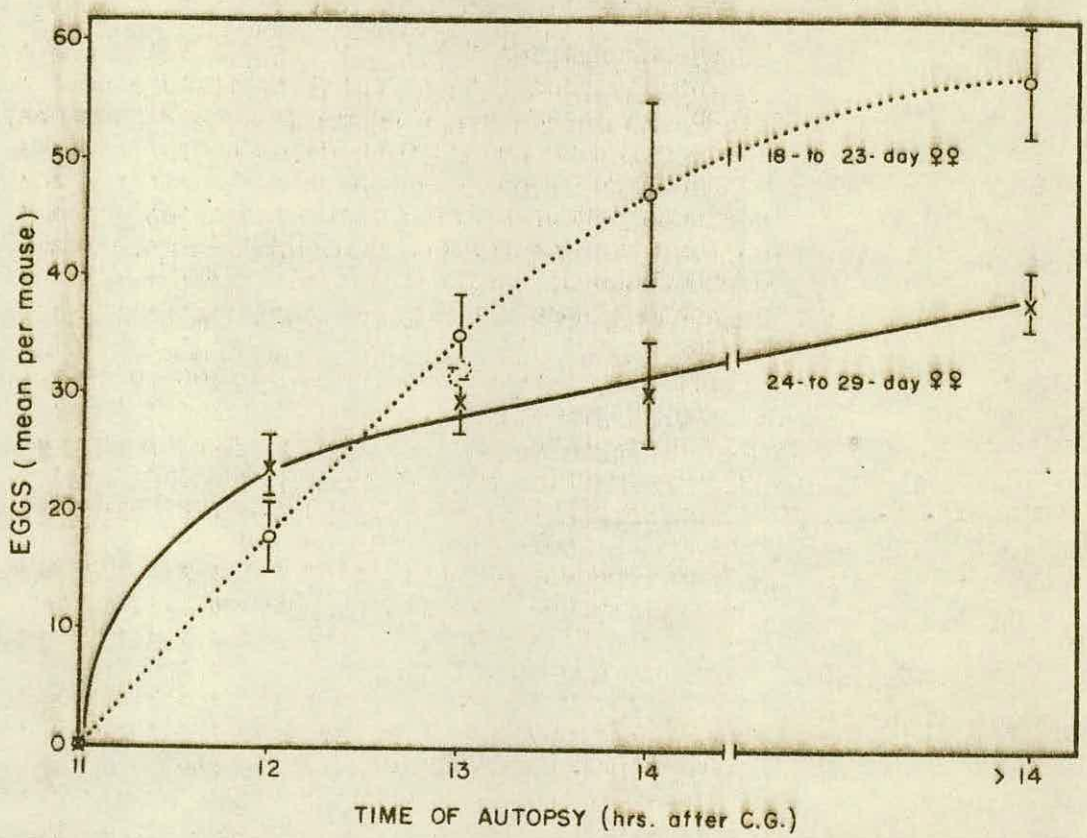
variation between females as to the duration of ovulation. The evidence from numbers of unruptured follicles has suggested that, whereas some females had completed ovulation within the interval between 11 and 12 hours, in other mice (whether potential yielders of high or low numbers of eggs), ovulation lasted from before 12 hours to after 14 hours from injection of CG.

Rate in females as a group. Although ovulation was apparently still taking place in some mice of the two age groups at 14 hours, the mean number of eggs recovered from each group at 14 hours was significantly greater in the younger group (Table III.1). As can be best seen in Figure 4, mice with a potential for high yields of eggs seem to ovulate at a faster rate between 12 hours after CG and the completion of ovulation.

Maximum rate in individuals. The maximum numbers of ova recovered at each autopsy interval provide some indication as to the maximum rate of superovulation possible in the hybrid stocks used. The greatest numbers of tubal ova recovered from females with no mature follicles left unruptured in autopsies at 12, 14 and 16 hours after CG were 47, 69, and 79, respectively.

Time of Superovulation Relative to Time of Day

For convenience, all females in experiments so far described in this chapter were induced to ovulate during the day. Thus, the data did not allow the preclusion of diurnal control as a factor influencing the onset and rate of superovulation. In an experiment



RATE OF SUPEROVULATION FOR MICE OF TWO AGE GROUPS

Mean number of eggs (± standard error) recovered at various intervals after chorionic gonadotropin.

FIGURE 4

designed to test this possibility, two groups of mice were induced to superovulate, the second group receiving PMS and CG about 12 hours behind the injection schedule of the first group. The hormone dosages and injection intervals were those mentioned under "Materials and Methods". The actual times of CG administration were 11:30 pm and 11 am, respectively. At the time of the experiment, the hybrid P, Q, and R stocks of mice were not available and instead, outbred J stock mice (described in Chapter II) were used. These were 29-32 days old when treated with PMS, and 13.9 to 21.9 gms. in weight at the time of autopsy. The mice for each series were divided, before injection, into 4 groups to be autopsied at 10-11 hours, 12 hours, 14 hours, or at 19-20 hours after CG. Groups with the same CG-autopsy interval were made as nearly comparable as possible in the two series by utilizing pairs of sibs.

The data, as shown in Table III.3, indicate that ovulation which has been induced to occur in immature outbred mice at about noon instead of just after midnight, as in spontaneous ovulations among adults, is not appreciably retarded in time of onset. Regardless of whether ovulatory hormone was injected near midnight or at midday, in each group only one of the females autopsied 10 or 11 hours later had tubal ova (one and four ova, respectively). In both groups ovulation had begun in all females by 12 hours. The mean numbers of ova recovered at 12, 14, and 19-20 hours did not differ significantly between midday and midnight ovulators, suggesting that rate, as well as onset of superovulation was not affected by any diurnal periodicity.

Table III.3

TIME OF SUPEROVULATION WITH RESPECT TO TIME OF DAY OF HORMONE TREATMENT

Immature outbred J stock mice

Time of autopsy (hours after CG)	Midday ovulation (CG at 11:30 pm)			Midnight ovulation (CG at 11:00 am)		
	Total ♀♀ autopsied	Number ♀♀ ovulated	Mean ova per ♀ ovulated	Total ♀♀ autopsied	Number ♀♀ ovulated	Mean ova per ♀ ovulated
10 - 11	12	1	1.0	11*	1	4.0
12	10	10	12.7	11*	10	14.3
14	10	10	15.3	9*	8	20.8
19 - 20	10	10	19.8	10	10	18.7

*Includes one unovulated female with apparent hypo-stimulation by CG.

DISCUSSION

Although the term "ovulation" refers specifically to the rupture of follicles from the ovary, the main criterion used in the present discussion has been the arrival of ova in the Fallopian tubes. Whether or not these two events are as nearly synchronous in the immature superovulated mouse as in the normal adult is not definitely known. However, the question of the length of time spent by the freshly ovulated ovum in the ovarian bursa is largely one of academic interest. Nevertheless, it is of practical importance to have accurate knowledge of the time at which ova enter the tubal ampulla, the site of fertilization in the mouse, especially in studies timing the development of the egg from fertilization onwards.

No completely satisfactory methods for accurate determination of the time of ovulation in mammals are available from the previous literature. Burr et al. (1935) detected ovulation in the intact rabbit by recording bodily electropotential changes. They stated that the number of surges of potential corresponded to the number of follicles ruptured. However, Nicholas and Carmosino (1944), working with rats, found no correlation of sign, amplitude or number of voltage peaks with the number of ova shed. Walton and Hammond (1928) and Hill et al.(1935) were able to observe ovulation in the anaesthetized rabbit, but the possibility can not be excluded that anaesthetization and operative interference may alter the timing of ovulation. This is suggested by Walton and Hammond's report that ovulation failed to occur in two does operated on at about 9 hours p.c. (or about one half

to one hour before the start of ovulation). Blandau (1955) made use of paralysis (inflicted by sectioning the spinal cord between cervical vertebrae) to permit observation of superovulation in the living rat. Unfortunately, the method of direct observation of the ovary is not ideally suited to determination of the rate and duration of ovulation for the following reasons: (1) not all surfaces of the exposed ovaries can be easily watched, (2) the rupture of follicles other than those located peripherally is difficult, if not impossible, to observe, and (3) observations cannot always be started before the onset of ovulation.

Time of Onset of Superovulation

In the present investigation, the gonadotropin treatment of 18- to 29-day old sexually immature mice of a hybrid stock was found to provide a means of controlling with great accuracy the time of onset of ovulation. Whereas no treated females had tubal ova when killed up to 11 hours, 10 minutes after CG, 93% had ovulated by 12 hours after CG. This uniform response in time of onset of superovulation, which was repeatable in the hybrid stocks of mice used, has made it possible to make reasonable estimates of the rate and duration of ovulation for the superovulated females as a group.

The time of onset of superovulation among the hybrid mice used in this study is compared in Table III.4 with that reported herein for outbred mice and reported in the literature for rats and inbred strains of mice. In the outbred J stock of mice (column 5, extracted from Table III.3), the majority of animals had begun to ovulate between

Table III.4

TABULATION OF VARIOUS INVESTIGATORS' RESULTS ON TIME OF OVULATION INDUCED IN THE IMMATURE RAT OR MOUSE BY INJECTIONS OF PMS FOLLOWED BY CG

AUTHORS	Rowlands (1944)	Austin (1951)	Runner & Palm (1953)	Gates	Gates
ANIMAL USED	Rat	Rat	Mouse (inbred)	Mouse (outbred)	Mouse (hybrid)
AGE OF ANIMAL	immature (40-50 g.)	40-55 days	28-32 days	29-32 days	18-29 days
HOURS AFTER CG	a b* c	a c	a* b* c	a b c	a b c
10	- - -	- -	0% 0 24	8% 1 12	0% 0 15
11	- - -	12% 25	- - -	9% 4 11	0% 0 15
12	10% 1 10	62% 29	42% 2 24	95% 14 21	93% 23 43
13	- - -	83% 23	- - -	- - -	96% 32 27
14	50% 11 10	95% 21	83% 5 24	95% 18 19	96% 37 24
16	100% 20 10	- -	100% 8 24	- - -	- - -
18-20	100% 27 10	- -	100% 7 24	100% 19 20	100% 43 25

a = % of ♀♀ with tubal ova
b = Mean ova per ♀ ovulated.
c = Total No. of ♀♀ used in subgroup.
* Approximate figures.

11 and 12 hours after CG, but ovulation as early as 10 hours was noted. Four inbred strains of mice (C57BL, BALB/c, C3H, and C57BR) were found by Runner and Palm (1953) to have a range in time of induced ovulation of more than two hours for over 80% of females. In work on the rat by Rowlands (1944) and Austin (1951), superovulation appeared to commence in individual animals anywhere from 11 to 14 hours after CG. Summarizing the comparisons made in Table III.4, the range in time of onset of superovulation reported for outbred mice, 4 inbred strains of mice, and rats was appreciably greater than that for the 18- to 29-day old hybrid P, Q, and R stock mice used in the present study.

Edwards and Gates (in preparation) have shown that when ovulation induced hormonally in mature mice occurs over a narrowly defined time interval, the final stages of oogenesis leading to completion of the first maturation division may also be accurately timed. Accordingly, one would expect superovulation in hybrid mice, such as those used in this study, to be preceded by stages in the first maturation division which occur at readily predictable times. Thus, superovulation in the immature hybrid mouse may be of potential usefulness in experiments involving chemical, mechanical, or radiation treatment of ova before, as well as after, ovulation.

Rate and Duration of Superovulation

It is possible to make a limited comparison as to rate and duration of ovulation between data herein presented for the superovulated mouse and that reported in the literature following different methods of estimation. Blandau's (1955) observations of superovulation in living rats give some indication as to rate of ovulation, with the limitations stated at the beginning of this discussion. In his data based on three "typical" ovulators, the mean interval between rupture of individual follicles (13 ovulations observed per female) was 6.1 minutes. Near the beginning of ovulation, follicles were often seen to rupture simultaneously, whereas, a maximum interval of 48 minutes was seen to occur between the last two ovulations in one of the rats. The rupture of 13 follicles from one ovary of each of these three rats took place over an interval of from 1 hour, 14 minutes to 1 hour, 28 minutes. The duration of ovulation was probably somewhat longer, since several ovulations had been completed before the observations were begun. In the Elephant shrew, a mammal which spontaneously ovulates very large numbers of ova, Van der Horst and Gillman (1941) surmised that ovulation of a mean of 120 ova took a considerable time, possibly a few hours, as judged by the appearance of stages, from ripening follicles to fresh corpora lutea, present in the ovary at one time. Therefore, in the present work, although some immature females must have completed ovulation within one hour, the estimation that superovulation may have taken as long as 3-5 hours for females as a group is reasonable in view of results reported by other authors.

Despite the seemingly long duration of superovulation in the immature hybrid mice as a group, most of their ova were ovulated over a comparatively short time interval. Just over 60% of the number expected at completion of ovulation were ovulated within 50 minutes of the onset of ovulation (i.e., by 12 hours after CG), and about 90% of the ova expected to be ovulated were in the Fallopian tubes within 1 hour, 50 minutes. This rapid initial rate in ovulation makes it possible to time superovulation so accurately in mice such as the immature hybrids used here, that the majority of ovulations (90% of ova) for females as a group can be established at 12 hours $\pm$ 1 hour after injection of CG.

CHAPTER IV

VIABILITY AND DEVELOPMENTAL CAPACITY OF SUPEROVULATED EGGS PRIOR TO IMPLANTATION

INTRODUCTION

That gonadotropic hormone treatment results in the ovulation of fertilizable ova in many species has long been established. The earliest reports for various species are as follows: mouse (Engle, 1927), rat (Cole, 1936 and 1937), rabbit (Paducheva et al., 1935), sheep (Cole and Miller, 1933), and cow (Zavadovskii et al., 1935). Fairly high rates of fertilization after superovulation have been noted for some species: 76% for the mouse (Runner and Gates, 1954a), 60% for the rat (Austin, 1950), 81% for the rabbit (Warwick et al., 1943) and 74% for the mature ewe (Casida et al., 1944). However, the above rates of fertilization for the immature rat and rabbit, when compared with these for untreated adults, were shown to be below normal by Austin and by Warwick et al., respectively. Also, Austin's work (1950) suggests that further loss among superovulated eggs occurs through fragmentation during tubal development. Thus, he found that in superovulated immature rats the percentage of normally developing eggs, in rats with fertilized eggs, decreased from 60% in recoveries made at less than 24 hours post coitum (p.c.) to 45% at two days p.c. Austin concluded that in addition to a subnormal fertilization rate there must be abnormalities inherent in superovulated eggs, perhaps

due to precocious maturation.

Evidence from the literature indicates that abnormalities of follicular maturation are not uncommon for the immature rodent. Kingery (1914) described abnormal maturation spindles in atretic follicles of the immature mouse. Lane (1938), studying ovaries of immature rats, found binuclear and polyovular follicles but could not increase their incidence by gonadotropic hormone treatment. Simulated, or pseudoparthenogenetic segmentation of unfertilized eggs was found to be more common among prepuberal than among mature animals. This has been reported for follicular ova of the immature rat, rabbit, and guinea pig (Bacsich and Wyburn, 1945), and for tubal ova recovered from the superovulated rat (Austin, 1949).

This chapter reports the results of examinations of eggs recovered from superovulated immature mice during fertilization and at 1½ days before implantation to provide information on both viability and rate of development of the eggs at these preimplantation stages.

MATERIALS AND METHODS

Viability and Development before Cleavage

Female mice of the J stock (described in Chapt. II) were injected at 21-32 days of age with 2 i.u. PMS, followed 36 hours later (midnight ± 1 hour) with 2 i.u. CG. At 9 hours after CG, the females were paired with fertile strain A males and examined for vaginal plugs during the next two hours at intervals of between 10 minutes and one hour. Groups of mated females (141 in all) were autopsied at hourly

intervals from 11 to 18 hours and at 20, 22 and 23-24 hours after CG. Tubal eggs, after recovery in saline, were prepared following the method of Austin and Smiles (1948) for examination under a phase contrast microscope at magnifications of from 400 to 2000 times. The aims of the examination were twofold: to determine for superovulated eggs (1) the rate of sperm[1] penetration (through the vitellus) and of formation of pronuclei and (2) the incidence of eggs showing evidence of aberrant development during various precleavage stages.

Viability and Development at 3½ Days P.C.

A comparison was made of the eggs from immature and mature mice as to the percentage survival and stage of development reached at 3½ days p.c. (approximately 1½ days before implantation). In one of the two stocks of mice used (the R stock), development of superovulated ova after delayed fertilization as well as after normal fertilization was investigated.

The method was as follows. Ovulation was induced in 83 R stock (see Chapter II) females, aged 18 to 28 days, by the injection of 2 i.u. PMS at 12:30 pm ± 1 hour, followed 36 hours later by 2 i.u. CG. At 9 to 12 hours after CG (mean, 10.3 hours, or just before ovulation), 29 of these females were paired with males of either the 101 or CBA inbred strains. (Subsequent analysis showed no effect of strain of male on rate of cleavage of eggs.) These females were checked at hourly intervals and

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[1]Although "spermatozoon" is etymologically more proper, common custom is invoked to justify the use of "sperm" in this text.

15 of them were removed as soon as vaginal plugs were found. Those that had not mated by 13 hours after CG (approximately one hour after onset of ovulation) were removed, and they, plus 54 previously unpaired females were put with males at 18 to 23 hours (mean 19.8 hours) after CG. These females subjected to delayed matings were examined at intervals of one to two hours up until midnight (about 24 hours after CG), and the final check for copulation plugs was made early the next morning. Twenty females mated in this group in which fertilization had been experimentally delayed.

For each of the 9 days that normal and delayed matings were scheduled, a control group was run concurrently to verify the time of ovulation in unmated females. Thus, following each series of hormone treatments, one to three (total for all series, 18) females were autopsied at 10 to 11 hours after CG to confirm previous findings that ovulation does not occur this soon. Similarly, for each series, one to three females (total for all series, 22) were autopsied at 12 hours after CG to be assured that ovulation had begun in most females by then (see Chapter III).

In order to permit comparisons of the development of eggs from normal and late mated superovulated females with the development of eggs from spontaneous ovulations, a second control series was run. Uniparous or diparous R stock females 2 to 5 months old were paired with strain 101 or CBA males at 5-11 pm and examined for vaginal plugs the following morning. Altogether, 25 females mated and were used in this control series.

The superovulated and spontaneously ovulated females which had mated were autopsied at an estimated 3½ days after ovulation. Thus, superovulated females were killed at 92 to 96 hours after CG injection and the adults were killed between 11 am and 3 pm, three days after the respective mornings on which their vaginal plugs were found. The uteri of these females were excised and their contents were flushed with Pannett-Compton saline (Pannett and Compton, 1924) into small concave watch glasses. After the eggs had been flushed out, they were concentrated near the center of the watch glass by gently swirling the saline containing them. The Fallopian tubes were also examined, but tubal eggs were rare at this time.

Examination of the eggs was carried out under a wide-field binocular dissecting microscope, using 60 times magnification. The eggs from each female were classified individually as one of the following: medium blastocyst (round, with large blastocoel and small inner cell mass), early blastocyst (with very small blastocoele), morula (approximately 8-16 cells, normal cleavage) or inviable (cytolyzed or fragmented). The eggs were then fixed in aceto-carmine and nuclear and chromosomal stains[2] were made according to the Feulgen squash technique, as modified by Slizynski (1949). To assess the rate of cleavage up to 3½ days p.c., the numbers of cells, as determined by counts of stained nuclei, were recorded for the individual eggs of each female.

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[2]The results of the chromosomal counts in these eggs have been the subject of a previous report (Gates and Beatty, 1954).

A group of J stock females was used for a study of the development attained at 3½ days p.c. by eggs from induced and spontaneous ovulations. The animals used for this series were the donors of eggs for the transplantation experiment described in the next chapter. The numbers of blastocysts, morulae, and inviable eggs were recorded for 54 immature females (27-36 days old) and 60 adults (2-5 months old and uniparous).

DESCRIPTION AND ANALYSIS OF RESULTS

Precleavage Stages

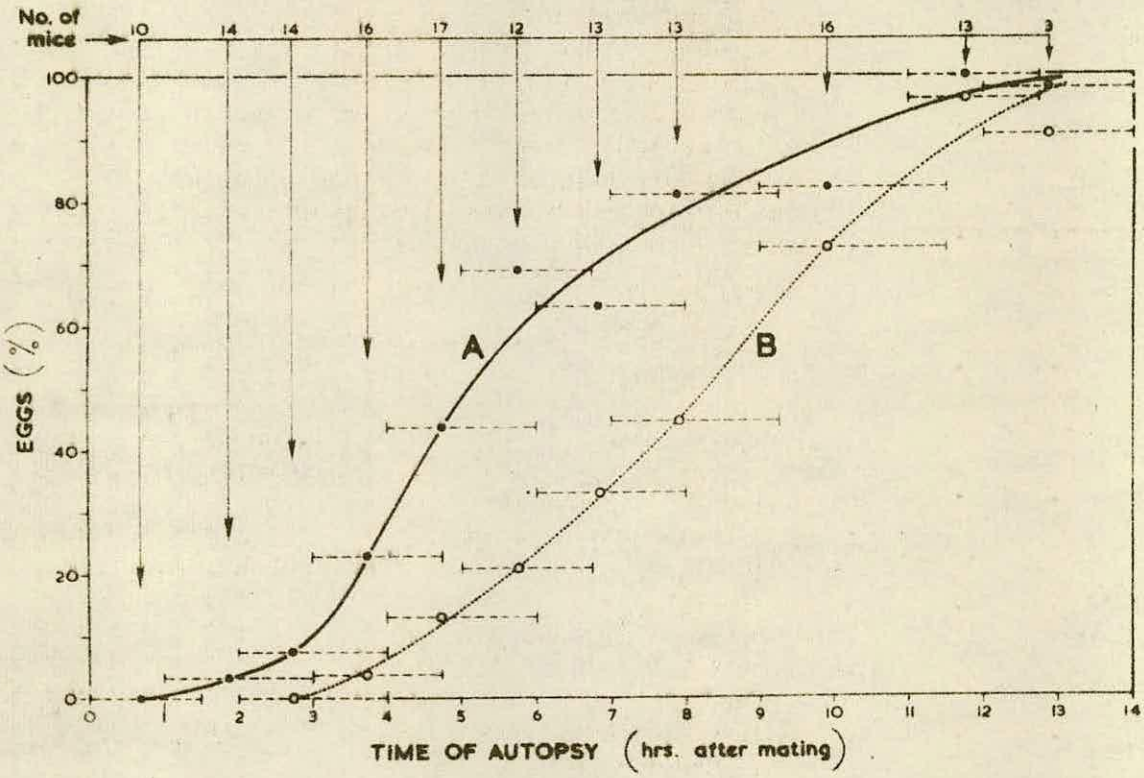
Fertilization. Results of the study to determine the rate of sperm penetration and of pronucleus formation in superovulated eggs are tabulated in Table IV.1. The times of autopsy are expressed both with relation to the time of CG injection and to the time of copulation. The precise time of copulation for each female was not known, but was arbitrarily set at the midpoint of the mating interval (the time elapsing between the last two examinations for a vaginal plug). Since the longest mating interval was one hour, the maximum discrepancy possible between estimated and actual time of copulation was less than half an hour. The average interval between mating and autopsy for each group of females autopsied (Table IV.1, 2nd column) was estimated from the mean of the midpoints of mating intervals of the individual females.

In Figure 5, the percentages of penetrated and pronucleate eggs recovered at each time of autopsy are plotted at points represent-

Table IV.1

THE PERCENTAGE OF SUPEROVULATED MOUSE EGGS PENETRATED BY SPERMS OR PRONUCLEATE, ACCORDING TO BOTH TIME OF CG INJECTION AND TIME OF MATING. Data presented graphically in Fig. 5.

Time of autopsy (Hrs. after CG)	11	12	13	14	15	16	17	18	20	22	23-24
Interval between autopsy and mean for midpoints of mating periods (Hrs:mins)	0:41	1:52	2:44	3:47	4:44	5:47	6:51	7:54	9:56	11:46	12:53
Range: time between last vaginal plug & autopsy to time between first female paired & autopsy (hrs)	0 to 1 1/2	1 to 3	2 to 4	3 to 4 3/4	4 to 6	5 to 6 3/4	6 to 8	7 to 9 1/4	9 to 11 1/2	11 to 12 3/4	12 to 14
No. of females autopsied	10	14	14	16	17	12	13	13	16	13	3
Total eggs recovered	81	205	220	251	234	170	214	229	263	189	53
% eggs with sperm in vitellus	0	3.6	7.7	22.7	43.6	68.7	63.2	81.8	82.1	100	98.1
% pronucleate eggs	0	0	0	4.0	12.8	20.5	33.0	44.2	72.2	96.3	90.4



PERCENTAGE OF SUPEROVULATED EGGS PENETRATED BY SPERMS (A) OR PRONUCLEATE (B) FROM OUTBRED MICE AUTOPSIED AT VARIOUS TIMES AFTER MATING.

FIGURE 5

ing the mean mating-to-autopsy intervals, with broken lines indicating the shortest and longest possible intervals between copulation and autopsy in individual females. The time required for completion of sperm penetration in the immature superovulated females, as a group, was approximately 11 hours, beginning at one to two hours and ending at about 12 hours after mating. This compares favourably with the 12 or more hours required for the mature, untreated mouse (Braden and Austin, 1954a). It may be of interest to note that the mean number of eggs, 15, in the present series was about twice that, mean of 8 eggs, in Braden and Austin's untreated mice. The average distance between lines A and B in Figure 5. suggests there is a mean interval of 2½ hours between sperm penetration and pronucleus formation. Braden and Austin reported this to be 2 3/4 hours for the spontaneously ovulating mouse. From these results, it would seem that the time relationships involved in fertilization of superovulated ova are consistent with those of ova from untreated mature mice.

Of importance to the subsequent discussion is the high percentage of superovulated eggs which were fertilized. The 16 females which were autopsied at 22 to 24 hours after CG (11 to 14 hours after mating) had superovulated a total of 242 eggs, 99.2% of which bore sperms in the vitellus and 94.6%, male and female pronuclei. Thus, fertilization and the second maturation division of the egg was seen to have proceeded normally in a very high percentage of the eggs of these superovulated immature females.

Development before fertilization. The incidences among the eggs of immature females of those anomalies which occurred independently of fertilization are shown in Table IV.2. Most common among the abnormalities were eggs that had been ovulated as primary oocytes (shown by Noyes, 1952, not to be fertilizable). The large clear germinal vesicle in the centre of each such egg was evidence that nuclear differentiation leading to the first maturation division had not yet occurred. The 0.5% incidence (11 out of 2,028 eggs) of primary oocytes among the superovulated mice in the present series was lower than that found by Austin and Braden (1954b) among adult mice of an unspecified strain. The incidence of unfertilized uninucleate eggs (0.1%) and eggs having undergone "immediate cleavage"[3] (0.2%) were similarly low. The total percentage frequency of superovulated eggs undergoing abnormal development independently of fertilization was less than one percent.

Development after fertilization. The abnormalities noted in superovulated eggs just after fertilization are shown in Table IV.3. A comparison with the incidence of similar anomalies in adult mice is presented from the data of Braden (1957b). The stocks of females and males used for both sets of data were the same. Of the 1,719 eggs examined in the work of this chapter, 16 (0.9%) had two sperms within the vitellus. Fertilized uninucleate eggs were less common (0.5%), and of the 9 found, two were gynogenetic. In each case, a

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[3] See Braden and Austin (1954b) for a definition of their proposed term "immediate cleavage".

TABLE IV.2

ABNORMALITIES, INDEPENDENT OF FERTILIZATION, IN EGGS OF SUPEROVULATED MICE

Incidence compared with that in published data[b,c] for untreated adults.

Abnormality	Incidence in immat. ♀♀ [a] No. abnormal eggs / Total examined	%	% incidence in adult ♀♀
Primary oocytes	11/2028	0.5	1.4[b]
Immediate cleavage	4/2028	0.2	0.2[c]
Uninucleate, unpenetrated eggs	2/2028	0.1	---

a. Eggs examined from 21-32 day old superovulated J stock females mated to A strain males.

b. From Austin and Braden (1954[b]), based on 1120 eggs from adult mice of an unspecified strain.

c. Data from Braden (1957), based on 1335 eggs from matings of adult J stock females to males of various strains.

TABLE IV.3

ABNORMALITIES IN RECENTLY FERTILIZED EGGS OF SUPEROVULATED MICE

Incidence compared with that in published data[a] for adults, same stocks.

Abnormality	Incidence in immat. ♀♀ [a] No. abnormal eggs/Total examined	%	% incidence in adult ♀♀[b]
Dispermy	16/1719	0.9	0.6
Uninucleate, penetrated eggs	9/1719	0.5	4.5
Polar body suppression	1/1719	0.06	0.0

a. Fertilized eggs examined from 21-32 day old superovulated J stock females mated to A strain males.

b. Data from Braden (1957), based on 160 fertilized eggs from adult J stock females mated to A strain males.

sperm and two pronuclei (one of them large and presumably a male pronucleus) were seen within the second polar body, and a single, small female pronucleus remained in the vitellus. In the one observed case of polar body suppression, there was one large pronucleus and two small, presumably female, pronuclei in an egg which was penetrated by only one sperm. The total incidence of superovulated eggs that were developing abnormally after recent fertilization was only 1.5%.

In addition to the aforementioned anomalies, all of which have been previously described (Austin and Braden, 1953 and 1954b, and Braden and Austin, 1954b), four cases of an obviously delayed second maturation division were found. The author knows of no previous reports of this for mouse eggs. In three of these eggs, the male pronucleus was fully formed, but the chromosomes of the second maturation division were still in late anaphase, the spindle not yet having rotated prior to extrusion of the second polar body. In the fourth egg, a well-formed male pronucleus was present when the egg was only just completing the second maturation division. These 4 eggs with retarded second polar body extrusion were not classified as abnormal since no predictions about their subsequent development could be made.

3½ Days P.C.

Viability. A summary of percentage survival and the stage of development reached at 3½ days by eggs of prepuberal and adult R stock mice is given in Table IV.4. The percentage of inviable eggs is based

Table IV.4

SURVIVAL AND DEVELOPMENT TO 3½ DAYS GESTATION OF SUPEROVULATED EGGS FROM HYBRID R STOCK MICE

Comparison between immature and mature mice and between immature mice superovulating relatively low and high numbers of eggs.

Maturity of ♀	Range in No. eggs per ♀	No. ♀♀	Mean No. eggs	% eggs inviable	Mean No. blastocysts	Blastocysts as % of living eggs
Immature	7-91	15	42.9	34.4	12.7	45.0
Mature	4-16	25	10.2	27.0	3.8	50.3
Immature	7-33	7	23.6	23.0	9.4	52.0
Immature	35-91	8	59.8	38.3	15.5	42.0

on eggs that were neither blastocysts nor apparently normally cleaved morulae. It was felt that the number of inviable eggs which was missed was insignificant since (1) great care was taken in examining uterine flushings and tubal contents and (2) the total number of eggs recovered compared wery favourably with that found in the same stock 2-4 hours after completion of superovulation using the same gonadotropin treatment.

The percentage of inviable eggs from immature R stock females (34.4%) was slightly greater than that of eggs from adult females (27.0%). However, there was a great difference between the number of eggs superovulated (mean 42.9 and range 7-91 eggs) and the number spontaneously ovulated (mean 10.2, and range 4-16). When percentage mortality was analyzed on the basis of numbers of eggs superovulated, in half of the animals, those that superovulated only 7-33 eggs, the percentage of dead eggs (23.0%) did not exceed that of eggs from mature animals (27.0%). On the other hand, in those females which superovulated between 35 and 91 eggs (mean 59.8) the mortality of eggs was 38.3%. A χ^2 test based on the total number of eggs in each group ($\chi^2 = 12.65$, $P<0.001$), indicated that there was a significant difference between mortality in eggs of immature hybrid R stock females that ovulated relatively few eggs and those that yielded high numbers of eggs.

The results of analysis of numbers of living and dead eggs recovered from J stock females are shown in Table IV.5. In this stock, the egg yield after superovulation (mean 16.7) was considerably

Table IV.5

SURVIVAL AND DEVELOPMENT TO 3½ DAYS GESTATION OF SUPEROVULATED EGGS FROM OUTBRED J STOCK MICE

Comparison between immature and mature mice and between immature mice superovulating low and relatively high numbers of eggs.

Maturity of ♀	Range in No. eggs per ♀	No. ♀♀	Mean No. eggs	% eggs inviable	Mean No. blastocysts	Blastocysts as % of living eggs
Immature	4-48	54	16.7	6.2	10.9	69.3
Mature	2-14	60	10.2	4.8	8.4	86.9
Immature	4-14	26	9.2	5.9	6.8	78.2
Immature	15-48	28	23.7	6.0	14.6	63.0

less than that in the hybrid R stock females (mean 42.9). The percentage mortality in the outbred J stock did not differ between 3½-day eggs from immature and mature females nor between eggs from females superovulating 4-14 eggs and those superovulating 15-48 eggs.

Developmental capacity. The relative numbers of blastocysts and morulae recovered at 3½ days provide a basis for comparison of the development reached at this stage in eggs from prepuberal and adult mice. Since, in R stock mice, the percentage of inviable eggs varied in the different groups, as previously shown in Table IV.4, development was scored in terms of the number of blastocysts as a percentage of living eggs only. The percentage of blastocysts was similar between relatively poor ovulators (7-33 eggs) and mature animals: 52.0% and 50.3% blastocysts, respectively, but was somewhat lower (42.0%) in superior ovulators (35-91 eggs), with $\chi^2 = 3.54$ ($P = 0.05 - 0.1$). Therefore, as with percentage survival to 3½ days, developmental rate tended to show a decrease with increasing numbers of eggs superovulated by the immature hybrid females.

The results of appraising development in terms of number of nuclei in 3½-day eggs from superovulated R stock hybrids are shown in Table IV.6. Whereas 10.4% of superovulated eggs had not yet begun their fourth cleavage by 3½ days p.c., there were no eggs from mature animals that were equally retarded. Also, the percentage of eggs having completed 5 or more cleavages was significantly less for immature females (60.7%) than for mature females (75.1% with $\chi^2 = 4.87$, $P<0.05$), further indicating the retarded development of a large

Table IV.6

COMPARATIVE CLEAVAGE RATE TO 3½ DAYS GESTATION OF EGGS FROM IMMATURE AND MATURE MICE

Frequency, according to number of nuclei and cleavages, of eggs recovered at 3½ days p.c. from R stock mice. (Percentages in parentheses.)

No. nuclei-	4	8±2	10-13	16±2	19-29	32±3	36-60	64±3	68-125
No. cleavages-	2	3		4		5		6	
10 immat. ♀♀	1	9	13	25	39	40	72	16	6
	(0.5)	(4.1)	(5.8)	(11.3)	(17.6)	(18.1)	(32.6)	(7.2)	(2.7)
15 mature ♀♀	0	0	0	6	12	29	21	3	1
	(0)	(0)	(0)	(8.3)	(16.7)	(40.3)	(29.2)	(4.2)	(1.4)

percentage of superovulated eggs by 3½ days p.c.

The developmental rate up to 3½ days for superovulated ova from the J stock is summarized in Table IV.5. Development, expressed as the percentage blastocysts among all living eggs was found to be retarded in eggs of superovulated females (69.3% blastocysts) as compared to adult controls (86.9% blastocysts). Even when developmental rate was considered only in those females which superovulated numbers of eggs (range 4-14, mean 9.2) similar to that of adults (range 2-14, mean 10.2), the percentage of blastocysts, 78.2%, was still lower than that of adults, 86.9% (χ^2 on total eggs, 9.36, $P<0.005$). Retarded development at 3½ days was found to be associated with increasing numbers of eggs shed in this stock, as it was with R stock females. Thus, J stock females superovulating eggs in high numbers (range 15-48, mean 23.7) had a lower percentage of blastocysts (66.0%) than did the group ovulating low numbers (4-14 eggs) cited above (χ^2 on the totals, 11.55, $P<0.001$).

Effect of delayed fertilization. The effect of experimental delay in the time of mating upon percentage survival of superovulated eggs to 3½ days p.c. was striking. The degree in delay of fertilization was estimated as follows. It was shown in Chapter III that superovulation in this stock is virtually complete by 14 hours after CG and in this chapter, that the earliest recorded sperm penetration occurred more than one hour after mating (Fig. 5). Since the females for this series were paired with males more than 4 hours after ovulation (between 18 and 23 hours after CG), it follows that the earliest that any of the eggs in this series could have been fertilized was 5 hours after ovula-

tion. However, the mean time at which females were exposed to males was 5.8 hours after ovulation (i.e., 19.8 hours after CG). It may be seen in Figure 5 that 50% of eggs were not penetrated until 5 hours after mating. This sets a minimum estimate for the time at which a majority of eggs could have been penetrated at about 11 hours after ovulation.

The over-all percentage mortality after delayed fertilization was 68.4% at 3½ days p.c. This differs significantly from the 34.4% mortality for eggs from superovulated females with a normal mating time. In Table IV.7, the females which were subjected to delayed mating are divided into 4 groups of equal size on the basis of number of eggs recovered from each. The percentage mortality of eggs is shown to increase with increasing number of eggs ovulated from 48.7% (range of 8-32 eggs recovered) to 76.1% (in females with 63-98 eggs). The rate of development attained by these eggs after delayed mating did not, however, change with number of eggs ovulated. As a result, the effect of delayed fertilization was such that the mean number of blastocysts recovered from females ovulating a mean of 75.4 eggs (2.8 blastocysts) did not differ appreciably from that in females ovulating only a mean of 23.4 eggs (2.2 blastocysts).

DISCUSSION

Any cessation or retardation of the normal development of an egg prior to its implantation in the uterus could possibly be attributed to one or more of the following factors: (1) aberrant maturation before fertilization, (2) retarded or abnormal fertilization,

Table IV.7

EFFECT OF DELAYED FERTILIZATION ON SURVIVAL AND DEVELOPMENT OF SUPEROVULATED EGGS TO $3\frac{1}{2}$ DAYS GESTATION

Relationships of viability and development to number of eggs superovulated by hybrid R stock mice subjected to >10 hours delayed coitus.

Range in No. eggs per	No. ♀♀	Mean No. eggs	% eggs inviable	Mean No. blastocysts	Blastocysts as % of living eggs
8 to 32	5	23.4	48.7	2.2	18.3
36 to 45	5	40.0	65.0	3.2	22.9
47 to 62	5	52.4	67.6	2.6	15.3
63 to 98	5	75.4	76.1	2.8	15.6
Totals	20	47.8	68.4	2.7	17.9

(3) failure to be fertilized, or (4) failure to cleave either at a normal rate or at all. In the following discussion, the contribution of each of these factors to the viability or development of superovulated eggs prior to implantation is assessed from the experimental evidence just presented.

Precleavage stages

The low incidence of abnormalities occurring independently of fertilization in superovulated eggs from immature mice (0.8% of all eggs) suggests that there was no appreciable degree of abnormal maturation prior to fertilization as far as could be determined by microscopical examination of the internal details of the eggs. All the types of abnormalities found in superovulated eggs after fertilization (Table IV.3) would probably have led ultimately to inviability of the eggs affected. However, their total incidence (1.5%) was not as great as that found by Braden (1957b) in identical stocks of adult mice (5.1% abnormal eggs). Sperm penetration and the formation of pronuclei (in other words, the completion of the second maturation division) was found to proceed at a rate comparable to that reported by Braden and Austin (1954a) for adult mice. By 11 to 14 hours after mating, about 95% of the eggs of superovulated immature mice had been fertilized and had reached the stage of pronucleus formation. In summary, the viability and development reached by superovulated eggs from immature mice just prior to the beginning of cleavage appears to have paralleled that of eggs spontaneously ovulated by adult mice.

The high percentage of fertilization among superovulated eggs (95%) which was obtained in the present series of experiments is not in accordance with the low percentage reported by Austin (1950) for eggs of superovulated immature rats. In 30 rats mated after superovulation, the percentage of total eggs fertilized was 45%, and even among females with one or more fertilized eggs, only 63% were fertilized. This contrasted with the high fertilization rate in adult control rats of 89%. If a comparison is justified between the data herein reported for immature mice and Austin's data on the immature rat, the discrepancy in rates of fertilization may be partly accounted for by the smaller mean number of ova shed, 15, in the mice of this series as contrasted to 37 ova in Austin's rats. The decrease in the sperm/egg ratio with increasing numbers of eggs superovulated may be an explanation for the low rate of fertilization reported by Austin for immature rats, rather than some inherent deficiency in the superovulated ova.

Data presented by Braden and Austin (1954c) indicate that the number of sperms reaching the site of fertilization in the mouse may be quite limited. They counted an average of only 17 sperms (range 2-75) in each tubal ampulla at 10-15 hours after ovulation. If this approximates to the absolute number of sperm available for fertilization, a decreased percentage of eggs fertilized might be expected to accompany the superovulation of excessive numbers of eggs. However, the recovery, in this study, of a maximum of 82 blastocysts and morulae from one superovulated mouse at $3\frac{1}{2}$ days p.c. demonstrates that

fertilization of a large number of eggs is not impossible when adequate numbers of sperm are present at the site of fertilization. One important point to be made from results presented in this chapter is that in view of the high fertilization rate obtained (95%), treatment of immature mice with gonadotropins, in itself, does not lead to ovulation of nonfertilizable ova.

3½ Days p.c.

Viability. A decreasing percentage survival of eggs to 3½ days with the superovulation of numbers in excess of about 35 has been demonstrated in the hybrid stocks of mice used. Possible explanations for the relationship with increasing numbers of eggs are: (1) decreased fertilization rate due to decreasing sperm/egg ratio, (2) increasing percentage of abnormal eggs shed, or (3) an association with immaturity of the female (due to the inverse relationship between age and number of eggs superovulated -- see Chapter II).

Evidence against the third possibility has been provided in recent, unpublished work. In two groups of females, both 21-22 days old, the numbers of eggs superovulated were controlled by the PMS dosage. The corresponding rates of survival for eggs from females ovulating 51.1 ± 5.1 eggs (range 33-73) and those ovulating 10.6 ± 1.06 eggs (range 4-17), were 68.7% and 86.6%, respectively ($\chi^2 = 15.8$, $P<0.001$). Thus, a statistically significant negative relationship between the number of eggs superovulated and the percentage survival to 3½ days was demonstrated to exist in the absence of an effect of age.

That immature females ovulating excessive numbers of eggs (i.e., 35 or more) may be shedding a correspondingly large number of potentially inviable eggs is a possibility that needs further investigation. Unfortunately, at the time cytological examinations were being made of precleavage eggs, the conditions for getting consistently high egg yields were still unknown. However, in spite of the proportional increase in mortality with increasing numbers of eggs, the yield of normally developing 3½ day eggs by superovulation greatly exceeds that possible from mature females. Thus, for the R stock, the mean number of living eggs recovered at 3½-days was 36.9 for immature females superovulating more than 35 eggs (mean 59.8), compared to only 7.5 living eggs from mature females at 3½ days. Among J stock mice, in which the egg yield after superovulation was 50% greater than that after spontaneous ovulation, the survival rate of eggs from immature females was not less than that for eggs from adults at 3½ days p.c. Therefore, it would seem that superovulation per se does not result in decreased viability of eggs up to 3½ days of gestation.

Developmental capacity. One characteristic of eggs recovered at 3½-days p.c. from superovulated mice was the great variation in degree of development attained. Thus, as shown in Table IV.6, whereas the modal number of cleavages for 3½-day eggs from immature mice was over 5, eggs with only three cleavages were not too uncommon. Casida et al. (1944) had likewise noted a great range in development of superovulated eggs, one ewe having eggs with from 2 to 16 cells.

These authors suggested that either (1) eggs of the earlier stages might have been dead but not yet recognizably fragmented upon examination or (2) there were variations in length of development accounted for by differences in time of ovulation. It is doubtful, however, if either of these possibilities could account for the variations in development reported in this chapter. Firstly, fixation in aceto-carmine prior to making nuclear counts had disintegrated the non-living eggs, and secondly, the duration of ovulation was shown in Chapter III to be, at most, only about 4 hours.

As with the percentage of survival, the rate of development up to 3½ days p.c. of eggs from superovulated mice as a group was found to be retarded with increasing numbers of eggs shed. Despite the over-all retardation, however, the absolute number of well developed eggs (i.e., blastocysts) was directly proportional to the number of eggs superovulated. Since development has already been shown to proceed at a normal rate during maturation and fertilization, in J stock mice, the slight retardation in development of superovulated eggs before implantation at least in that stock, is apparently a result of a delayed rate of cleavage in some of the eggs.

Effect of delayed fertilization. The importance of regulating mating time with respect to time of ovulation is shown by the results of experimentally delayed matings. When fertilization was not allowed to occur until about 11 hours or more after ovulation, the percentage of eggs surviving to 3½ days (32%) was about half that of eggs following undelayed mating (66%). The findings of Blandau (1952) would seem

to indicate that much of this loss can be attributed to reduced fertilization rate and increased incidence of abnormal development. Blandau found that in rats inseminated 9-12 hours after spontaneous ovulation only 71% of their eggs were fertilized, of which 43% were developing abnormally. Data presented in this chapter demonstrate that the effect of reduced survival rate following delayed fertilization was such that, with increasing numbers of eggs superovulated (from mean of 23.4 to mean of 75.4 eggs), there was no appreciable increase in number of developing eggs at 3½ days (from a mean of 12 to mean of 18 eggs, respectively). Therefore, the large yields of eggs possible from superovulation are nullified in terms of the number that can survive to 3½ days when fertilization is delayed to more than 10 hours after ovulation.

The results of the experimentally delayed matings may provide an explanation for the decreasing percentage survival (and possibly, for the retardation in development) among 3½-day eggs with increasing numbers of eggs superovulated in the hybrid stocks of mice used. The effect of increased numbers of ova, relative to the limited numbers of sperm at the site of fertilization may be to extend the interval between the time of onset and completion of sperm penetration of ova in any given superovulated female, thus resulting in a considerable delay in fertilization for the last of the ova to be penetrated.

CHAPTER V

VIABILITY AND DEVELOPMENTAL CAPACITY OF SUPEROVULATED EGGS FROM 3½ DAYS P.C. TO TERM AS STUDIED BY TRANSPLANTATION

INTRODUCTION

It is well known that eggs from superovulated immature animals can develop into what appear to be normal young. What is less certain from the literature is the percentage of such eggs that are able to develop and the degree of development achieved at birth compared to that of foetuses from spontaneously ovulated eggs of adults.

Superovulated eggs have developed to term in the uteri of their sexually precocious mothers. Developing in this environment, the eggs usually have a low rate of survival (Engle, 1931b, for the mouse; Cole, 1940, and Austin, 1950, for the rat; and Parkes, 1943, for the rabbit). Even when the frequency of pregnancy is increased by progesterone therapy, prenatal mortality in the prepuberal animal is high (Smithberg and Runner, 1956). The development of superovulated eggs within the uteri of their immature mothers does not, however, allow distinction between subnormal viability due to factors associated with possible immaturity of the host's reproductive system and that due to possible innate deficiencies of the superovulated eggs.

Attempts to estimate postimplantation viability of eggs from animals treated with gonadotropins have been made by transplantations to adult hosts. Warwick et al. (1943; see also, Murphree et al.,

1942) reported 10% survival of 3-day post coitum (p.c.) eggs transplanted from immature rabbits to 5 adult hosts that became pregnant. However, the survival of transplanted eggs from mature donors was equally low, 7.1%. Runner and Palm (1953) obtained a 14.2% survival for eggs from immature mice following transfer before fertilization to adult hosts. They concluded, since Runner (1951) had reported 20% survival of ova artificially ovulated from pregnant mice, that "... unfertilized ova from prepuberal donors may survive less frequently than do ova from pregnant [mature] donors."

In the present work, viability of mouse eggs from immature and mature donors is compared after development in the uteri of genetically similar adult hosts. The findings were reported in a preliminary publication (Gates, 1956) and confirmatory results have been published by McLaren and Michie (1956).

Previous evidence regarding the capacity for normal embryonic development of the eggs of superovulated prepuberal animals has largely been based on gross appearance of the developing young. In addition to Warwick et al., and Runner and Palm (cited above), Chang (1948) and Adams (1954), both working on rabbits, comment on the normal appearance of young developing from superovulated eggs of immature animals after transfer to mature hosts. However, both Willett (1953) and Adams (1954), after reviewing previous literature, conclude that more data are needed to establish the capacity of superovulated ova for normal development. The present study, in addition to estimating viability, utilizes a quantitative measure of the growth of foetuses to compare eggs from

immature and mature mice as to rate of development in the same uterine environment.

MATERIALS AND METHODS

Direct comparison of the development attainable by foetuses derived from immature and mature females was made possible by the transplantation of eggs from superovulated donors to adult hosts already bearing their own fertilized eggs. A second group of transplants, from mature donors to mature hosts, had a twofold purpose: (1) it permitted a comparison of the survival rate of superovulated and spontaneously ovulated eggs developing under similar conditions (i.e., after transplantation), and (2) it permitted a study of the effect upon subsequent embryonic growth of handling of the eggs during transfer. Finally, a group consisting of mated, intact females served as a control for any general effects which the transplant operation might have had on eggs native to transplant hosts. The two experimental groups, involving transplantations from immature and mature donors, were run concurrently.

The Experimental Design

Donor and recipient matings were so arranged that foetuses from transferred and native eggs, though distinguishable by presence or absence of eye pigmentation, were otherwise similar in genetic background. The females used as donors and recipients were of the albino J stock, noted for its high fertility. The two types of males used for matings of the donors and recipients were closely related. One

was of the albino inbred strain A, the other was from a homozygous non-albino stock derived by repeated back-crosses in which segregation was forced at the albino locus. To compensate for any possible effect which genes at- or closely linked to- the albino locus might have upon embryonic development, the genetic marker (pigmented eyes) was alternately applied to embryos from transferred and native eggs. The experimental design for the egg transplantation operations, including number of animals used, is outlined as follows.

Type of donor	No. hosts receiving eggs	No. donors of eggs	Genotype of foetuses native to the host	Genotype of foetuses from transplanted eggs
Immature	37	33	+ c	c c
Immature	37	30	c c	+ c
Mature	41	32	+ c	c c
Mature	45	35	c c	+ c

Treatment of Donors and Recipients

The females used as donors of superovulated eggs were 27-36 days old. They were given one injection of 2 to 4 i.u. PMS between 5 and 10 p.m., followed about 42 hours later by 2 i.u. CG injected between 11 a.m. and 2 p.m. The adult donor and recipient females were all uniparous and 2-5 months old. These, together with the immature donors, were paired with males of the appropriate genotype between 8 and 12 p.m. on the day of CG injection and were examined for vaginal plugs on the following morning. At the time of the transplant operations, the eggs of the donor and recipient females were approximately

3½ days old, dated from the estimated time of ovulation. (Spontaneous ovulation in adult females of the C stock, closely related to the J stock was found by Braden (1957a) to begin at midnight and to be completed in 50% of females by 5 a.m.

Handling of Eggs Prior to Transplantation

The uteri of donors were flushed with Pannett-Compton saline (Pannett and Compton, 1924) and the eggs were collected in watch glasses as described in Chapter IV. Eggs that appeared as normal morulae or blastocysts (under 60 times magnification) were drawn into thin-walled capillary pipettes (inner diameter about 200μ) which were operated by mouth suction. An air bubble, between two short columns of saline, was first drawn into the pipette to mark the position of the egg-containing column of fluid. The latter was approximately 0.75 cm. long, the eggs being positioned near the opening of the pipette.

An attempt was made to equalize the numbers of transferred and native young competing in the same host. The average litter size in J stock females is about 9. Thus, in most of the operations, each uterine horn of a host received 4 eggs, the total of 8 per host usually coming from a single donor. Occasionally an adult donor yielded less than 6 eggs; in such a case, transfer of her eggs was made to a single uterine horn.

The Transplantation Technique

The method of egg transfer was that used, but not described,

by Runner and Gates (1954b). Propsective recipiants were anaesthetized with a 3:1 mixture of chloroform and aether. A mid-dorsal incision (0.5 cm. long) was made through the skin, followed by incisions through the peritoneal wall directly over each ovary. The remainder of the operation was conducted under a binocular microscope of 7 times magnification. The ovarian fat pad and ovary were pulled through the skin incision until the mesentery of the utero-tubal junction was exposed. This mesentery was held with forceps while the anterior tip of the uterine horn was pierced with a fine-pointed syringe needle. The capillary pipette containing the eggs was inserted through the opening thus made. The eggs were slowly expelled until the air bubble beyond the egg-containing column of fluid could be seen through the uterine wall to reach the tip of the pipette. During the actual transfer, and as the pipette was withdrawn, a pair of fine watchmeker forceps were clamped over the site of puncture through the uterine wall. Before the ovary and its fat pad were replaced they were moistened with physiological saline to reduce the chances of adhesions. The incision through the skin, but not those through the peritoneum, was closed with a single 11 mm. suture clip.

The egg medium (Pannett-Compton saline) was prepared under sterile conditions and stored in a refrigerator between operations. The watch glasses, syringes and needles were boiled for more than 10 minutes in distilled water. At the start of each day's operations, the egg pipettes were rinsed in 70% alcohol, absolute alcohol and

aether, in that order. Immediately after each transfer, the pipettes were rinsed in 0.03% sodium citrate to remove tissue and blood cells. This was followed by a rinse with sterile distilled water. The pipettes were re-used at each day of operation.

Autopsy of Pregnant Recipients

It was considered advisable to autopsy recipients at 18½ days gestation (about one day prior to full term) for the following reasons: (1) this permitted assessment of viability by individual uterine horns as well as by hosts, (2) the incidence of early embryonic mortality could be estimated from the number of resorption masses, (3) foetal weight is almost surely less variable than weight at birth, which varies with length of gestation, time of discovery of the litter, number of urinations (frequent just after birth), and presence or absence of milk in the stomach, and (4) neo-natal loss by cannibalism was avoided.

At autopsy of the recipients, foetuses were dissected out of each uterine horn, and their umbilical cords were severed. All surface fluid was removed by submersion of each foetus in warm water, followed by drying with blotting paper. Foetuses developing from transplanted eggs were distinguished from those native to the host by presence or absence of eye pigmentation. The uterine horn from which a foetus came, as well as its sex, was recorded. It was felt that the sexing of foetuses at 18½ days p.c. equalled the high reliability with which sex was determinable at birth. The very few questionable

cases of sex assessment were confirmed by dissecting out the gonads. Finally, body weights of the foetuses were taken on a chain-o-matic balance, the weights being accurate to within 0.01 gm.

DESCRIPTION AND ANALYSIS OF RESULTS

Effect of Various Factors on Viability after Transplantation

Reproductive capacity of the hosts. The percentage of egg recipients becoming pregnant, 79.6%, was not significantly different from the 85.5% pregnancy among unoperated females of the same stock ($\chi^2 = 0.91$, $P > 10\%$). Therefore, default of pregnancy was apparently due to general reproductive failure of the host, rather than to lack of viability of both the transplanted and untransplanted eggs. For this reason, viability is assessed in the following studies only among those eggs that were transplanted to hosts that subsequently became pregnant.

Stage of development of the eggs at transplantation. The survival rate of morulae after transplantation was markedly lower than that of blastocysts as seen in the summarized data of Table V.1. In the few transplants of morulae from adult donors, the percentage survival was not appreciably different from that of morulae transplanted from immature donors. The combined data for transplants from immature and mature donors show that the survival rate of transplanted morulae, 10.8%, is significantly lower than that of transplanted blastocysts, 44.7%.

Genotype of embryos. Embryos with different genetic markers, cc or +c, were found not to differ in their ability to survive after

Table V.1

EFFECT OF STAGE OF DEVELOPMENT OF EGGS AT TRANSPLANTATION ON SUBSEQUENT SURVIVAL

Comparative survival rate of blastocysts and morulae to 18½ days gestation after transfer to hosts subsequently becoming pregnant.

Stage at transfer	Maturity of egg donor	No. of eggs transplanted	% survival to 18½ days
morula	immature	69	11.59
	mature	14	7.14
	immature and mature	83	10.84*
blastocyst	immature	373	41.02
	mature	320	49.06
	immature and mature	693	44.73*

* χ^2 for difference between these %'s = 35.16, $P < 0.001$

transplantation (see Table V.2). This comparison is based on transplant operations in which the number of eggs transplanted was held constant, i.e., 4 blastocysts per uterine horn. The percentage survival to 18½ days in pregnant hosts only, when based on combined data from immature and mature donors was 35.4% for embryos of the cc genotype and 40.7% for +c type embryos ($\chi^2 = 1.08$, $P>10\%$). Since the percentages are not significantly different, in subsequent analyses of viability, data are combined for transplants using the two genetic tags.

Whole inoculum loss of eggs. A substantial portion of the over-all mortality of transferred eggs was found to occur as losses in which all of the eggs from particular transplant operations were lost. This can be ascertained from Table V.4, which will be discussed in more detail later. Out of all transplants of blastocysts, involving 173 uterine horns, in 42 or 24.3% of the horns there were total losses of the transferred eggs. In order to test whether probability of survival was constant for all transferred eggs, a test for goodness of fit to a binomial distribution, with "p" representing the rate of survival for all transplants (i.e., total eggs survived, 138 divided by total eggs transplanted, 364), was made (Table V.3). The test is based on transfers utilizing a constant number (4) of blastocysts. The resultant high value for χ^2 (24.68, $P<0.001$) demonstrated the lack of constancy in probability of survival. The greatest contribution to the total χ^2 was made by horns with no survivors. The indications

Table V.2

EFFECT OF GENOTYPE ON PERCENTAGE SURVIVAL OF TRANSPLANTED EGGS

Percentage of 3½-day blastocysts surviving to 18½ days gestation in transplants of 4 eggs per horn to hosts subsequently becoming pregnant

Genotype of trans. eggs	Maturity of egg donor	No. blastocysts transplanted	% survival to 18½ days
c c	immature	120	33.33
	mature	72	38.39
	immature + mature	192	35.42*
+ c	immature	88	40.91
	mature	84	40.48
	immature + mature	172	40.70*

* χ^2 for difference between these %'s = 1.08, P>5%

Table V.3.

TEST FOR GOODNESS OF FIT TO A BINOMIAL DISTRIBUTION OF THE NUMBER OF TRANSFERRED EGGS SURVIVING.

Frequency of uterine horns with 0 to 4 embryos surviving after transfer of 4 blastocysts to each horn.

No. transplanted eggs survived per horn (X)	Total transplanted eggs survived in all horns	Total eggs transplanted to all horns	Frequency (No.) of horns with "X" eggs surviving		χ^2
			Observed	Expected[a]	
0	0	104	26	13.52	11.52
1	19	76	19	33.03	5.96
2	46	92	23	30.24	1.73
3	57	76	19	12.31	
4	16	16	4	1.88	
(3 & 4)	(73)	(92)	(23)	(14.19)	5.47
Totals for X = 0 to 4	138	364	91	90.98	24.68[b]

[a]Expected frequency based on survival rate in all transplants, ($p = 138/364 = 0.3791$).

[b] χ^2, with 3 degrees of freedom, has $P < 0.001$.

are that in the transplants herein reported, like those of McLaren and Michie (1956), there is a tendency toward whole inoculum loss of eggs which acts in addition to the random loss of individual eggs.

A study of those donors whose eggs were transplanted to more than one uterine horn was made to see whether the eggs of some donors were more prone to whole inoculum loss than the eggs of others. Seventeen out of all the donors used in the transplant experiments provided blastocysts for more than one inoculum (range, 2-6 inocula per donor; and total inocula, 56). In no case was there total inoculum loss in all of the inocula of any of these 17 donors. Thus, there was no indication that whole inoculum loss was the result of a tendency for some donors to provide eggs, 100% of which were inviable.

Survival in individual uterine horns. The transplantation data were analyzed to determine whether transplants to individual horns of a given host should be regarded as completely independent operations. In 31 hosts that had received equal numbers of eggs in both horns, the correlation in percentage survival between left and right horns was found to be low and not significant ($r = 0.138$, with 29 degrees of freedom). This would tend to indicate that survival after transfer, rather than being dependent largely on the host proper, was more a function either of (1) the immediate environment of the uterine horn in which it implanted or (2) some factor associated with the actual transfer technique.

Transuterine migration of transplanted eggs was found to be rare in the stocks used. Only one case of migration had been observed

among 124 genetically tagged eggs which survived after 194 eggs had been transferred in unilateral operations to 41 uterine horns. This incidence of transuterine migration was considered to have a negligible effect on the assessment of survival rate in individual horns.

Survival of Superovulated Eggs from 3½ to 18½ Days P.C.

The comparison of viability of eggs from immature and mature females after transplantation is shown in Table V.4. For reasons given in the preceding discussions (1) the results of transfers using eggs tagged with the genotype +c and those with cc eggs are combined, (2) only data from transplants of blastocysts are included, and (3) viability is assessed by individual horns, rather than by hosts. The percentage survival of eggs from immature and mature donors in all transplants, regardless of the number of eggs per injection, was 41.02 and 49.06, respectively. However, the frequency of horns with no surviving transplanted embryos was greater in transfers of superovulated eggs (27.6% of horns) than in transfers of eggs from adult mice (20.9% of horns). When survival was assessed only in horns containing one or more transplanted embryos, the survival rate of 56.25% for eggs from immature donors was not significantly different from that of 61.57% for eggs from mature donors ($\chi^2 = 1.54$).

The difference between rates of survival in all transplants (involving 2 to 6 eggs per horn) may possibly result from the fact that the mean number of eggs transplanted from immature donors was greater than that from mature donors (means of 4.29 and 3.72 eggs,

Table V.4

COMPARATIVE VIABILITY OF EGGS FROM IMMATURE AND MATURE MICE

Percentage survival to 18½ days gestation of blastocysts transplanted to mated adult recipients

Maturity of egg donor	No. of eggs transplanted per horn	No. horns receiving eggs	Total eggs transplanted	% survival to 18½ days
		SURVIVAL IN RECIPIENTS BECOMING PREGNANT		
Immature	2 - 6	87	373	41.02
Mature	2 - 6	86	320	49.06
Immature	4	52	208	36.54
Mature	4	39	156	39.74
		SURVIVAL IN HORNS WITH ONE OR MORE FOETUSES FROM TRANSPLANTED EGGS		
Immature	2 - 6	63	272	56.25
Mature	2 - 6	68	255	61.57
Immature	4	35	140	54.29
Mature	4	30	120	51.67

respectively). Transplants of 4 eggs each were most commonly employed, and when calculated from these alone, the survival rate did not differ significantly between transplanted eggs from immature and mature donors: 36.54% and 39.74% survival, respectively: ($\chi^2 = 0.33$). When bias due to both whole inoculum loss and variable numbers of eggs transplanted was eliminated (Table V.4, last column, last two rows), the respective percentage survivals of eggs from immature and adult donors were 54.3 and 51.7. These data provide strong evidence for the normal viability of 3½-day eggs from ovulations induced in immature mice.

Capacity for Normal Embryonic Growth from 3½ to 18½ Days P.C.

Effect of sex, genotype and development at transplantation. Body weights of the foetuses at 18½ days of gestation were used as the measure of embryonic growth of transplanted and untransplanted young. Foetuses developing from the few transplants of morulae which were made, appeared to be retarded in growth and therefore only weights of foetuses from transplanted blastocysts were used in subsequent analyses. The genotype of the foetuses appeared to have no effect on body weight, as shown in Table V.5. Weights did differ for the sexes, however, the mean body weight of the males, 1.258 gms., being 3.3% greater than the mean weight for females, 1.218 gms. (standard error of difference between means = 0.011, $P<0.001$). Thus, in the following analyses of postimplantation development, results were combined from the two types of transplants employing different genetic markers, but weights were always analyzed separately for males and females.

Table V.5

INFLUENCE OF GENOTYPE AND SEX UPON FOETAL WEIGHT

Body weight of 18½-day non-transplanted foetuses from J stock (albino) females mated to albino or non-albino strain A mice.

	c c genotype		+ c genotype		Genotypes combined	
	No. foetuses	Mean wt. (gms.)	No. foetuses	Mean wt. (gms)	No. foetuses	Mean wt. (gms.)
Females	241	1.226	284	1.212	525	1.218*
Males	260	1.251	284	1.265	544	1.258*

* Standard error of difference between means = 0.011, $P < 0.001$.

Methods of data analysis. The design of the experiment enabled comparisons to be made within hosts of the developmental (growth) capacity of eggs transplanted from immature donors with that of spontaneously ovulated eggs, native to the host. Hosts to 3½-day eggs transplanted from adult donors provided a control for the effect of transplantation on subsequent development. The analyses shown in Tables V.6 and V.7 were carried out according to the methods outlined by Snedecor (1956) for an R X 2 table involving disproportionate subclass numbers. Comparisons were made within litters, and for each sex, of differences between mean body weights of the 18½-day foetuses from transferred and native eggs. From these within-litter differences, statistically weighted according to relative numbers of foetuses from the two sources, calculations were made for all litters of the best estimates of the mean differences in weight (and their standard errors) between foetuses from transferred and native eggs (see last column of Table V.6).

Results of data analysis. The mean difference in weight between foetuses developing from eggs of immature donors and those from eggs native to the adult hosts did not differ significantly from zero. The corresponding differences for female and male foetuses were -0.005 ± 0.018 gms. and -0.017 ± 0.014 gms., respectively. Furthermore, results of a completed analysis of variance with a correction for disproportion (Table V.7) showed that when variations due to hosts and to interaction were taken into account, the variance in foetal weight

Table V.6

DEVELOPMENTAL CAPACITY OF 3½-DAY EGGS TRANSPLANTED FROM IMMATURE AND MATURE MICE.

Comparisons within litters of weights of 18½-day foetuses from transplanted eggs (immature and mature donors) and eggs native to the adult recipients.

Maturity of egg donor	Sex of foetuses	No. litters for weight comparisons	No. foetuses from trans. eggs (T)	No. native foetuses (N)	Best estimate of mean difference in weight (T-N) and S.E. (gm.)
Immature	♀	33	75	96	- 0.005 ± 0.018
Immature	♂	35	68	121	- 0.017 ± 0.014
Mature	♀	35	70	119	- 0.013 ± 0.019
Mature	♂	36	70	129	- 0.011 ± 0.018
Immature and mature	♀	68	145	215	- 0.009 ± 0.013
	♂	71	138	250	- 0.014 ± 0.012

Table V.7.

COMPLETE ANALYSES OF VARIANCE FOR FACTORS AFFECTING MEAN DIFFERENCE IN WEIGHT BETWEEN FOETUSES DEVELOPING FROM EGGS OF IMMATURE DONORS AND MATURE HOSTS

From data presented in Rows 1 and 2 of Table V.6.

Sex	Source of variation	Degrees of freedom	Mean square X 1000	Variance ratio	P
	Egg source, trans. or native	1	0.988		
♀♀	Recipients	32	74.588	6.294	<0.001
	Interaction	32	15.452	1.304	>0.10
	Individuals	72	11.853		
	Egg source, trans. or native	1	11.210	1.468	>0.70
♂♂	Recipients	34	87.830	11.499	<0.001
	Interaction	34	13.109	1.716	0.05-0.025
	Individuals	84	7.638		

due to source of eggs (superovulated or spontaneously ovulated) was not significantly greater than that between individuals. In the analysis for males, despite the presence of interaction (at the unconvincing level of P = 0.05 - 0.025), it is quite obvious from the mean squares that variance due to egg source was not of significance. Thus, there is substantial evidence that the capacity for normal embryonic growth of blastocysts transplanted from superovulated immature mice was not inferior to that of spontaneously ovulated eggs.

Effect of Transplantation on Embryonic Growth Rate

Of secondary interest was the question of whether the transplantation technique employed could have a retarding effect on subsequent embryonic growth. The 3rd and 4th rows of Table V.6 illustrate that the development reached at 18½ days by embryos transplanted from adult donors apparently was not retarded relative to that of embryos native to the adult hosts. On the basis of data from all transfers (immature and mature donors) the unbiased estimate of mean difference in weight between foetuses from transplanted eggs and those developing in situ was -0.012 gms. (for both sexes combined). There is less than a 5% probability that any retarding effect of transplantation of 3½-day mouse eggs on 18½-day foetal weight would exceed 0.04 gms., which is only 3% of the mean 18½-day weight of untransplanted foetuses.

Survival Rates Obtainable following Transplantation

Under favourable conditions, very high rates of survival were obtained following egg transplantation. When those operations were excluded which involved undue trauma or injection of air into the uterus, there was a 52.3% survival rate in 36 pregnant recipients which had received 310 eggs in bilateral transplants. Similar "non-traumatic" operations involving 16 unilateral transfers resulted in 64.4% survival of 59 eggs transplanted to hosts which subsequently became pregnant. Equally promising results were obtained in a small series to determine percentage survival in transfers of 3½-day eggs to 2½-day pseudopregnant hosts (stocks similar to those in the present study). In this series, 60.2% of 113 eggs transplanted bilaterally to 10 hosts had survived to term and 80.5% had survived to implantation, as indicated by the number of resorbed, as well as living, embryos. These high survival rates provide good evidence for the efficacy of egg transplantation using superovulated eggs.

DISCUSSION

Survival of Superovulated Eggs from 3½ to 18½ Days P.C.

Determination of the absolute rate of survival for eggs from immature mice is impossible in the absence of a technique which, while allowing development in a mature reproductive system does not in itself affect viability. However, the work reported on in this chapter compares the relative rates of survival of eggs from immature and mature

females following transfer of both types of eggs to similar environments.

Results from the work of other investigators involving large numbers of transplants of mouse eggs as well as results presented in this chapter have indicated that the rate of survival after transplantation seems to be affected by the stage of development of the eggs at the time of transplantation relative to the post-coital stage of the recipient. McLaren and Michie (1956) found that in the 4 possible combinations of transplants involving 2½-and 3½-day eggs transplanted to 2½-and 3½-day hosts, the best rates of survival of transplanted eggs were obtained when the donors' eggs were advanced in age over recipients' eggs. Similarly, Boot and Muhlbock (1953) reported that transplants of 3-day eggs to 2-day recipients were more successful than other (unspecified) combinations. These results indicate, as McLaren and Michie suggest, that "...the implantation of the precocious group hinders the subsequent implantation of their backward foster-siblings." This would seem to have been the case in the present study in which morulae from either immature or mature donors had a lower survival rate (10.84%) after transplantation to 3½-day hosts than did blastocysts (44.73% survival). Since it was shown in Chapter IV that the ratio of morulae to blastocysts at 3½ days was greater in superovulated immature J stock donors than in mature J donors, an assessment of survival rate based on transplants of all living eggs recovered would need to make some allowance for the effect of developmental stage at transplantation on subsequent survival.

For this reason, the comparison of viability of superovulated and spontaneously ovulated eggs is based only on transfers of blastocysts in the present investigation.

As mentioned in the introduction to this chapter, the results presented here have appeared in a preliminary report. Supporting data for the reported findings on survival rate of superovulated eggs from immature mice (Gates, 1956) have been published by McLaren and Michie (1956). Unfortunately, there is no mention in McLaren and Michie's report as to the degree of immaturity of the donors they used, nor are the majority of control transplants with mature donors done concurrently with the rest of the experiments. The mice used as recipients were "miscellaneous adult females". In transfers of 3½-day eggs to 2½-day recipients (a combination favouring the transplanted eggs), they reported an average of 2.03 foetuses surviving per host (15 hosts) from superovulated eggs compared to 2.1 per host (16 hosts) from spontaneously ovulated eggs. The resultant survival rate was similar for all transfers of both types, and though low, about 22%, it was in agreement with the preliminary report (Gates, 1956) of the results here given in full -- that rate of survival from 3½ days to term is comparable for eggs from superovulated immature and spontaneously ovulated mature mice.

The Capacity of Superovulated Eggs for a Normal Embryonic Growth Rate

The only published report to the author's knowledge which compares the postimplantation rate of development of superovulated and

spontaneously ovulated eggs of laboratory animals is that of Adams (1954), using rabbits. He found that the birth weight (33.3 ± 2.5 gms.) of 17 small breed young developing in situ in superovulated immature (90 days old) rabbits was significantly less than that (39.5 ± 0.9 gms.) of 138 young from mature small breed females. Significantly greater birth weights of young from the superovulated eggs (mean 48.9 ± 1.6 gms.) were possible following transfers to a mature large breed host. However, Adams' study lacks the control data needed to demonstrate the comparative growth rate of superovulated and spontaneously ovulated eggs developing in the same uterine environment.

Venge (1950) concluded from his transfers of spontaneously ovulated eggs in rabbits that, "...it is only necessary when calculating the modifications of the birth weight due to transplantation to segregate the influence of the litter size." In the present chapter, in comparing the growth rate (to 18½ days) of foetuses derived from eggs of immature and mature donors, litter size, as well as effect of maternal environment, has been eliminated as a variable. This has been possible since the design of the experiment enabled eggs from immature and mature mothers to develop side-by-side in the same hosts.

In Chapter IV it was seen that a portion of the eggs of superovulated immature mice were already beginning to show retarded development at 3½ days p.c. However, the present chapter demonstrates that those superovulated eggs which have undergone a normal rate of

development up to 3½ days in the immature animal will, if allowed to implant in the uterus of a sexually mature animal, parallel the prenatal growth rate of foetuses from mature mothers.

Efficacy of Transplantation of Eggs from Superovulated Mice

How do the results of the transfer of eggs from superovulated mice compare with those for eggs from spontaneously ovulated mice, or from rabbits? Venge (1950), using normally ovulated rabbits, obtained a 48.1% survival in inter-tubal transfers of 862 twenty-four hour old eggs to 142 subsequently pregnant hosts. Chang (1950) transferred superovulated eggs (maturity of the donors not specified) utilizing combinations of donors and hosts of several different post-coital stages. His best survival rates in transfers involving simultaneous matings of donors and hosts were 58.7% from 121 three and a half-day old eggs transferred to 13 hosts and 62.2% from 209 twelve-hour old eggs transfered to 21 hosts which subsequently became pregnant. Using superovulated immature rabbits, Adams (1954) reported 43.7% survival in 11 pregnant recipients of 64 eggs in the 16-cell stage. Among the investigators who have carried out large numbers of transfers of fertilized mouse eggs from mature donors, Fekete (1947) reports what is probably the largest series to date. In transplants of 1,941 two-day eggs to the uteri of 395 mice which subsequently became pregnant, she reported 35.4% survival. In another large series, Boot and Muhlbock (1956) obtained a survival rate of about 42% in inter-uterine transfers of more than 1,400

mouse eggs (spontaneously ovulated) to 169 subsequently pregnant hosts. Chang's (1950) successes, mentioned above, with 59-62% of rabbit eggs surviving after transfer, represent the highest published survival rates for transplanted mammalian eggs.

Previous reports on survival of superovulated mouse eggs after transplantation have been made by Gates and Runner (1952), Smithberg and Runner (1953), and Runner and Palm (1953), each of the three groups having transplanted eggs before fertilization. The best survival rates obtained by the first two groups of workers were 36 and 33%, respectively, but it must also be noted that unfertilized eggs seem to survive less well after transplantation than do fertilized eggs.

The survival rates obtained in this work, 52 to 64%, are at least as good as those previously reported for the rabbit or for the mouse, utilizing eggs from untreated mature donors. It has also been shown in the work of this chapter that the transplantation of 3½-day eggs from immature mice has no significant retardatory effect upon subsequent embryonic and foetal growth rate in a mature host. Therefore, as an outcome of the use of egg transplantation to demonstrate the normal viability and developmental capacity of blastocysts from superovulated immature mice, it is apparent that the transplantation of superovulated mouse eggs is to be highly recommended for experiments requiring manipulation and subsequent implantation and development of mammalian ova.

CHAPTER VI

GENERAL SUMMARY AND CONCLUSIONS

Effect of Age and Maturity on Superovulation and Induction of Oestrus

Number of eggs superovulated. A relationship has been shown in some stocks of mice between prepuberal age and the number of eggs that may be superovulated. In this work, egg number in the hybrid R stock of mice was found to decrease with increasing age at hormone treatment between 20 and 28 days. The relationship has been confirmed in subsequent work of the author on other stocks of mice and has been extended to show also that a positive correlation exists in the range between two and three weeks of age and that the number of eggs superovulated remains relatively constant with increasing age at treatment after 4 weeks. It is stressed that the relationship has not been demonstrated in all of the stocks of mice tested and that it may be dependent upon the health and preweaning nutrition as well as the genetic background of the animals used. The possibility that the relationship between age and number of eggs superovulated is determined by prepuberal patterns of oogenesis is discussed and warrants experimental evidence.

Injection of female mice at the optimum age of three weeks with an optimum PMS dose of 2 to 4 i.u., followed 36 hours later by 2 i.u. CG has resulted in an average yield of over 60 eggs per mouse

(the maximum obtained in this study was 98 eggs and in subsequent work cited herein, 117 eggs). Thus, the changing potentiality for egg production in prepuberal mice, according to age at hormone treatment, may be put to advantage in obtaining high yields of eggs consistently.

Percentage of superovulated females which mate. Data on outbred J stock mice indicated that, contrary to number of eggs ovulated, the percentage of superovulated mice which mate may be directly proportional to increasing prepuberal age at treatment between 3 and 4½ weeks. However, even though the age which is optimum for superovulation may not be the best for a full oestrus response, the 50% or so of animals that will mate after treatment at three weeks of age is a workable percentage in experiments requiring large numbers of fertilized eggs from few animals. Furthermore, the percentage of mice that are receptive to a male after superovulation may be higher among females judged to be precocious by early opening of the vagina. Two means of having the advantages of both abundant yields of eggs and fertilization in a large percentage of immature mice may be: (1) by artificial insemination of females superovulated at three weeks of age or (2) by selection for treatment of three-week old females judged precocious by early opening of the vagina.

Time Relationships of Superovulation

Time of onset. The onset of superovulation in immature females of the hybrid stocks of mice used was found to occur over a more

narrowly limited range of time than that previously reported for either untreated laboratory animals or for rats and some inbred strains of mice subjected to gonadotropin treatment or to reversed periods of light and dark. Ova had not reached the tubal ampullae of any of the superovulated females autopsied by 11 hours, 10 minutes after injection of CG. The data indicate that for over 90% of treated females, ovulation had begun during the 50 minute interval prior to 12 hours after CG. One hour later, virtually all females had begun ovulating. This uniformity in time of onset of ovulation has made it possible to estimate the rate and duration of superovulation in these mice.

Rate and duration. It was estimated that for any given group of treated females, over 60% of the ova expected to be superovulated were shed within one hour after onset, and over 85%, by two hours after onset of ovulation, i.e., by 13 hours after CG. For females that were potential yielders of eggs in high numbers, as determined by age at treatment, the duration of superovulation did not appear to be noticeable longer, but rate of ovulation (for females as a group) was faster after 12 hours from CG than that among females destined to ovulate low numbers of eggs. In individual females, the duration of ovulation ranged from less than 50 minutes to an estimated 3-5 hours. The maximum numbers of ova recovered from both tubal ampullae of females autopsied at 12, 14, and 16 hours after CG injection were 47, 69, and 79, respectively. Neither the onset nor rate of superovulation appeared to be influenced by the time of day at which the hormone injections were given. Since methods for detect-

ing follicle rupture in the intact animal do not exist, superovulation of the immature mouse is a recommended technique for experiments requiring control over the time of ovulation.

Viability and Rate of Development Prior to Implantation

Precleavage stages. A high percentage of superovulated eggs appeared to undergo normal maturation, sperm penetration and formation of pronuclei. By 11 to 14 hours after mating, about 95% of the eggs from immature mice were fertilized and had completed the second maturation division. Thus, it would seem that just prior to cleavage, the eggs of superovulated immature mice have rates of survival and development comparable to that of eggs from mature mice.

3½ days p.c. Examination of eggs from immature mice at 3½ days p.c. indicated that both the viability and rate of development attained shortly before implantation were inversely related to the number of eggs superovulated. However, in immature females superovulating excessively large numbers of ova, despite the decreased percentage survival and development of their eggs, the absolute number of normally developing blastocysts at 3½ days exceeded that from spontaneously ovulated mice. Only after experimentally delayed mating (to more than 10 hours after ovulation) was the developmental rate so retarded that even excessively large yields of eggs from superovulation resulted in only a slight increase in number of living eggs at 3½ days p.c. The fact that viability and rate of development to 3½ days of eggs from immature females which had ovulated relatively low

numbers of eggs was comparable to that of eggs from untreated adult mice, suggests that treatment of immature mice with gonadotropins per se does not lead to ovulation of subnormal ova.

Viability and Development of 3½-Day Eggs to Term, after Transplantation

Viability. The technique of egg transplantation has enabled a comparison to be made as to the rate of survival between eggs of immature and mature mice, following development in the uteri of genetically similar adult hosts. In transplants of 4 blastocysts per uterine horn, the corresponding percentages of survival for transplanted eggs (competing with the hosts' own eggs) were 37% and 40%, respectively, for eggs from immature and mature mice. Similarly, no significant differences in the rates of survival were found among transplants in which whole inoculum loss of eggs was eliminated as a variable. These data indicate that superovulated eggs which have reached the blastocyst stage of development at 3½ days p.c. are equally as viable as blastocysts having developed from spontaneously ovulated eggs.

Capacity for normal embryonic growth. The transplantation experiments also permitted comparisons to be made within hosts of the developmental capacity of blastocysts from immature donors with that of eggs native to the adult recipients. Development, or specifically growth, was assessed in terms of body weight attained by foetuses at 18½ days of gestation (about one day before parturition). The within-litter mean difference in weight between foetuses developing from eggs of immature donors and those from eggs native to the adult

hosts did not differ significantly from zero. Therefore, it may be concluded that 3½-day blastocysts developing from superovulated eggs have the capacity to undergo embryonic growth rates comparable to those of eggs from mature mothers.

Efficacy of Transplantation of Eggs from Superovulated Mice

The results of the egg transplantations done in this study demonstrate the practicability of egg transfer in the mouse. Firstly, the transplant operation does not appear to reduce the mated recipients' chances of becoming pregnant. Secondly, high rates of survival to term of mouse eggs transplanted at 3½ days p.c. are attainable. In operations not involving undue trauma to the uterus, the percentage survival of eggs was 52% in bilateral transplants to 36 hosts and 64% in unilateral transplants to 16 hosts all bearing their own, as well as the fostered young. Finally, the data provide substantial evidence that transplantation of fertilized eggs does not lead to a significant retardation in subsequent embryonic and foetal growth rate.

Conclusion

In conclusion, this study of superovulation in the sexually immature mouse permits an appraisal of the suitability of the technique for use as an experimental tool in specific research problems.

1) Superovulation of the immature mouse is highly recommended as an abundant source of mammalian ova for study and experimental treatment or manipulation. Under optimum conditions of gonadotropic hormone treatment, the yields of eggs from immature mice

are greatly in excess of those previously reported either for treated or untreated laboratory animals. The fertilizability and normal development of the eggs up to the beginning of cleavage makes the technique an excellent means of obtaining recently fertilized eggs. Likewise, the superovulated immature mouse is an abundant source of eggs in advanced stages of cleavage for, even though development of some of the eggs to 3½ days gestation appears to be somewhat retarded, the number of normally developing blastocysts attainable exceeds that from spontaneous ovulation. Furthermore, accurate timing of the onset of superovulation in the immature mouse, permits treatment of large numbers of eggs at any given stage of maturation such as for the induction of heteroploidy or parthenogenesis.

2) When applied to increased production of animals, pregnancy in the immature mouse, achieved by superovulation, mating and progesterone therapy, permits both a shortening of the generation interval and an increase in litter size. These features could be of great advantage, for example, in genetic linkage studies in the mouse. It can be expected, from the results presented herein, that those superovulated eggs reaching the blastocyst stage at 3½ days p.c. would have the capacity to develop into normal young at birth.

3) Induction of oestrus and ovulation in the immature mouse is -- perhaps most important of all -- well suited to the study of some

of the basic problems in early embryology and physiology of reproduction. Firstly, knowledge of the time of superovulation makes possible both a detailed study of the process of ovulation and precise timing of the development of the egg from fertilization onwards. Secondly, since the prepuberal mouse can be induced to come into oestrus, a more general application of gonadotropic hormone treatment of immature mice is as an approach to the study of sexual maturation in the female. Finally, in view of both the success of transplantation of fertilized mouse eggs attained in this study and recent reports of the feasibility of culturing mouse eggs, the three techniques of (a) superovulation of immature mice to provide large numbers of eggs for (b) treatment and culture in vitro, followed by (c) subsequent transfer to a mature host, when used together, can provide a very practical approach to experimental studies on early development of mammalian ova.

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