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THE APPLICATION OF QUANTITATIVE METHODS TO THE STUDY OF EARLY DEVELOPMENT AND OVARIAN STRUCTURE IN MAMMALS

A Thesis Presented for the Degree of Doctor of Science of Edinburgh University

by
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Contents

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INTRODUCTION

INTRODUCTION

"Measure what is measurable, and what cannot be measured make measurable". This advice, attributed to Galileo, appears to be followed more and more strictly as biology advances. In some departments of biology quantitative methods are more easily applicable and more immediately productive than in others. The most rapid progress in biological knowledge is now being made by methods that are essentially quantitative - biochemical and biophysical methods -, and if the sciences of morphology and embryology are to afford any information which may be correlated with information obtained by the biochemist and the biophysicist, strict quantitative methods must be employed by the morphologist and the embryologist. Even in morphology and embryology there are differences in the extent to which quantitative methods have been applied in different parts of the field. Non-Mammalian embryology offers disproportionately more examples of strict quantitative work than does Mammalian embryology.

The force of the need for detailed and therefore quantitative information regarding early Mammalian development was appreciated in the course of an attempt made by the author to correlate a Mammalian teratological specimen with the theories arising from the experimental production of abnormalities in non-Mammalian animals. (A copy of this study ("Posterior Duplicity in a Dog") is included as an appendix to this thesis.) The investigations here recorded have been made with a view to the extending of quantitative methods to the study of the earliest stages of post-ovulation development in a Mammal and to the study of the histology of a Mammalian ovary. A considerable part of the work has consisted in the devising and adapting of methods suitable to the

material studied - stained and mounted sections. The author feels that it is only by such means that the observational sciences dealing with form and changes of form may make contributions comparable in theoretical and practical value to the contributions of the experimental sciences, which are today the more prolific and the more popular.

General Methods

Quantitative methods may be classified as involving either counting or measurement, although any biometrical study tends to involve both.

Impressions gathered from a series of observations are apt to be erroneous, and the only satisfactory basis for a statement, for example, of the frequency of a structure or phenomenon is actual enumeration. Even the conclusions derived from enumeration are, however, very apt to mislead, and for this reason, where there is any variation from specimen to specimen, the application of statistical tests, usually very simple tests, is necessary to demonstrate whether the apparent conclusions are of significance or whether the occurrences observed might be due to chance. Where the material is limited in amount, like a collection of Mammalian ova, the application of such tests becomes essential, and for those who distrust statistical methods it should be reassuring that the tendency of these tests is in many instances to bring in a verdict of "not proven". Incidentally, experience of these methods creates a scepticism concerning many of the generalizations founded upon small data in embryological and histological literature.

As opposed to simple enumeration, the measurement of size of cells, nuclei and other structures in mounted sections involves

considerable difficulty, and there are some who think that the results are not sufficiently trustworthy to compensate for this difficulty and the laboriousness of securing them. It is pointed out how fixation and other treatment may modify the shape and size of the structures measured and how the position of the structures themselves makes it difficult to determine their true dimensions. Owing, for example, to their obliquity in the section, it is almost impossible to find the true length of connective tissue nuclei. In answer to such criticism it should be recalled that all histological observations are open to somewhat similar objections, and in all fine observational and quantitative work it is essential that the conditions, e.g. fixation, etc., in the various instances are the same, or at any rate to show that differences of technique have no significant effect. The object of all measurement is to refine the crude criteria of size: large, medium and small, and thus to show whether there is an appreciable difference between two sets of structures or specimens. Further discussion of these points is to be found in Section XI, and when the significance of the technique is appreciated it will be realized that the method of measurement of structures prepared in a simple manner such as formalin fixation is a mode of approach which is open in some instances when the approach by complex cytological methods is closed.

Tests of accuracy are necessarily applicable to measurements as well as to the results of enumeration. An important field for the application of such tests is in the estimation of the influence of fixation, staining and other processes. Many instances of this use of the tests are recorded in the following papers.

Scope

A thesis should be more than a collection of studies. It should possess a unity of purpose and subject. It should also be in

some way a complete treatment of the subject chosen.

While unity of purpose and general method may be readily conceded to the following records, the subject lacks in one way a unity. Most of the observations have been upon ferret material, but one set of investigations was carried out on the human ovary. The reason for this was largely the desire to work on material of direct value to human medicine.

That the work on the ovary is incomplete is obvious. Many more investigations along similar lines could be undertaken, for the methods are capable of almost indefinite extension. For example, the methods of volume measurement here used and tested might be employed profitably in determination of the sizes of ovarian ova and follicles.

In one sense, therefore, completeness is almost unattainable, and it is desirable to determine whether in any respect the following studies are sufficiently complete to form a thesis. Inasmuch as it forms a thorough investigation of the ferret ova between the stages of ovulation and the commencement of segmentation, this collection of records may be justifiably claimed to be complete. In another sense also a fair degree of completeness may be claimed for these studies. They may be considered as illustrating the application of refined and critical quantitative methods of various types. The methods of enumeration and of actual measurement are exemplified, and the different methods of measurement suitable to different structures are used, e.g., linear measurement of connective tissue nuclei, volume determination by calculation from linear measurements where the structures (pronuclei) conform sufficiently to a simple geometrical solid, and volume determination by the area-weight method where the structures (ova) are too

irregular ~~to apply~~ for the method just mentioned. The two opposed types of investigation are exemplified, viz, the type in which the material actually in existence is very limited (a collection of tubal ferret ova), and the type in which the material is unlimited (the connective tissue of a human ovary).

If a sufficient degree of completeness be granted to these investigations, it should next be asked whether the field surveyed is sufficiently large. At first glance it seems very small. Not even the whole course of tubal development of the ova has been surveyed. This limitation was self-imposed by the author, who preferred to make a minute study of a small field by new methods, even if these were very laborious, rather than add one more to the numerous embryological records which are based upon non-quantitative observation. Whether the positive results obtained justify this limitation may be judged from the records that follow. The value of the negative results is, however, apt to be overlooked, although the investigation was undertaken partly with a view to securing such results. For example, as has been mentioned, the application of statistical tests has a most important negative value as throwing doubt upon the generalizations of other untested reports. Moreover, as the work progressed, it was realized that the application of such quantitative methods to segmentation stages of Mammalian ova would only be satisfactory after a very great augmentation of the existing collections of ova, not only of the ferret, but probably also of any other Mammal.

Arrangement

In general the arrangement of the records is in the chronological order of their appearance for publication, and illustrates to some extent a development of the technique, for it commences with a study

which was not strictly statistical - a study of the pluriovular follicle. An exception to the chronological order of arrangement is the placing, in the last section of the thesis proper, of the study of ovarian connective tissue nuclei, which is a somewhat separate type of investigation.

General Conclusions

Apart from the specific conclusions reached in the various sections, the following records may, in the opinion of the author, reasonably be claimed to demonstrate, in respect both of ordinary histological material such as the ovary, and also of the ovum just after ovulation, the value of quantitative methods and of tests of accuracy, the size of collections of specimens suitable for these methods and tests, the importance of making due allowance for technique, and the influence exerted by technique upon the results.

THE PLURIOVULAR FOLLICLE, WITH REFERENCE TO ITS OCCURRENCE IN THE FERRET

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THE PLURIOVULAR FOLLICLE, WITH REFERENCE TO ITS OCCURRENCE IN THE FERRET

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One hundred years ago von Baer (1827) referred to his observations of pluriovular follicles in the ovaries of the dog and the pig. Recently was published Hartman's paper (1926), partly devoted to these follicles as they occur in the opossum. During the century which included these two dates, over sixty publications appeared, recording the finding of, or the search for, the abnormality in Mammals. To obtain data for a comparative study, analysis of very nearly all of these is here made, and certain of the data have been selected to form a brief historical summary, arranged according to: year of the publication in which the record is to be found; author; animal. An asterisk denotes a record of "tubes" containing ova.

1827: v. Baer (dog, pig); 1840: Hausmann (dog); 1842: Bidder (cow), Bischoff (rabbit); 1845: Bischoff (dog); 1861 and 1863: Klebs (man); 1863: Grohe (man), Pflüger* (cow, cat, dog, man), Quincke (man, cat, rabbit, cow), Schrön (cat, dog); 1867: Kölliker (man); 1869: Plihal* (cow, man); 1870: van Beneden (man, kangaroo), Slavjansky (man), Waldeyer (man, dog); 1874: Foulis (general: cow, cat, man were chiefly studied); 1875: de Sinéty (dog, cat); 1878: Balfour (rabbit); 1879: Balbiani (dog); Wagener (rabbit, dog); 1880: van Beneden (bat); 1881: Schulin (sheep, pig, cow, bat, man); 1887: Janosik (general: cat, dog, man; rabbit specifically); 1888: Nagel (man); 1891: Alexenko (man); 1893: Klien (man), Schottländer (man); 1895: Hellin (man; reference also to findings of Hensen, Kiwisch and Scanzoni, without detail); 1898: v. Franqué (man); 1899: Stöckel (man), Rabl (man); 1900: Bouin, P. and M. (dog), v. Schumacher and Schwarz (man); 1901: Honoré (rabbit), Loeb (guinea-pig), Rosner (armadillo); 1903: Ancel (dog), Cuénot (armadillo); 1905: Schottländer (man); 1907: Welch (man); 1908: Smyth (dog); 1908–9: v. Winiwarter and Sainmont (cat); 1909: Chappelier (mouse); 1911: Schmaltz (dog, pig, sheep); 1912: Arnold (man), Hansemann (man), O'Donoghue (*Dasyurus viverrinus*), Tur (cat, rabbit); 1913: Kingsbury (cat); 1915: Corner (pig); 1917: Loeb (guinea-pig); 1921: Häggström (man), Woerdeman (man; reference to findings of Strassman, man); 1924: Allen, Francis, &c. (mouse), Kennedy (dog, cat, rabbit, guinea-pig, fowl); 1926: Hartman (opossum, *Macacus rhesus*, cat, dog).

The analysis has revealed in many instances a lack of standardisation. A few observers have, in individual ovaries, estimated the proportion

of pluriovular follicles (Rosner, 1901; Cuénot, 1903; O'Donoghue, 1912; Häggström, 1921). The more important question of the frequency with which ovaries containing pluriovular follicles are met in a series of ovaries has also rarely been adequately answered. Such observations have been made by Schrön (1863: 400 ovarian "Präparate" of cat; 80 of dog), O'Donoghue (1912: serial sections of ovaries of 45 individuals of *Dasyurus viverrinus*), Patterson (1913: over 50 pairs of ovaries of Texas armadillo), and by Hartman (1926: ovaries of about 150 opossums). The widespread distribution of the pluriovular follicle in Mammalia is obvious; of its frequency in the different species we are still largely ignorant, and the possible relationship to twinning or to fertility in general is, in regard to many species, obscure.

After the example set by Hartman (1926) in his extensive observations, it seemed desirable to make a survey of another Mammal, a member of the group Carnivora—the common ferret, for which, apparently, no record of the abnormality has hitherto been published.

MATERIAL AND METHODS

In the present survey, 496 ovaries of adult ferrets were used, and 39 ovaries of young, i.e. non-mature, ferrets[1]. The animals had in most instances been killed by chloroform vapour; a few by coal-gas. The ovaries had been fixed immediately after death by one or other of the following fluids: strong Flemming, Lenhossek, Mann's picro-corrosive sublimate, Maximow, Perenyi, Zenker. Each ovary was cut as a complete series of paraffin sections at 10 or 7 microns. In most cases both ovaries from an individual could be examined. The stains employed were chiefly Mayer's haemalum and eosin, Heidenhain's iron-alum haematoxylin, Mallory's connective tissue stain. Heidenhain's "Azan" stain, and the picro-Ponceau S stain of Curtis have also been used.

As the adult animals were purchased for breeding from dealers, no information concerning the exact age could be obtained. In the following records, unless it is specially indicated that a statement refers to the ovaries of non-mature animals, the implication is that adults only are referred to.

Before the results of the present observations are studied, it is desirable to indicate the limits of the term "pluriovular" as here employed. I do not apply the term to those follicles which differ from the normal merely by the presence of other follicles in their thecae. This exclusion appears from the literature to be traditional. Hartman (1926) further objected to the inclusion of primordial follicles in the group, for these might or might not become pluriovular follicles. In the present numerical survey, I have excluded primordial follicles in which the ova are in contact or are separated by only a single layer of epithelium between their adjacent surfaces, unless the epithelium passes from the non-adjacent side of the one ovum to that of the other without leaving a gap or groove externally.

[1] The material used is the property of the Department of Anatomy, Edinburgh University.

In order to obtain a number of pluriovular follicles for systematic comparison, a survey of the collection of adult ovaries was first made. At the same time an attempt was made to form an estimate of the frequency of the condition. Groups of the different ovaries were formed by random choice, and each ovary in a group was subjected to the same type of examination.

RESULTS OF THE SYSTEMATIC EXAMINATIONS

A. Thirty-nine ovaries from 21 different animals. From each ovary two or three slides were examined, situated at equal intervals in the series. The numbers of sections examined averaged approximately one-tenth of the total.

Ovaries found to contain pluriovular follicles: 13.

Number of animals in which the follicles were found: 8.

Number of pluriovular follicles found: 25.

Maximum in any one ovary: 4 (two instances).

Maximum in any one animal: 6 (two instances).

B. Fifty-eight ovaries, probably from rather more than half that number of animals. (It was not possible in all cases to pair off the ovaries according to the animals to which they belonged.) Two methods were employed on the same ovaries.

Method I. From each ovary 10 follicles of volume about ·00016 c.mm. or over were examined from end to end. Those chosen were the first ten complete follicles of proper size met when the examination commenced at one end of the series of sections. The smallest of these possessed epithelium which had almost entirely passed from the early flat condition to the later cubical; the larger follicles were such as might reveal themselves as pluriovular only when traced through the series containing them.

Of the 580 follicles, 9 were pluriovular, from 8 different ovaries, i.e. about 1·5 per cent. of the follicles examined.

Method II. From each ovary 10 sections were examined minutely. The intervals between them were chosen so as to be as nearly equal as was practicable. The sections examined amounted on the average to about one from every 40.

In the 580 sections, 33 pluriovular follicles were found, from 19 different ovaries. This was, very roughly, 0·1 per cent. of the follicles examined. The difference in this percentage from that of Method I, is largely due to the fact that in the latter primordial follicles were not observed, and that the sections examined were in series.

Maximum number of pluriovular follicles found in one ovary by Methods I and II: 4 (two instances). (In only one instance was a follicle recorded under both headings.)

In Examinations A and B II, conducted on different ovaries, the ovaries found to contain pluriovular follicles were about one-third of those examined.

The remaining ovaries were examined less minutely than the preceding, but a number of pluriovular follicles were recorded in them, and there appeared

no reason to conclude that they differed in respect of the frequency of these structures from those more minutely examined.

COMPARISON OF PLURIOVULAR FOLLICLES

By these various methods, 154 pluriovular follicles were noted From them, 100 were chosen for examination, so that impressions gained in the previous survey should be verified or corrected. Complete follicles and those best preserved were selected; the choice was spread widely among the ovaries containing the specimens. Notes were made under the following headings: Stage of the sexual cycle in the animal; Number of corpora lutea, if the animal was pregnant; Position of the follicle in the ovary; Shape of the follicle; Size in cubic millimetres; Presence or absence of liquor folliculi; Follicular epithelium,—the number of layers and the shape of the cells; Degeneration in the follicular epithelium; Number of ova in the follicle; Degeneration of ovular nucleus or cytoplasm; Relative sizes of the ova; Position of the ova in the follicle and relationship to each other.

THE SPECIES DISTRIBUTION OF PLURIOVULAR FOLLICLES

As already indicated, generalisations concerning the frequency of the condition in ovaries of different species are not numerous. From a survey of the literature it appears that only the following conclusions are justified. Among Marsupials, the opossum presents the condition very frequently. It was found by Hartman (1926) in approximately two-thirds of the preparations he examined (ovaries from about 150 opossums). In another Marsupial, *Dasyurus viverrinus*, it does not seem to be frequent (O'Donoghue). In Eutherian Mammals of the Edentate group, its rarity in the Texas armadillo seems to have been established (Patterson, 1913). As regards Ungulates, the most satisfactory evidence is that based on the wide experience of Corner, who was led to conclude that in the pig the pluriovular follicle is uncommon (1915 and 1923). The view of Schmaltz is somewhat different, probably owing to less abundant material, possibly owing to some differences in the breeds of animals surveyed.

The extent to which some of the genera of Rodents are used for ovarian research, together with the infrequent appearance of reports upon the pluriovular follicle in their ovaries, might be thought to indicate the rarity of the phenomenon in this group. The common attitude of slight interest in the subject may, however, possibly be indicated by the illustration of one such follicle in the mouse ovary, shown by Allen, Francis, and their co-workers during discussion of another subject. Regarding Carnivores, on the other hand, there seems to be a fair consensus of opinion that the dog ovary frequently presents pluriovular follicles (Waldeyer, Balbiani, Ancel, Schmaltz). The only dissentient seems to be Schrön. There seems to be little evidence concerning the ovary of the adult cat in regard to the frequency of the phenomenon. The present investigations have indicated that in the adult ferret the condition is met with moderate frequency; but pluriovular follicles are seldom.

perhaps never, abundant in any ovary, relative to the normal follicles. In Chiroptera, it is of interest to note that van Beneden (1880) stated that the horseshoe bat presented pluriovular follicles more commonly than *Vespertilio murinus* did.

Although a fair number of individual human ovaries containing pluriovular follicles are on record, evidence of the frequency of the condition in the human ovaries is still not abundant. Welch instituted enquiries among many histologists and pathologists; their opinions agreed with that of Waldeyer (mentioned by v. Franqué) indicating the rarity of this follicular abnormality in human (presumably adult) ovaries.

Paucity of data prevents any extensive correlation between the species distribution of this condition and that of other phenomena; but such comparisons as are possible are of some value as introductory to the more fruitful study of individual instances.

CONCOMITANT PECULIARITIES OF OVARIAN STRUCTURE

(*a*) *Plurinucleated Ova*

It has been held by some authors, working with young ovaries, that the multiple nucleus is a precursor of the pluriovular follicle (Quincke; Klebs, 1861 and 1863; van Beneden, 1870; Balfour and Eismond inclined to a similar view). In adult human ovaries apparent stages of transformation from the former condition to the latter have been observed by Stöckel, Rabl, and v. Schumacher and Schwarz. Unfortunately our knowledge of the frequency of plurinucleated ova is as unsystematised as that of the frequency of pluriovular follicles. There is co-existence of the two conditions in the opossum ovary, in which plurinucleated ova are very frequent (Hartman, 1926). In the dog ovary there seems to be no evidence of this frequency. Von Winiwarter and Sainmont recorded the presence of both the follicular and the nuclear abnormality in the ovaries of very young cats. Criticism of this as a suggested mode of origin of pluriovular follicles has been advanced by a number of authors. Bischoff (1863) did not believe in the existence of plurinucleated ova. Waldeyer opposed Klebs' contention that egg division took place in follicles after their separation as such. Observation of individual ovaries from various species of animals, containing numbers of pluriovular follicles, has shown no evidence of association with plurinucleated ova (P. and M. Bouin, Honoré, Arnold, O'Donoghue, Hartman, 1926—a cat specimen).

The present investigations were commenced with the object of ascertaining the frequency of plurinucleated ova in the ferret, as well as of studying the pluriovular follicle; but careful scrutiny of thousands of ova in the adult has failed to reveal an instance of this nuclear abnormality. The series of ovaries from young animals is much more limited, but here also no evidence appears that multiple nuclei in the ova are connected with the formation of the abnormal follicles.

(*b*) *Abundance of Small Follicles*

Even in the anoestrous period, the opossum ovary contains a very large number of small follicles (Hartman, 1923). Individual cases in which pluriovular follicles were accompanied by an abnormal abundance of small follicles have been reported by v. Franqué (human), v. Schumacher and Schwarz (human), and Hartman (1926, cat). In the ferret, follicles of various sizes are to be found in the ovaries abundantly at all periods of the year, and I am not prepared to correlate any abnormality in this respect with the presence or absence of pluriovular follicles.

(*c*) *Epithelial Ingrowths*

Tube-like ingrowths of the germinal epithelium form a marked feature of the ovary of the dog (Waldeyer, Balbiani, Schmaltz). Hartman (1926) compared these to the "primordial egg masses" found in the opossum. (Balbiani, however, stated that in the dog they often contain no ova.) A further comparison is made by Hartman (1926) with the structures found in the ovary of an 18-year-old girl by Plihal, and diagnosed by him as persistent Pflüger's tubes. Individual instances of the co-existence of pluriovular follicles and epithelial ingrowths have been noted by v. Schumacher and Schwarz (adult human) and by Woerdeman (human, probably adult).

The ferret ovary contrasts with that of the dog in possessing a tunica albuginea that forms a definite narrow band lying close beneath the germinal epithelium. Where strands of ova and epithelium extend inwards from near the free surface, their admixture with spindle cells and fibrils is as a rule intimate. Tube-like ingrowths are rare. In the adult ferret ovary I have not found that pluriovular follicles are associated with abnormally numerous epithelial ingrowths or with follicular strands; but, as will be indicated later, it appears that the production of the abnormal follicle is dependent on the relationship of follicular tissue to connective tissue at this very early stage of follicular development. This question of ingrowths is, in fact, merely a part of the larger question now to be treated.

THE RELATIONSHIP OF THE OVARIAN CONNECTIVE TISSUE TO THE FOLLICLES

Two chief types of opinion have been held concerning the mode of action of the connective tissue in the production of pluriovular follicles:

(*a*) Secondary union of follicles owing to weakness of intervening connective tissue;

(*b*) Primary disproportionate development of the follicles and the connective tissue.

(*a*) *Secondary Union.* Three suggestions have been made concerning the sizes of the follicles concerned.

(1) Union during enlargement of primordial follicles suggested by Grohe. In the ferret ovary, two follicles of about primordial size are frequently seen close together, separated only in part by connective tissue, and in any single instance it is not obvious whether this tissue is penetrating or being expelled. The former alternative is favoured by our knowledge of the general tendency of the cells of this tissue to penetrate, and especially by the demonstration of that tendency kinetically by tissue-culture experiments. (See, for example, Fischer, p. 117.) The actual frequency with which the partial separation is seen, compared with the frequency of pluriovular follicles, indicates that invasion is proceeding in the vast majority of cases.

(2) Union of medium-sized or large follicles. Rosner published figures to represent stages in a supposed union of follicles larger than primordial, even containing liquor folliculi. In the ferret it frequently happens that two follicles are pressed closely together, and then the follicular epithelium and thecal interstitial cells become much flattened. I have, however, not found stages in which this was followed by breakage of the intervening connective tissue, and consequent union of follicles. Rosner cited lobulation of pluriovular follicles in support of his contention. In the series I have examined some tendency to lobulation is present in follicles without liquor folliculi and containing a larger and a smaller ovum; the smaller forming a projection. This appears explicable by the growth of the larger. The internal tension of the smaller ovum would prevent its being flattened out over the surface of its growing neighbour, and it would come to project as a lobule. This suggestion also would account for the thinning of the epithelium over the smaller ovum.

(3) Union of a small with a large follicle. Loeb (1917) discussed the "hypotypical" ovary of a guinea-pig which had been used in underfeeding experiments, had had its thyreoid gland removed and the lobe of another thyreoid engrafted subcutaneously. Pluriovular follicles were formed here, the author claimed, mostly by "concrescence"—a process by which small follicles, especially primordial, pushed their way into larger ones, perhaps even into degenerating follicles. In the ferret I have noted a number of instances of the presence of a small follicle in the theca of a larger one, but I find no evidence that the former ever loses its integrity. In the guinea-pig discussed by Loeb, the thickness of the granulosa surrounding the different eggs in a follicle was stated to accord with the character of the egg, and this was thought to be compatible only with the view of secondary union. Thinning of epithelium over the smaller of two eggs that are close together seems explicable by differences in the rate of growth, as mentioned above in connection with lobulation of the follicles (fig. 1).

(*b*) *Primary Disproportionate Development* of the follicles and the connective tissue. Suggested by Schrön, and considered possible by Balfour. This theory was advocated at greater length by Schottländer (1893 and 1905), who stated that pluriovular follicles resulted from the irregular growth of connective tissue in the formation of follicles from egg balls. Hellin, the Bouins, Honoré,

O'Donoghue, and Hartman (1926) adopted a similar explanation (imperfect subdivision of Pflüger's tubes). It is this lack of balance in development between follicular and connective tissue that appears to account for the pluriovular follicles of the ferret. As the theory has sometimes been advanced without detailed consideration of the actual mechanism, it may be of value to mention five factors which have been suggested by specimens observed in the ovary of the adult ferret.

(1) Rate of growth of ova. Two ova of just beyond primordial size may be seen lying very close together, with their adjacent sides parallel and separated by epithelium that is greatly flattened. It appears that in such cases growth of the ova has been too rapid for them to be accommodated by displacement of neighbouring structures, and the follicles, instead of forming two separate spheres, tend to form a single one, which may be then surrounded by a common layer of connective tissue.

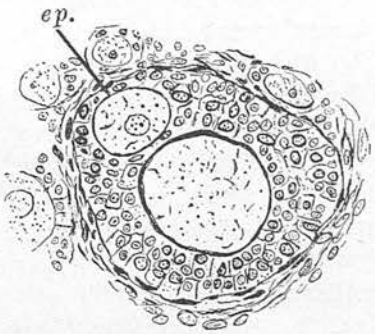

Fig. 1. Two ova in one follicle. The smaller ovum forms a projection, and over it the epithelium, *ep.*, is thin and flat. (× 410.)

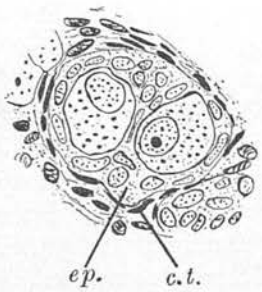

Fig. 2. A stage in the formation of a bi-ovular follicle. The connective tissue, *c.t.*, is excluded from the interovular sulcus by the epithelium, *ep.*, which forms a projection. (× 525.)

(2) Distribution of the follicular epithelium. In the ferret, each primordial ovum is usually surrounded by a single layer of flattened epithelial cells, but, sometimes, only a single layer of epithelium lies between two such ova, and this layer may be very thin, perhaps absent over a part of the surfaces of the ova. This condition is more frequently met than is the small pluriovular follicle, and it therefore appears probable that some other factor is necessary for the production of the latter, even if the single epithelial layer plays a part. Small pluriovular follicles, indeed, frequently show only one layer of flat epithelium between two ova.

(3) Growth of follicular epithelium. The sulcus around the area of contact of two primordial follicles is in some instances filled with epithelium, which may even form a projection outwards instead of the usual depression. Alongside lie connective tissue cells. It appears that these are excluded from the interovular sulcus by the abundance of epithelium (fig. 2).

(4) Slowness of growth or lack of penetrative power of the connective tissue. Evidence of deficient power of the connective tissue was manifested, according to Loeb (1917) by atypical atresia of follicles in the ovary of the guinea-pig, mentioned above, which showed pluriovular follicles. I have not observed such a generalised deficiency associated with pluriovular follicles in the ferret. Factor (3), above, implies a local defect of the connective tissue, at least relative to the epithelium.

(5) Distribution of the connective tissue. In some regions of certain ovaries the connective tissue cells are not so evenly distributed as in other regions. They are arranged in strands along the sides of a row of several follicles, and few at right angles to this, and thus appear less favourably placed than usual for surrounding each follicle before the epithelium of two or more primordial follicles has united to form a common syncytium. In some instances the factor responsible for this distribution seems to be the rate of ingrowth of strands of follicles and epithelium from near the germinal epithelium. Comparison with the ingrowths of the dog ovary is suggested.

Whichever of the suggested factors may be responsible, the essential attainment appears to be an unbroken epithelial surface. There is thus no capillary interval left between the follicles. Instead, there is a capillary interval between the follicular surface and the surface of neighbouring structures; and we may suppose that, under the capillary and surface forces here acting, the young connective tissue cells are compelled to migrate, and thus form the rudiments of a theca common to the epithelium which surrounds the several ova. (For the importance of these forces in the migration of cells, see Fischer, p. 39.)

THE DEGREE OF THE ABNORMALITY

(*a*) *The Number of Ova in a Follicle.* From previous records it appears that the minimal deviation from the normal number of ova is the commonest—the bi-ovular condition. Records of follicles containing a considerable number of ova have been made: about 10 ova (the Bouins: dog); 12 (Hartman, 1926: *Macacus rhesus*); 13 (Arnold: human); over 100 (Hartman, 1926: opossum; this is exclusive of "egg masses").

The importance of serial sections in an estimation of this kind need hardly be emphasised. From the 100 pluriovular follicles that have been completely examined in ferret ovaries, the following figures were obtained:

Seventy-four follicles each contained two ova; 18 contained three; 2 contained four; 3 contained five; 2 contained six; 1 contained at least 13, probably 14 ova, all of them small. (This follicle showed no liquor folliculi.)

(*b*) *Relation of the Epithelium to the Ova within the Follicle.* Van Beneden (1880) described in the horseshoe bat two types of compound follicle: one in which the follicular epithelium separated the ova; the other in which two or three ova were in direct contact. Both types have been recorded in subsequent publications. The results of the examination of the 100 pluriovular follicles in the ferret may be summarised as follows:

Ova in contact: 37 follicles. Of these, 14 showed the presence of more or less liquor folliculi; in one the liquor had probably been absorbed.

Ova separated by epithelium: 63 follicles. Of these, 38 showed liquor folliculi; in one the liquor had probably been absorbed.

Although the distinction is not of great intrinsic value, the presence or absence of epithelium between two growing ova is important in regard to the theory of origin of the zona pellucida. Foulis (1874) and van Beneden (1880) showed that two ova in contact had developed the zonae pellucidae, just as did ova surrounded by epithelium—an indication that the ovum and not the epithelium was the responsible factor. The observations of O'Donoghue, and of Hartman (1926) support this view, as do some of my own. The other possibilities (migration or degeneration of the epithelium after it had formed the zona) were not considered likely by Hartman (1926). In most cases degeneration can easily be dismissed. To prove that emigration does not occur in any individual instance would be difficult; but the numbers given above, so far as they go, suggest that invasion of the interval between ova occurs as the follicle enlarges, after the zona is at least partly formed, the index of size being the presence or absence of liquor folliculi. Among the more developed follicles, disproportionately more show separation of ova by epithelium than among the less developed. From observation it is clear that the epithelium between ova can undergo great flattening as the ova grow, without losing its continuity; and this suggests that where epithelium does not intervene between ova, it has been absent from the beginning.

In the larger pluriovular follicles that have been described, various degrees of separation of the ova have been noted, from those follicles in which a common cumulus contained two or even more closely apposed ova (O'Donoghue) to those described by Welch and by Smyth, in which the ova lay at widely separated parts of a large follicle. Of the 100 follicles specially examined in the ferret ovary, there were 30 in which the cumulus was observable, either completely or partly formed. In 28, the ova lay in separate cumuli or in cumuli which were in the act of separating (see fig. 5, p. 150).

THE RELATIONSHIP OF THE AGE OF THE ANIMAL TO THE OCCURRENCE OF PLURIOVULAR FOLLICLES

Among the observations briefly summarised near the commencement of this article, some were concerned with the pluriovular follicle in young animals only. In this category are the records of: Bidder, Klebs, Grohe, van Beneden (1870), Foulis, Nagel, Klien, Hellin, Loeb (1901), Smyth, v. Winiwarter and Sainmont, Chappelier, and Kingsbury.

Klebs (1861) and Grohe emphasised the comparatively great frequency of the condition in the ovaries of young children.

Of the 39 ovaries of young ferrets that have been examined, about three-quarters dated from the earliest days after birth, and showed principally fol-

licles in the process of formation. The remainder were from animals aged from 4 weeks to about 3 months. The animals contributing to this second group were not all born of one mother. The ovaries of this group showed fully formed follicles, and in all of these ovaries some of the follicles were pluriovular.

The six ovaries of three animals about 3 months old were examined systematically, the sections chosen varying from about one-twentieth to about one-thirteenth of the total number. The pluriovular follicles found varied from 10 to 23 per ovary. Some of these were in the group of deeply placed, cord-like follicles; others were among the more peripheral, large follicles, and were often piriform or lobulated.

The relationship of these types to the pluriovular follicles of adult ovaries is raised by the article of Woerdeman (1921), who described a human ovary, probably adult, in which some of the pluriovular follicles had arisen, apparently, from medullary cords and were therefore homologous with seminiferous tubules.

Elongated follicles, each containing several ova in a row, mentioned by v. Schumacher and Schwarz, are apparently common in the opossum (Hartman,

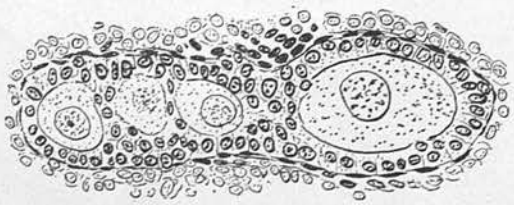

Fig. 3. One of the elongated cord-like pluriovular follicles, rarely found in the adult ferret. (× 410.)

1926). They occur, but are very uncommon, in the adult ferret (fig. 3). Those I have seen are small follicles, lying in the cortex, and do not differ, except in shape, from the other pluriovular follicles. In the adult ferret I have found no evidence that pluriovular follicles arise from medullary cords. V. Winiwarter and Sainmont considered it questionable whether the medullary structures in the adult cat were the same as those of the young animal.

The prevalence of degeneration in the pluriovular and other follicles in the young ferret ovary (as in that of the young cat, v. Winiwarter and Sainmont) indicates that we must account for the pluriovular follicles of the adult by factors present there, as has been attempted above. Nevertheless, the young ovary is of value in this study, because, as Kingsbury pointed out, it illustrates the effects of varying relationships of follicular tissue and stroma. The young ferret ovaries indicate that the lack of balance between these two groups, incident to growth and development, is responsible for the greater frequency of the pluriovular follicle, and also for the greater variety of shape. One of the most striking facts in the three-months' ovary is the comparative infrequency of pluriovular follicles among the small follicles closest to the periphery—

those which have been produced when the relation of connective tissue to follicular tissue resembles most closely that of the adult.

GROWTH AND DESTINY OF THE PLURIOVULAR FOLLICLE

(*a*) *Conversion to Uniovular Follicles* by the ingrowth of stroma—a view entertained, as regards small, especially primordial follicles, by a number of authors, some of whom, however, regarded other fates as also possible. It has been held by Quincke, Schottländer (1893), Hellin, Stöckel, Rabl, and Hansemann. In the adult ferret it does not appear to be at all common for even the smallest pluriovular follicles, as defined earlier in this paper, to be converted in this way. The process appears more probable in young ovaries, but there it is likely to be accompanied by degeneration.

(*b*) *Degeneration of the Contents.* Instances of this were described and discussed by Honoré and by Hansemann; and atresia was accepted as the common fate by Janosik, Schmaltz, and by Hartman (1926).

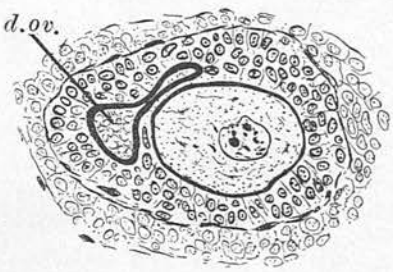

Fig. 4. A bi-ovular follicle. The ovum, *d.ov.*, with thicker zona pellucida, is much degenerated. (× 410.)

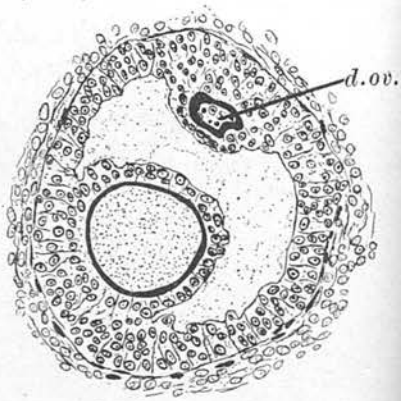

Fig. 5. A follicle showing ova in separate cumuli at opposite sides of the follicular cavity. One ovum, *d.ov.*, is much degenerated. (× 155.)

The evidence of degeneration in ovum, follicular epithelium, or both, noted during the examination of the 100 ferret pluriovular follicles, may be summarised as follows:

Follicles with no liquor folliculi (total: 46): 12 with signs of degeneration; 4 doubtful.

Follicles with a trace of liquor folliculi (total: 19): 11 with signs of degeneration; 4 doubtful.

Follicles with liquor folliculi in moderate or large quantities (total: 33): 31 with signs of degeneration.

From the remaining two follicles the liquor had apparently been absorbed in the process of degeneration.

Great differences in the degree of degeneration in different ova in the same follicle have been noted (figs. 4 and 5).

(*c*) *Growth.* (1) Growth of the ova. The ova in a pluriovular follicle may be all equally developed (Schrön, O'Donoghue, Arnold). Non-degenerate biovular follicles may contain ova unequally developed (Loeb, 1901). Primordial ova may be contained in advanced follicles along with larger ova (Schottländer, 1893; Loeb, 1917; Hartman, 1926). It was as small ova that Statziewitch (see Alexenko) and also Schottländer (1893) interpreted the "Nährzellen" of Nagel. (Hartman, 1926, suggested that Schottländer, Nagel and others had before them different structures—true ova and swollen granulosa cells. The latter are common in or near the cavity of atretic follicles in the ferret (fig. 6), but the resemblance to ova is usually only superficial.)

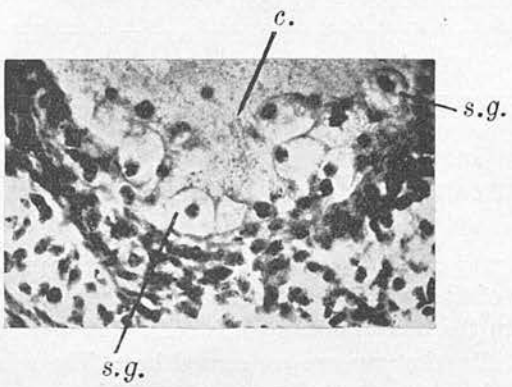

Fig. 6. Swollen granulosa cells, *s.g.*, near the cavity, *c.*, of a large atretic follicle. The nuclei stain more uniformly than do those of ova. (Fixed in Maximow; stained by Heidenhain's Iron-haematoxylin. × 400.)

In the examination of the 100 ferret pluriovular follicles, examples of the various types just mentioned were met. As revealed by the examination of serial sections, without actual measurement of ova, 37 out of the 100 follicles contained ova that were obviously different in size from their companions.

Attempts to account for this disparity are various:

(i) Inclusion of a small follicle in a larger one (Loeb, 1917). This theory has already been discussed, and has not been substantiated for the ferret.

(ii) Arrested development of the smaller of two ova in the same follicle (Arnold). This might account for some cases, but in some follicles the more developed ovum is the one showing obvious degeneration, the less developed being apparently healthy (fig. 4, p. 150).

(iii) Different thresholds of response to growth stimuli, whatever these may be (Hartman, 1926). This appears to be the most satisfactory explanation. Near pluriovular follicles, it is quite common to see ova which differ in size, and yet, from their position, may be presumed to have arisen at the same time, and now appear to be in a similar environment. The finest environmental differences escape histological demonstration, but it appears probable that

individuals differ in their capabilities even at this early stage of their life history. Similar considerations apply to the variation in extent of degeneration, noted above.

(2) Growth of the follicle, and maturation. Many of the pluriovular follicles described in the literature are small, even primordial (e.g. those of Stöckel, Rabl, Hansemann). Large follicles have, however, been recorded (e.g. those of Schulin, v. Franqué, Welch, Smyth, O'Donoghue).

The sizes of 100 ferret pluriovular follicles were estimated in cubic millimetres. They varied from 0·000,041 c.mm. to 0·74 c.mm. The smallest had one layer of cubical epithelium; the largest had many layers of polygonal epithelium and abundant liquor folliculi.

The following ripe pluriovular follicles are on record: one in a dog (Bischoff, 1845); two in *Dasyurus* (O'Donoghue); one in a pig (Corner, 1915).

I have not seen a mature pluriovular follicle in the ferret.

THE RELATIONSHIP OF PLURIOVULAR FOLLICLES TO FERTILITY

"Fecundity" was the term used by Pearl "to designate the innate reproductive capacity of the individual organism, as denoted by its ability to form and separate from the body mature germ cells." It is with this, rather than with general fertility that we are concerned here. The relationship of pluriovular follicles to fecundity might be one of two chief types:

(*a*) *Co-existence with Diminished Fecundity* or sterility (Loeb, 1917). The evidence from ovulation rate in the ferret (mentioned below) inclines in this direction, but is quite inadequate.

(*b*) *Co-existence of the Follicles with Increased Fecundity.* This might imply either direct influence upon the fecundity, or an indirect relationship—the presence of a common cause.

(1) Direct influence upon the fecundity.

(i) Monochorionic twinning was held by Rosner and Ancel to be due to the liberation of more than one ovum from one follicle. This contention was criticised by Cuénot and, as regards the armadillo, with which species Rosner was concerned, it has not survived before the constructive work of Newman and Patterson on specific polyembryony (Newman and Patterson, 1910; Patterson, 1913; Newman, 1917).

(ii) Dichorionic twinning. V. Baer suggested that rupture of a pluriovular follicle would account for cases in which the (total) number of ova liberated was greater than that of the corpora lutea. Cases of this nature have been recorded (Bischoff, 1842; Corner, 1915; Lillie). In none of these, however, is it clear that monozygotic twinning or polyembryonic budding can be excluded. Adequate proof of the liberation of two ova from one follicle can emerge only from a complete examination of ova and ovaries soon after rupture of the follicles. Two animals in the series at present under consideration furnish useful

evidence in this respect. From each, both ovaries with oviducts were preserved as a complete series of sections, with the recently liberated ova still within.

Animal No. 1. Corpora lutea of the one ovary numbered 5; with 5 ova corresponding. Corpora lutea of the other ovary numbered 3; the ova numbered 4. Three of these were at the 7- or 8-cell stage of segmentation; the other was a well preserved unsegmented ovum of about one-third of the volume of each of the others; its nucleus was clearly defined, but showed no signs of maturation.

Both ovaries contained pluriovular follicles.

Animal No. 2. The left ovary contained 7 newly ruptured follicles; the ova corresponding numbered 7. The right ovary contained 3 newly ruptured follicles; the ova corresponding numbered 4. Of these, three were in the oviduct in the pronuclear stage; the fourth was in the periovarian space, was of similar size to the others, but was probably degenerating. No pluriovular follicles were seen in a thorough examination of both of these ovaries.

(2) Indirect relationship of fecundity and the pluriovular follicle. Hellin, v. Franqué and Hartman (1926) have held that the tendency to fertility, manifest in twinning or in large size of litter, conduces to the production of numerous ova and to the formation of pluriovular follicles.

Various points in this view call for discussion.

(i) Concomitance of numerous small follicles with pluriovular follicles. Even if this concomitance were accepted as a general rule, which is not supported by evidence from the adult ferret, it is not clear that a tendency to fertility is manifested by simple abundance of small follicles. It is equally possible that the latter merely indicates a retention to some degree of a non-mature condition by the ovary, and in the non-mature state pluriovular follicles are fairly frequent. Hellin, indeed, admitted this relationship between abundance of follicles, presence of pluriovular follicles and immaturity; but looked upon more fertile species (multiparous) as less highly evolved, as regards reproduction, than the less fertile (uniparous). The ovaries of the multiparous were more embryonic in structure. Against this, it may be urged that, within a species, the fertility of younger animals is, as a general rule, less than that of somewhat older ones (Marshall, 1922). It appears that this lower fertility is actually due to lower fecundity (Hammond). Unless, therefore, the term "tendency to fertility" be taken as synonymous with "abundance of oocytes," there appears to be, as the work of Pearl showed, little relationship of this abundance to fecundity. Some other evidence must, therefore, be sought, if it is desired to prove a relationship between pluriovular follicles and fecundity.

(ii) Ovulation rate. In the opossum, there is, undeniably, co-existence of the follicular abnormality with high ovulation rate, stated to be 22 per animal (Hartman, 1926; see also Hartman, 1919). For the dog, in which it seems probable that pluriovular follicles are frequent, statistics of the ovulation rate are not extensive. The averages from three small sets of figures, given by widely separated observers, have been calculated, and are as follows:

5·4 per animal (Bischoff, 1845: ova, corpora lutea or recently ruptured follicles from 11 animals); 5·2 (Bonnet, 1897: corpora lutea from 11 animals); about 6·4 (Marshall and Halnan, 1917: corpora lutea from 7 animals). On account of the variation in prenatal death in different species (Robinson, 1921; Corner, 1923), litter size is not a satisfactory substitute for ovulation rate; but it may be mentioned that Heape gave the average litter for large breeds of dogs as 7·5 (1464 litters), and for terriers as 5·4 (1158 litters). The dog cannot be looked upon as an extremely fertile animal, and it is improbable that the actual fecundity is very great.

On the other hand, species may be mentioned in which high fecundity is unaccompanied by frequency of the pluriovular follicle. The collection of pig ovaries concerning which we have the most definite pronouncement upon the rarity of pluriovular follicles, is that of Corner. The ovulation rate of these animals (Corner, 1923) suggests that they belong to breeds of rather under the average fertility of British animals, as indicated by Wallace, who held that a sow ought to rear 10 or more pigs in each litter after the first. In view of the size of the animal, however, the fecundity of the pigs studied by Corner is still to be looked upon as high, the lowest average ovulation rate being $8{\cdot}109 \pm {\cdot}118$ per animal (in a group of 156 sows).

Again, Hill gave data from ovulation rate which led him to consider that *Dasyurus viverrinus* had a great tendency to fertility. Yet, in this species, ovaries containing pluriovular follicles do not seem to be frequent in a series (O'Donoghue).

In the ferret, the number of ova shed at ovulation is 9·95 (Robinson, 1921); breeders consider the average birth rate per animal to be 6. Compared with the dog, the ferret has by no means a low fertility, and yet it does not show pluriovular follicles very frequently.

Individual instances of abnormally high fertility accompanied by pluriovular follicles in the ovary have seldom been reported, and the records are not convincing. Ten children had been borne by a woman of 41 whose ovary showed the follicular abnormality (v. Schumacher and Schwarz). Smyth's case of a fertile Gordon setter is sometimes quoted. The evidence has been criticised by Hartman (1926); the chief fault, as regards the present question, is that the ovaries of some of the offspring and not of the mother were examined. Woerdeman made an indirect reference to Strassman's case, in which it was alleged that the ovary of a woman who died while giving birth to twins was found to contain two ova in nearly every follicle. (I have been unable to consult the actual record.)

In the ferret series that has been examined, it has not always been possible to group the ovaries in pairs according to the animals to which they belonged; but the number of corpora lutea has been noted in the various ovaries. The average number of ova shed by each ferret ovary at one ovulation is just under 5. With this may be compared the following figures, obtained from the ovaries which showed the most abundant pluriovular follicles: Two ovaries

from one animal contained respectively 5 and 3 corpora lutea. Two from another contained respectively 4 and 3. Single ovaries, all from different animals, contained corpora lutea as follows: 4 (3 ovaries); 3 (2 ovaries); 1 (1 ovary).

It appears that the tendency to the production of pluriovular follicles may exist in the ovary of an animal which cannot be considered as very highly fecund.

Apart, therefore, from the rare possibility of two mature healthy ova being liberated from a follicle, there seems to be no necessary association of pluriovular follicles and increased fecundity.

THE SIGNIFICANCE OF THE UNIOVULAR CONDITION OF FOLLICLES

The most obvious disadvantage of the pluriovular follicle, as a mode of reproduction, is exemplified by the instance given previously, in which, of two ova liberated from one follicle, one was by no means ready for liberation. The differences in growth rate between ova render the uniovular follicle a more suitable structure for ovulation than the pluriovular.

THE IMPORTANCE OF THE PLURIOVULAR FOLLICLE IN RESEARCH

In the first place, the possibility of rupture of a pluriovular follicle has always to be borne in mind in the study of corpora lutea counts in regard to migration of ova, even in animals where the pluriovular follicle is no more frequent than it is in the ferret.

Secondly, if the chances of liberation of more than one ovum from a follicle are greater than in the ferret, statistics of prenatal death are apt to be affected, these being based on a comparison of the number of corpora lutea with the number of ova found at various stages of pregnancy.

SUMMARY

Analysis has been made of the majority of the previous records of the pluriovular follicle in the ovaries of Mammals. This abnormality is widely distributed; but there has been great lack of standardisation in the accounts given, and there is little knowledge of the frequency of the condition in different species. The conclusions which appear justifiable are that the abnormality is frequently met in the opossum and dog; but is rare in *Dasyurus viverrinus*, in the Texas armadillo, in the pig (at least of some breeds), and probably in *homo*.

Four hundred and ninety-six complete ovaries of adult ferrets have been preserved as serial sections, and these have been surveyed. Certain groups of the ovaries, chosen at random, have been systematically examined. The con-

clusions drawn from them are: (1) pluriovular follicles occur with moderate frequency in the adult ferret, one or more being present in about one-third of the ovaries; and (2) the abnormal follicles are not abundant in any one ovary.

One hundred ferret pluriovular follicles have been investigated, and the data, in part numerical, form the basis of the subsequent discussion.

There appears to be no connection in the adult ferret between the presence of pluriovular follicles and (1) the presence of plurinucleated ova (of which no example was observed); (2) abnormal abundance of small follicles; (3) abnormal abundance of tubular ingrowths from the germinal epithelium.

The pluriovular condition does not appear to be due to secondary failure of the connective tissue intervening between follicles; but to the lack of balance in development between the follicular tissue and the connective tissue at the stage when the small follicles normally become separated from each other. Various conducing factors are suggested by specimens observed (p. 146).

In the 100 follicles examined, the bi-ovular type is the commonest; the greatest deviation from the uniovular being a follicle with at least 13 small ova.

The theory of origin of the zona pellucida is discussed in connection with the absence of the follicular epithelium between growing ova. The conclusion is, on the whole, in favour of the production of the zona by the ovum.

Thirty-nine ovaries of young (non-mature) ferrets have been examined. In this series ovaries containing pluriovular follicles were much more frequent than in adults; and the abnormal follicles in the individual ovaries were also more abundant. The conclusions with regard to the cause were confirmed by these specimens.

Differences in growth and degeneration between the ova of the same follicle are exemplified. The cause is apparently differences in the response of the various individual ova to the stimuli which promote growth or degeneration, as the case may be. Degeneration seems to be the usual fate of these follicles.

The relationship of the follicular abnormality to fertility, in particular to fecundity (Pearl), is treated. Two instances of the rupture of a pluriovular follicle are recorded in the ferret. Apart from the mere possibility of the liberation of two healthy mature ova from a follicle, there seems to be no necessary association of pluriovular follicles and increased fecundity in the ferret. The available data suggest that this conclusion is true of Mammals in general.

The most obvious disadvantage of the pluriovular follicle as a mode of reproduction, is exemplified by the liberation of a non-mature ovum.

The possible importance of the pluriovular follicle in research is mentioned (p. 155).

All the specimens used for the figures are from adult ferrets. Fig. 6 is from an untouched photograph. All the rest are from camera lucida drawings.

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While the above article was in the press there appeared a numerical study of the pluriovular follicle in the mouse by E. T. Engle (*Anat. Rec.* vol. xxxv, p. 341). Evidence is furnished that the condition is not frequently seen in the ovaries of this Rodent.

THE TECHNIQUE OF ESTIMATING SMALL IRREGULAR AREAS IN BIOLOGICAL RESEARCH, WITH NOTES ON THE TESTS OF ACCURACY

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THE TECHNIQUE OF ESTIMATING SMALL IRREGULAR AREAS IN BIOLOGICAL RESEARCH, WITH NOTES ON THE TESTS OF ACCURACY

By DONALD MAINLAND, M.B., Ch.B. (Edin.)
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INTRODUCTION

In biological investigations there is an increasing tendency to establish numerical relationships between phenomena. While following this tendency the morphologist meets with considerable difficulties which arise from the complexity of the objects that he studies, and which materially reduce the accuracy of the results obtained. Investigations which entail difficulties of this kind are those in which surfaces of irregular outline are to be measured with a view to estimating, for example, the relative amounts of different tissues in sections of an organ, or the ratio of nuclear volume to cytoplasmic volume in a cell, or, again, the rates of growth of various organs. In a valuable contribution to this technique, Scammon and Scott (1927) have analysed some of the sources of error inherent in different methods. They have shown, in the first place, that a 7-inch Amsler polar planimeter is by no means sufficiently accurate, especially for small areas (circles of about 1 cm. in diameter).

A second method commonly used for the determination of irregular areas is the area-weight method, whereby pieces of paper of the required shapes and sizes are cut out and weighed, and, if the paper is of uniform thickness, values proportional to the areas are obtained. This method Scammon and Scott (1927) consider to be more accurate than the planimeter method, but still liable to error. Finally, these authors have discovered that sheets of celluloid composition are less liable to introduce errors than is paper.

Probably owing to its tediousness, the use of squared paper in such estimations is not by any means common. It is, however, sometimes necessary to ascertain very accurately the relative sizes of small irregular areas, such as the images of chromatin particles, of small groups of cells, or of bacteria, in a microphotograph or *camera lucida* drawing, or, perhaps, the cerebral convolutions of very small animals; and in such cases the question arises whether the accuracy of the squared-paper method would compensate for its tediousness.

In the course of researches now being conducted upon the early development of Mammals, it was desired to examine in this way drawings of chromatin particles, the areas being about 4 or 5 sq. mm. The two planimeters examined (Amsler's No. 3, and Ott's compensating polar planimeter) were obviously not of sufficiently fine calibration for such areas. It was therefore decided to

compare as regards accuracy the squared-paper method with that in which celluloid is used, with particular attention to areas smaller than those treated in Scammon's and Scott's investigations. Moreover, these investigators purposely confined attention to the variability due to the material (paper or celluloid). Thus, their paper and celluloid discs were not traced by hand and then cut, as would usually be done in investigations of organs or tissues, but were punched out by hollow steel cylinders. Upon the results of Scammon's and Scott's minute and carefully controlled observations are based the present tests; but in these the conditions have resembled to a fair extent those under which the methods would be actually practised.

METHOD OF TESTING ACCURACY

To determine the relative accuracy of different techniques in such a case, elementary statistical methods must be employed. Wherever cells or tissues are compared numerically, tests should be carried out, in order that it may be fully appreciated how far the results are really significant, and it is regrettable that even within recent years records have appeared which show no evidence that tests of error have been performed. Since such tests frequently involve the elementary statistical treatment employed in the present investigation, it will perhaps be of use to record this treatment in some detail.

In some instances, as will be shown, the observations were made by weighing, in others by measurement of areas; but in either case twenty-five observations were made, and from them the arithmetic average or mean was calculated. The object was to ascertain how far the readings were liable to deviate or vary from the average—a variation due to a number of causes, such as lack of uniformity in the material (squared paper and celluloid) and imperfections in manipulation (tracing and cutting). For this purpose the average deviation might be calculated, i.e. a simple average of the differences between the various readings and the mean, all the deviations being considered of positive sign. Instead of this, however, the "standard deviation" is commonly used, especially in biology. Although more difficult to calculate, it has the advantage of laying rather more stress on the larger deviations than does the average deviation. The standard deviation is obtained (Elderton, 1927, pp. 50–1) by finding the differences between the various observations and the average; squaring these differences; adding them together; dividing by the number of observations, and extracting the square root of the result[1]. Roughly, three times the standard deviation on either side of the arithmetic average

[1] An apparently more cumbrous, but actually a simpler method is the following, quoted from King (1921, p. 149): "Select some whole number approximating to the arithmetic average; compute the deviations therefrom; square each; summate; subtract therefrom n times the square of the difference between this number and the true average; divide by n; extract the square root of the quotient." (n is the number of observations, here 25.)

It may be remarked that even those of us whose interests or daily pursuits are non-mathematical find this and other of the simpler statistical methods soon repay the labour involved.

will include all the variates or various observations of which the sample (here 25) has been taken (Pearl, 1923, p. 274).

Certain of the results, however, are expressed in milligrammes and others in square millimetres, and it is impossible immediately to state whether there is greater liability to deviation in the one set or in the other. For this purpose the "coefficient of variation" is employed, which is the standard deviation expressed as a percentage of the average of the series (Pearl, 1923, p. 275).

The mean itself is liable to error. This liability will be small in cases where there are many observations closely equivalent to the mean, i.e. when the standard deviation is small (Elderton, 1927, p. 76), and therefore its liability to deviation can be expressed partly in terms of the standard deviation of the series. The accuracy of the mean naturally also depends on the number of observations. It has been found that this relation is not direct but that the accuracy depends on the square root of the number of observations. Taking four times the number of observations merely doubles the accuracy. Hence the deviation to which the mean is liable is obtained by dividing the standard deviation by the square root of the number of observations. Usually 0·6745 times this number is employed, and is called the "probable error" of the mean. The probable error is of value because, when the difference between two means exceeds three times its own probable error, it is considered significant (Elderton, 1927, p. 79; cf. Pearl, 1923, p. 214). (It may be pointed out that the standard deviation itself is of equal value as a test of significance. The use of the probable error is largely a matter of custom.)

In Test I (*a*) below, the standard deviation of the series of twenty-five readings is ± 0·311. Therefore the deviation to which the mean is liable is $\pm \frac{0{\cdot}311}{\sqrt{25}} = \pm 0{\cdot}062$. The probable error of the mean is therefore

$$0{\cdot}6745 \times (\pm\, 0{\cdot}062) = \pm\, 0{\cdot}042,$$

and the average is expressed as 4·144 ± 0·042.

The probable errors of the standard deviation and of the coefficient of variation have also been calculated (*v.* Pearl, 1923, pp. 274–5), and are inserted at the appropriate places below.

MATERIALS

In the subsequent tests the materials used were:

Thin paper ruled in square millimetres.

A hard pencil for the drawing of the outlines on the squared paper.

Sheets of "Eastman Kodaloid No. 3," the celluloid preparation employed by Scammon and Scott (1927). This material is used in the making of photographic films, and No. 3 has a thickness of approximately 0·135 mm.

Sharp pointed styles, for marking the kodaloid.

A swivel knife. This type of knife, employed by show-card writers in cutting out curved figures, is manufactured by the Paasche Airbrush Co. of Chicago;

it can be more accurately manipulated than scissors, and is to be recommended especially where irregular figures have to be cut.

An analytical balance sensitive to one-fifth of a milligramme.

A fine pair of draughtsman's compasses, capable of being set by a screw.

A hollow steel cylinder or punch.

TESTS AND RESULTS

The tests performed were as follows:

I (*a*). Twenty-five circles were drawn on the squared paper by means of the compasses. The areas were then estimated by counting the squares and fractions. (In this and the subsequent tests, only the parts completely within the boundary lines of the areas were counted.)

Average area (sq. mm.) $= 4{\cdot}144 \pm 0{\cdot}042$.
Standard deviation (sq. mm.) $= \pm 0{\cdot}311 \pm 0{\cdot}030$.
Coefficient of variation (per cent.) $= 7{\cdot}51 \pm 0{\cdot}720$.

I (*b*). A tracing on kodaloid of each of the circles of I (*a*) was made by means of a style. The discs were cut out with the swivel knife and weighed.

Average weight (mgm.) $= 0{\cdot}952 \pm 0{\cdot}054$.
Standard deviation (mgm.) $= \pm 0{\cdot}398 \pm 0{\cdot}038$.
Coefficient of variation (per cent.) $= 41{\cdot}78 \pm 4{\cdot}63$.

The coefficient of variation in I (*b*) is very great. (Compare also the results of Test III (*a*) below.) Under the circumstances described, therefore, the conclusion is that the use of squared paper leads to much less variable results (7·5 per cent. variability) than those obtained by the use of kodaloid (42 per cent. variability). The squared-paper method was actually found to be the quicker and less laborious method for such small areas.

II (*a*). As it was thought possible that the compasses used in Test I had introduced an error affecting both I (*a*) and I (*b*), small round areas were marked on the squared paper by pressing it against a block of cork by the sharp end of a metal cylinder or punch. Twenty-five observations were made.

Average area (sq. mm.) $= 5{\cdot}87 \pm 0{\cdot}057$.
Standard deviation (sq. mm.) $= \pm 0{\cdot}425 \pm 0{\cdot}041$.
Coefficient of variation (per cent.) $= 7{\cdot}24 \pm 0{\cdot}694$.

There is therefore no significant difference between this result and that of I (*a*), and thus the use of compasses was a sufficiently satisfactory means of making test circles.

II (*b*). The same style, made slightly sharper, was used to punch out twenty-five kodaloid discs, each of which was then weighed.

Average weight (mgm.) $= 1{\cdot}584 \pm 0{\cdot}0073$.
Standard deviation (mgm.) $= \pm 0{\cdot}054 \pm 0{\cdot}0052$.
Coefficient of variation (per cent.) $= 3{\cdot}43 \pm 0{\cdot}327$.

The variation in this series is under half that of the corresponding series of actual areas (II (*a*)). It will be noted that a difference of 0·1 mgm., which

the balance used is not designed to detect, would correspond to a difference in area of about 0·35 mm., which would certainly be detected. Relative insensitiveness of the balance may therefore produce a fictitious uniformity of results.

It will further be observed that the variation in this series is very much less than is found in series of kodaloid discs of similar or even of considerably greater size, when these have been traced on to the kodaloid and then cut from it, as in I (*b*) and III (*a*) and (*b*). This test, therefore, indicates the large element of variability introduced by the manipulations, as compared with the variability due to the substance employed. It demonstrates the importance of introducing all the relevant conditions into a test of accuracy before dependence is placed on a method in further investigations.

III. Test I illustrates probably the method most useful in such observations, namely, the direct tracing of the image on to squared paper. This might seem to be a method specially unfavourable to the accuracy of the kodaloid, on which a secondary tracing was made. (To use kodaloid for the preliminary drawing of a *camera lucida* image is not very practicable.) In many cases, moreover, it is desirable to trace from a drawing or photograph on to the squared paper for measurement. For these two reasons the following series of observations (III) were made. Several more or less circular or oval outlines of different sizes were drawn on cardboard with Indian ink, and of each area twenty-five tracings were made on the squared paper, and twenty-five on the kodaloid. The estimations of area and weight were carried out as before.

Results of Test III

		Average	Standard deviation	Coefficient of variation (%)
III (*a*)	Area (sq. mm.)	4·81 ±0·071	±0·526 ±0·050	10·94 ±1·73
	Weight (mgm.)	1·02 ±0·057	±0·419 ±0·040	41·26 ±4·56
III (*b*)	Area (sq. mm.)	9·50 ±0·115	±0·855 ±0·082	9·00 ±0·865
	Weight (mgm.)	3·37 ±0·042	±0·313 ±0·030	9·31 ±0·895
III (*c*)	Area (sq. mm.)	14·11 ±0·075	±0·554 ±0·053	3·93 ±0·375
	Weight (mgm.)	4·70 ±0·029	±0·213 ±0·020	4·52 ±0·432
III (*d*)	Area (sq. mm.)	20·04 ±0·128	±0·951 ±0·091	4·75 ±0·454
	Weight (mgm.)	6·80 ±0·049	±0·367 ±0·035	5·25 ±0·502
III (*e*)	Area (sq. mm.)	49·89 ±0·173	±1·28 ±0·122	2·57 ±0·245
	Weight (mgm.)	14·77 ±0·079	±0·589 ±0·056	3·99 ±0·381

From Test III it will be seen that as the area increases the liability of both methods to error decreases and that the difference between the variability of the two methods (area and weight) becomes less. At the same time the tediousness of the squared-paper method increases. Therefore under conditions such as these, the use of squared paper, although of great value for the smallest areas, is to be recommended only for these.

The larger squares of squared (coordinate) paper are usually bounded by lines which are thicker than the rest. This inequality in thickness of the lines

would naturally be expected to interfere with the accuracy of the measurements. The actual numerical effect of this was determined as follows.

IV (*a*). Twenty-five areas marked by the metal cylinder on squared paper enclosed thin lines, and only in some instances thick lines also. (This series is the same as the one given under II (*a*) above.)

IV (*b*). Twenty-five areas similarly marked enclosed only thin lines.

IV (*c*). Twenty-five areas similarly marked enclosed always thick lines as well as thin.

Results of Test IV

Series	Average (sq. mm.)	Standard deviation (sq. mm.)	Coefficient of variation (%)
IV (*a*)	5·87 ±0·057	±0·425 ±0·041	7·24 ±0·694
IV (*b*)	6·23 ±0·052	±0·385 ±0·037	6·17 ±0·591
IV (*c*)	5·26 ±0·047	±0·350 ±0·033	6·65 ±0·637

The difference in the coefficients of variation, even between IV (*a*) and IV (*b*), cannot be proved significant because it (1·07) is not even twice the value of its probable error (0·91, obtained by extracting the square root of the sum of the squares of the two probable errors 0·694 and 0·591). On the other hand, the difference between the averages even of IV (*a*) and IV (*b*) (= 0·36) is more than four times its probable error (= 0·077), and therefore the difference is significant: the introduction of thick lines has what may be called an appreciable effect on the average. Hence it is to be recommended that for such small areas perfectly uniform squared paper be used.

SUMMARY

A comparison has been made of two methods of determining the sizes of very small irregular areas, such as photographs or drawings of chromatin particles, of small groups of cells, or of bacteria, or the cerebral convolutions of very small animals—(1) the method of measurement by squared paper, and (2) the method by which pieces of the required shape and size are cut out in celluloid and weighed.

Most of the tests have been designed to reproduce closely the conditions under which actual investigations would be carried out.

The numerical results have been treated by elementary statistical methods.

It has been concluded that for small areas (4 or 5 sq. mm.) the squared-paper method is much the more accurate, and is even less laborious than the celluloid-weight method.

As the areas examined are made larger, the value of the squared-paper method rapidly diminishes.

The inaccuracy of the celluloid method appears to be due principally to the manipulations (tracing and cutting), and not to the substance.

The fact that in most squared paper the larger squares are bounded by

thicker lines than the small squares is of importance, for this lack of uniformity has an appreciable effect on the results in the case of small areas.

Owing to the importance of quantitative methods in these and many other biological problems, the elementary statistical treatment used here has been discussed in some detail.

ACKNOWLEDGMENT

To Dr C. H. Goulden of the Dominion Rust Research Laboratory, Winnipeg, I desire to express my thanks for reading the section of this paper that deals with general statistical methods.

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THE EARLY DEVELOPMENT OF THE FERRET:

THE PRONUCLEI

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Embryological literature contains numerous studies of the pronuclear stages of mammalian ova, and the addition to this collection of a report of any considerable size necessitates a word of explanation. The investigations communicated herein were carried out on the ova of the ferret, both of the light coloured and of the dark or "polecat" variety. The ferret belongs to the Carnivore group, and in this group no very complete study by modern methods of the transformations of the pronuclei has previously been carried out. In the second place, the application of statistical methods to a study of this type of specimen has apparently never before been attempted. Finally, in the present records emphasis has been laid on the influence of histological technique in the production of the phenomena observed in the specimens. The statistical treatment of the results of varying techniques in histology is still largely an unexplored field.

MATERIAL AND METHODS

From 30 different ovaries and 19 different animals 101 ova were examined. The animals were kept in captivity and fed on a mixed diet of bread, biscuit, milk and flesh. They were inseminated by the male while they were in oestrus, and were killed at a selected time after mating. The specimens (ovaries with uterine tubes or uterine tubes alone) were fixed, some in Zenker's fluid, others in strong Flemming's fluid, others in Perenyi's fluid, a few in Mann's fluid and a few in Mann's fluid with formol. Paraffin sections were prepared in series at 10 microns. The staining methods were Heidenhain's iron-haematoxylin with eosin or orange G; in other cases Mayer's haemalum and eosin. Most of the Flemming-fixed specimens were tinted with eosin. All the available Flemming-fixed ova were not chosen for examination because their lack of staining did not permit much detail of pronuclear structure to be demonstrated. This absence of random sampling had to be taken into consideration when statistical methods were applied, for random sampling is essential in statistical work. All the other available ova were chosen, except a few badly fixed specimens and a few specimens in which a fair number of sections was lost.

As will be subsequently seen, all the ova observed were not at the pronuclear stage; some were at the polar spindle stage and some at the stage of the first segmentation. The actual numbers of pronuclei taken into consideration are recorded in the appropriate sections below. In all, 71 out of the 101 ova were at the pronuclear stage of development.

STATISTICAL METHODS

In biological work it is becoming more and more widely realised that any phenomena that are capable of enumeration are capable of statistical treatment, and that any proofs based on these enumerations, unless they are statistical proofs, are unsatisfactory. A large part of the present investigations has involved the employment of one of the simpler statistical tests, namely the Chi Square test. As the description of this test in several of the commoner manuals is a little difficult to follow without a greater experience of statistical methods than many investigators possess, it may not be out of place to show how the test has been applied in one of the instances in the present research.

The purpose of the Chi Square test is to demonstrate association or independence between two sets of events or phenomena, e.g. between the number of hours that have elapsed after insemination of the animals and the position of the pronuclei in the ova obtained from those animals; or between the type of fixative used and the size of the chromatin particles in the pronucleus. The former of these two examples will be considered in detail here.

The pronuclei in the ova of the present collection of ferret specimens can be arranged according to the position of the pronuclei in the ovum, whether central, peripheral, or sub-central (i.e. between the central and peripheral positions). At the same time the pronuclei can be arranged according to the number of hours that elapsed between the insemination of the animals from which the specimens were obtained and the killing of those animals. By use of this combined classification the Chi Square test can be employed to ascertain whether there is any significant association between the position of the pronuclei and the number of hours that elapsed between the insemination and the death of the animal. That such an association is bound to exist is undeniable, but the object of the present investigation is to determine whether the present series of specimens is adequate to demonstrate the association.

In Table II, p. 266, there are 21 non-central pronuclei out of a total of 65. The factors, whatever they were, responsible for the distribution of the pronuclei found in this series have operated so as to cause 21/65ths of the total to be non-central. There are 14 pronuclei in the time period (A). Unless some additional factor entered in, it would be expected that the proportion of non-central pronuclei in this group would be the same as in the series as a whole, i.e. 21 : 65. The number of non-central pronuclei in that case would be 21/65ths of 14, i.e. 4·523. This may be called the "theoretical" or "expected" number. The actual number of non-central pronuclei in the group (A) is 9, an excess of 4·477 over the theoretical number. It is natural to suppose that this excess is due to the fact that ova found earlier after insemination will be at an earlier stage of development than those found later. On the other hand, the difference might be due to chance—the accidental presence of an excess, which might not be found if the total number of pronuclei in group (A) were larger. To eliminate this possibility and to show whether there are also unexpected distributions

in the other groups, (B) and (C), the following operations are performed. The expected numbers of pronuclei for the other squares or "cells" are calculated as above, and are shown in Table III. The expected or theoretical number is lettered t and the actual number a. The difference $a - t$ is found. Some of these differences are negative and some are positive. The differences are then squared, a process that eliminates the negative sign and so enables the numbers to be added without their counterbalancing each other's effects. The squaring also gives weight to the larger differences. The squared difference is divided by the corresponding theoretical number and is thus expressed as a proportion of t, since a large difference might mean very little if t also were large. The sum of all the numbers thus obtained is called χ^2, the method being named the Chi Square method. If χ^2 is large it implies that there is a large discrepancy between the expected arrangement and the actual one, some factor or factors having entered in to produce an irregularity in the distribution. In the present instance the two phenomena, time and the position of the pronuclei, are correlated or associated. The arrangement found is not due to mere chance. It is necessary to have some standard by which to decide whether χ^2 is large enough to be significant. This decision can be made with the aid of such a table as Fisher's (1928) Table III. It is clear that with a larger number of squares or cells than those of Table II (p. 266) the value of χ^2 might be equally large and yet not be as significant as if it were the product of a few calculations. The significance is therefore dependent on the number of cells, exclusive, of course, of the squares containing the totals and those containing the headings. If the group (A) in Table II and the first two columns of Table III are examined, it will be seen that the total 14 has been used in the calculation of t, and has therefore influenced the resulting χ^2, and when this total is fixed and one cell, say the (A) N.C. cell, is filled with the number 9, the other cell, (A) C., must be filled with the number 5. Similarly if the total 21 is fixed and two cells in the column N.C. are filled, the number in the remaining cell is determined. In calculating n, based on the number of cells, this fact is taken into consideration, and n represents the number of cells that can be filled arbitrarily. It is spoken of as the number of "degrees of freedom" and is found by taking the number of rows and the number of columns, exclusive of headings and totals, subtracting one from each and multiplying the two together. In Table II, $n = 1 \times 2$. With n and χ^2 Fisher's Table III can be used to find P, i.e. the proportion of cases in which the value of χ^2 will be exceeded, or the probability that χ^2 shall exceed the given value. Thus if the actual number of cells in a Chi Square test is 4, $n = (2 - 1) \times (2 - 1) = 1$, and if $\chi^2 = 0{\cdot}455$, $P = 0{\cdot}50$, and the chances are equal that a greater value of χ^2 should be found in such a case. If there are 6 cells, $n = (2 - 1) \times (3 - 1) = 2$. If χ^2 then $= 5{\cdot}991$, $P = 0{\cdot}05$. The chances of this value of χ^2 being exceeded are 0·05 to 0·95 = 1 to 19. With this value of P (0·05) and with any lower values, χ^2 is taken as significant, for the probability is so small that it should be due to chance. With any higher value of P, χ^2 is considered insignificant. In the present case $n = 2$, $\chi^2 = 9{\cdot}26$

and therefore P is lower than any value in Fisher's table. Hence there is a definite association between the time after insemination and the position of the pronuclei.

For further detail regarding the Chi Square method reference should be made to Yule (1927), pp. 64 *et seq.*, and especially to Fisher (1928), pp. 75 *et seq.*

MEASURES OF DEVELOPMENT OF THE PRONUCLEI

In any embryological study it is most desirable to express stages of development ultimately in terms of time, rather than in terms of contemporary phenomena. To do this directly with mammalian ova is not easy. The event from which time periods are measured in the ferret specimens now under consideration is not the actual fertilisation of the ovum, but the insemination of the animal. Robinson (1918) has shown that ovulation in the ferret may occur at any time between $30\frac{1}{2}$ and $93\frac{1}{2}$ hours after insemination; and, as fertilisation takes place at a varying time after ovulation, the time of insemination is obviously an unsatisfactory initial point from which to measure development. In the present series of specimens this lack of precision is further increased by the fact that in some cases more than one insemination took place during the time when the male and female animals were caged together.

Since it is impossible to obtain directly any close relationship between the duration of development and the phenomena that occur in its course, an attempt may be made to relate certain of the phenomena to others which are known to possess a definite time-sequence. For example, it is known that ova that possess two central pronuclei are at a more advanced stage of development than those in which the two pronuclei are only half way between the periphery and the centre of the ovum, and these ova are in turn more advanced in development than those in which the pronuclei are peripherally placed. This phenomenon—migration of the pronuclei—may be taken as a scale by which to measure other phenomena, e.g. changes in the cromatin of the pronuclei.

It might be thought that with our present embryological knowledge the study of a series of ova from an animal such as the ferret which has not previously been so studied, would consist merely in observing the manner in which the new specimens fitted into series of developmental stages described for other Mammals. As can be seen from such articles as that of O. van der Stricht (1923), however, the Mammals already so studied differ from each other in details, and it is therefore clear that caution is necessary. This is especially obvious when it is remembered that in the Carnivore group, to which the ferret belongs, there has never been described in detail the sequence of changes observed in the pronuclei. In the present study the object is in the first place to ascertain how far the series of specimens shows correlation between the time after insemination and the actual stages of development as represented by the approach of the pronuclei to the centre of the ovum.

Table I. *Position of pronuclei arranged according to the number of hours after insemination.*

(The numbers represent ova.)

Hours after insemination	One present; peripheral	Both present; peripheral	One central; one peripheral	Both subcentral	One peripheral; one subcentral	One present; central	Both central	Totals
41	1	.	.	.	.	.	.	1
41⅓	1	.	.	.	1	.	.	2
41½	1	3	1	1	.	1	4	11
44⅔	.	.	.	1	.	.	.	1
47½	.	4	.	1	1	.	13	19
51¾	.	.	.	.	.	.	2	2
53½	.	.	.	.	.	.	1	1
64¼	.	3	.	.	.	.	12	15
68	.	.	.	1	.	.	.	1
76½	1	.	.	.	.	.	6	7
116½	.	.	.	.	.	.	5	5
Totals	4	10	1	4	2	1	43	65

Table I[1] shows the ova arranged according to the position of the pronuclei and the time that elapsed between the insemination and the killing of the animal. For the purpose of determining whether there was any correlation between the recorded time-intervals and the positions of the pronuclei the Chi Square method was employed.

Table II. *Association between the post-inseminal period and the position of the pronuclei.*

Hours after insemination	Pronuclei not central (N.C.)	Pronuclei central (C.)	Totals
(A) 41 –41½	9	5	14
(B) 44⅔–53½	7	16	23
(C) 64¼–116½	5	23	28
Totals	21	44	65

Table II contains most of the figures from Table I grouped in a manner suitable for this determination. The resulting value of χ^2 is 9·26. From consultation of Fisher's (1928) Table III this is found to be highly significant, since $n = 2$, and therefore P is lower than any value in the table. Moreover, it will be seen from Table II that in the middle period of time (B) the proportion of ova with non-central pronuclei to those with central pronuclei is 7 : 16 (roughly 1 : 2), i.e. about the same as the ratio between the totals (21 : 44). In the earlier group (A) the number of ova with non-central pronuclei is

[1] In the collection of ova studied there were several specimens obtained from an animal 7 days after insemination and several obtained from another animal 9 days after insemination. While the exclusion of these in the investigations upon time which are recorded in the text may be open to criticism, the author feels justified in this selection by his experience of the mating of ferrets for the purpose of obtaining tubal ova.

greater than those with central, while the reverse is the case in the group (C) with a longer period after insemination. One is therefore justified in concluding that in this series treated as a whole the length of the post-inseminal period is a measure of the progress of development, but it should be noted that this does not necessarily apply to the comparison of individual specimens or to groups smaller than the whole series here considered.

Table III. *Details of the calculation of χ^2 from the data of Table II.*

Designation of cell	Theoretical number (t)	Actual number (a)	$a - t$	$\frac{(a-t)^2}{t}$
(A) N.C.	4·523	9	+ 4·477	4·431
(A) C.	10·16	6	− 4·478	2·116
(B) N.C.	7·106	7	− 0·430	0·025
(B) C.	14·89	15	+ 0·430	0·012
(C) N.C.	9·046	5	− 4·046	1·809
(C) C.	18·95	23	+ 4·05	0·865

In subsequent sections of this article the time after insemination is used as a possible measure of the progress of development of the ova; but where, for one reason or another, the series of ova is not exactly the same as in the test just made, it is not assumed without further investigation that the times recorded actually indicated development.

PRONUCLEAR CHROMATIN

(*a*) *Early transformations*

The course of pronuclear development after fertilisation in animals generally, consists, as is well known, in the approach of the two pronuclei to each other, their enlargement, the change of the chromatic material from a more or less uniform basiphilic mass to particles of various sizes, and the final transformation of this material into the spireme of the first segmentation spindle. In the mouse and rat, Kremer (1924) has recently laid stress on a preliminary aggregation of chromatin before the division of it preparatory to the formation of the first segmentation spindle.

In the earlier phases of development, the two pronuclei approach each other and migrate toward the centre of the ovum. Their position, therefore, may be taken as an indication of the degree to which they have developed, and thus be used in studying the transformation of the chromatin. Fourteen ova from the series under examination illustrate various degrees in the migration of the ovum, and a brief description of their pronuclei is given to indicate the range of possibilities in the process of transformation.

In one ovum (no. 11) two pronuclei were found in the peripheral part of the ovum, both stained a uniform dark blue with haematoxylin, one spherical in shape, the other more ellipsoidal. Two other ova (nos. 36 and 48) possibly represented earlier stages than this. They each showed a peripheral deep purple

mass, of irregular shape in the one, spherical and lobulated in the other. These masses, it appeared, might be stages in the conversion of the daughter chromosomes of the second polar spindle into the female pronucleus.

In one ovum (no. 9) both pronuclei were spherical and stained a uniformly deep purple; but while one was peripheral the other was placed between the periphery and the centre of the ovum. Migration had commenced before any visible change in the chromatic material had occurred. On the other hand, in one specimen (no. 7) both the pronuclei were peripheral and of spherical shape; but while one formed a ring with a pale centre and a faintly purple lobulated periphery, the other was a uniformly deep blue mass (fig. 4). In another ovum (no. 10) both the pronuclei were peripheral; one was a spherical homogeneous purple body, while the other showed lobules at its edge which were more deeply stained than was the centre. In ovum no. 13 the two pronuclei were peripheral and somewhat lobulated; one was almost uniformly stained, in the other the centres of the lobules were more faintly stained than were the outer parts. In several ova (nos. 21, 30, 91 and 97) the pronuclei were peripheral and either of spherical or of broad ellipsoidal shape, while the chromatin was in the form of globules and granules of various sizes. A reticulum was found in most of the last-mentioned pronuclei. Ovum no. 90 also belongs to this group, but a full view of its pronuclei was not obtained because it was obscured by a foreign body in the preparation. In ovum no. 43 one pronucleus was peripheral, the other had commenced to migrate towards the centre; both contained globules and granules of chromatin of various sizes. In ovum no. 15 one pronucleus was near the centre of the ovum, the other was still peripheral. Both showed chromatin in globular form (fig. 6). A possibility never to be overlooked is that in a small series like the present one it is difficult or even impossible to determine whether specimens are perfectly normal or not; but with this proviso it appears justifiable to summarise the course of development as follows.

The pronuclei pass from a condition of uniformly stained chromatic material to one in which this material is in the form of globules and granules of various sizes. The change, in at least some cases, occurs by way of an intermediate stage in which the periphery of the pronucleus is in the form of lobules more deeply stained than the centre. The change may occur while the pronuclei are still in the outer part of the ovum, but the pronuclei may commence to migrate to the centre of the ovum before the change occurs.

It will be noted that no attempt has been made in this section to distinguish the development of the male pronucleus from that of the female. A consideration of the differences between the two pronuclei is reserved for a subsequent section.

(*b*) *Size of chromatin particles*

After the pronuclei have come together near the centre of the ovum, there ceases to be any satisfactory criterion by which to determine whether they are at an early or a late stage of development. It has been shown that in this series there is an association between the position of the pronuclei and the

number of hours that elapsed between insemination and death; but one is not therefore justified in assuming that of any two ova the one obtained shortly after insemination is at an earlier stage of development than the other which was obtained later. The majority of the pronuclei in this series of ferret ova were centrally placed. This central stage is a later one than those already considered in which the pronuclei were either peripheral or sub-central. In the central pronuclei the chromatin was always in the form of granules and globules. Some pronuclei showed various sizes of particles—large, medium and small. Others were remarkable because of the large size of their chromatin masses; others showed no very large masses, but some medium-sized and some small; others showed chiefly small particles. These standards are not numerical, but represent in a rough way the differences between the pronuclei in this respect. The question then arises how far one can arrange in order these types so as to conform to the stages described by other authors. In such a study it is necessary to bear in mind the possibilities of: (1) transient alterations in the constituents of the pronuclei, not directly connected with the main course of their development; and (2) the different effects of different fixatives.

At first the pronuclei, central, sub-central and peripheral, were arranged according to the size of the chromatin particles under the four classes: (1) those in which the particles were of various sizes; (2) those in which large particles were specially noted; (3) those in which medium-sized particles were noted, large particles being absent; and (4) those in which small particles were most notable. The pronuclei were at the same time further grouped according to the time that had elapsed after insemination; for it has been shown that the time-intervals in this series considered as a whole correspond to a real progress in the development of the ova. The Chi Square test was applied to demonstrate any association between the time and the size of the chromatin particles. Only specimens fixed in Perenyi's or in Zenker's fluid were selected. Owing to the smallness of the numbers in some of the classes, groups were made as shown in Table IV. The results of this test ($\chi^2 = 13{\cdot}78$, $n = 1$; P much less than 0·01)

Table IV. *Association between time and size of chromatin particles.*

Hours after insemination	Pronuclei with particles of various sizes (V.)	Pronuclei with large particles, medium particles, small particles (L.M.S.)	Totals
(A) 41½–51¾	31	5	36
(B) 64¼–116½	16	20	36
Totals	47	25	72

show that there was a very high degree of association between the size of the chromatin particles and the time which had elapsed after insemination. In the later stages there was an excess of pronuclei in the cell marked "L.M.S." Analysis of the original data revealed that there were no pronuclei with large

particles in the earlier stages, whereas in the later stages there were many with large and many with medium-sized particles, but none with small particles. The conclusion indicated is that the excess of pronuclei in the cell "L.M.S." in the later stages is really an excess of pronuclei with large and medium-sized particles. A possible source of error in these results is the use of two different fixatives—Perenyi's fluid and Zenker's fluid. Another table (Table V)

Table V. *Association between time and size of chromatin particles, specimens fixed in Perenyi's fluid being excluded.*

(The lettering is as in Table IV.)

Hours after insemination	(V.)	(L.M.S.)	Totals
(A)	20	4	24
(B)	12	6	18
Totals	32	10	42

was prepared with the same time-groups and size-groups but with all the specimens fixed in Perenyi's fluid excluded. It will be noted that in one cell only four individuals are found. If less than five individuals are found in any one cell the tendency is for the association to become apparently significant; and therefore if less than five instances are found in any one cell and the association is not significant, the result can be accepted as in no way vitiated by the smallness of the number of instances. In other words, one can produce valid negative results even with less than five instances in a cell. In the present case, where specimens fixed in Perenyi's fluid were excluded there was no significant association between the time and the size of the chromatin particles ($\chi^2 = 1{\cdot}57$; $n = 1$; $P > 0{\cdot}20 < 0{\cdot}30$). A further application of the Chi Square test showed that there was a highly significant association between the size of the particles and the fixative (Perenyi or Zenker), for $\chi^2 = 5{\cdot}30$; $n = 1$; $P < 0{\cdot}05 > 0{\cdot}02$. This association was not so highly significant as that between time and size of particle. It was noted that the significance of the result was largely due to the proportions of the Perenyi specimens, which were equally distributed between the groups representing size of particles, whereas the proportions which held in the series as a whole were 47 with particles of various sizes to 25 with particles large or medium or small. From the original data it appears that this excess of Perenyi specimens in the second group is due to a preponderance of Perenyi specimens in which the particles were large.

Finally it was determined that there was no significant association between the time and the fixative ($\chi^2 = 2{\cdot}06$; $n = 1$; $P < 0{\cdot}20 > 0{\cdot}10$). Since this is so, there was obviously not a sufficient preponderance of Perenyi specimens in the later stages to account for the association between time and size of particle. Similarly the highly significant association between fixative and size of particle could not be accounted for by a preponderance of Perenyi specimens in the later group, where large and medium particles were more commonly found.

The results obtained in this section may be summarised as follows:

(1) There is a highly significant association between the size of the particle and the time after insemination (larger and medium-sized particles being found in later periods). This association exists only with specimens fixed in Perenyi's fluid.

(2) There is a highly significant association between the size of the particle and the fixative, Perenyi's fluid tending to give larger particles.

(3) There is no significant association between the time after insemination and the fixative. The specimens have therefore been chosen in a sufficiently random manner in this respect to render the results statistically sound.

It should be noted that these investigations on the size of the chromatin particles did not include all the specimens for which in an earlier section (p. 266) there was shown to exist a correspondence between the time after insemination and the development, as indicated by the position of the pronuclei. Before it could be definitely stated that there was any association between the development and the size of the particles, it would be necessary to prove this association either directly by reference to the position of the pronuclei in the specimens under consideration, or indirectly, by showing that in these particular specimens the time after insemination was a measure of the progress of development. Unfortunately the smallness of the series of specimens will not allow either of these methods to be employed.

(*c*) *Staining of the chromatin*

Out of a total of 49 central pronuclei, some fixed in Zenker's fluid, some in Perenyi's, and all stained with Heidenhain's iron-haematoxylin, 31 showed globules which were stained pink with eosin or were colourless or faintly stained in contrast to the deep purple or blue-black stain of the remainder of the chromatin. These differences might be due to several factors: differences in fixation, differences in staining technique, or differences arising from the actual structure or development of the pronuclei. Whatever the cause, wherever the pink, pale or colourless globules are present in a pronucleus, it must indicate some difference between those globules and the rest of the pronuclear particles. In the mouse pronuclei Lams and Doorme (1908) described a colourless plasmosome; in the pronuclei of the same animal Sobotta (1895) described the remains of the chromatin nucleoli which appeared as rings filled with karyolymph after the scattering of the chromatin over the other parts of the pronucleus. The exact nature of the pale, colourless or eosinophilic globules in the ferret has not been determined. To eliminate certain of the factors mentioned above as possibly responsible for the differences between the pronuclei in this respect, the Chi Square method was employed.

It was shown that there was no significant association between the fixative and the coloration of the chromatin particles ($\chi^2 = 1{\cdot}408$; $n = 1$; $P < 0{\cdot}20 > 0{\cdot}30$). Thus, neither the pronuclei fixed in Perenyi's fluid nor those fixed in Zenker's fluid showed such an excess of the pink, pale or colourless globules as could not

be accounted for by chance. The Chi Square test was also applied to prove whether there was any association of the colour of the pronuclear particles with the depth to which the section as a whole was stained with haematoxylin. There was no dependence of the one on the other ($\chi^2 = 0{\cdot}562$; $n = 1$; $P < 0{\cdot}50 > 0{\cdot}30$). Finally it was shown that the coloration of the particles was independent of the time after insemination ($\chi^2 = 1{\cdot}306$; $n = 1$; $P < 0{\cdot}30 > 0{\cdot}20$). So far, therefore, it has been impossible to demonstrate any cause of the pink, pale or colourless particles in the pronuclei. It is possible, however, that the time after insemination is not a sufficiently fine indication of the stages of development of the pronuclei; for out of 11 well-defined peripheral pronuclei in which the contents were granular or globular, not one showed any pink or pale or colourless particles, whereas out of 49 central pronuclei 31 showed such particles. Since the probability of the occurrence of a pronucleus with one or more of these particles is 31 : 49, and the probability of the occurrence of a pronucleus without such a particle is 18 : 49, then, on the assumption that the same factors are at work in the production of the staining reaction in both the central and peripheral pronuclei, it is possible to calculate the chances of all the particles being stained a deep purple or blue-black in the eleven peripheral pronuclei. Details of the theory and method will be found in Pearl (1923), pp. 236–7. In the present instance $p = \frac{31}{49}$; $q = \frac{18}{49}$. The last term of the expansion of the binomial expression $(p + q)^{11}$ gives the probability of the occurrence of 11 pronuclei all with purple particles. The value of this term is 0·000016, i.e. the odds are more than 50,000 to 1 against such an occurrence. Hence one may confidently assume that the factors responsible for the production of the eosinophilic or pale or colourless particles were not acting to anything like the same degree on the peripheral pronuclei.

It has already been shown that the large and medium-sized particles are characteristic of the pronuclei in the later stages after insemination, and therefore the Chi Square test was applied to prove whether there was any association between the size of the particles in the pronuclei and the presence or absence of the pink or pale particles. No such association was found ($\chi^2 = 1{\cdot}2$; $n = 1$; $P < 0{\cdot}30 > 0{\cdot}20$).

The conclusions to be drawn from these tests are as follows: eosinophilic, pale, or colourless particles are much more commonly found in central than in peripheral pronuclei. The occurrence of these in the central pronuclei is independent of the fixative, the depth of the basic stain and the time after insemination. It is not possible to show any association between the stage of development of the central pronuclei and the presence or absence of these particles.

(*d*) *Position of the chromatin particles*

Kremer (1924) described at a fairly late stage in the development of the pronuclei of the mouse an adhesion of chromatic material to the pronuclear membrane. In mouse ova and in those of the rat Kremer considered that an increase of the chromidial substance of the cytoplasm was connected with an

elimination of chromatin from the pronuclei, and suggested that the particles on the membrane might pass out from the pronuclei. Out of 97 of the ferret pronuclei under examination, 25 showed chromatin collected to a greater or less extent in the outer parts of the pronuclei (fig. 6); while in the rest the chromatin was central (4 pronuclei) or was scattered fairly uniformly (68 pronuclei). The Chi Square test showed that the position of the particles was independent of the time after insemination ($\chi^2 = 0{\cdot}96$; $n = 1$; $P < 0{\cdot}50 > 0{\cdot}30$). It was found that there was a highly significant association between the arrangement of the chromatin particles in the pronucleus and the presence of the eosinophilic or colourless particles ($\chi^2 = 8{\cdot}094$; $n = 1$; $P < 0{\cdot}01$). In the pronuclei, where the chromatin was peripheral, there was a much greater proportion showing only purple particles. The explanation of this is obscure, partly because there is no adequate measure of the time after fertilisation. There was no association between the position of the chromatin particles and the size of the particles predominating in the pronuclei ($\chi^2 = 0{\cdot}13$; $n = 1$; $P < 0{\cdot}80 > 0{\cdot}70$).

THE RETICULUM

A reticulum in the pronuclei of the ova of different Mammals has been described by various authors. Kremer (1924) has pointed out that during the very early stages of pronuclear development in the mouse there is no reticulum. Commonly it is not made clear in the literature whether the reticulum is an early or merely a later phenomenon. In the series of ferret specimens examined some of the pronuclei presented fine strands of reticulum, others a moderately coarse and some a very coarse reticulum (fig. 3). There were various differences also in the extent to which the different parts of the reticulum were continuous with each other. In some pronuclei there were long strands; in some the reticulum appeared merely as rows of granules (fig. 5). In a number of the pronuclei deeply stained chromatin particles formed varicosities on the reticulum (fig. 2). For the most part the medium-sized and larger chromatin particles showed no distinct relationship to the reticulum.

The formation of a reticulum can occur soon after the stage at which the pronuclei appear homogeneous. It was found in both male and female pronuclei when they were peripheral.

The reticulum was classified under the headings: Indefinite or Absent; Fragmentary or Granular; Fine; Medium; Coarse. These characteristics were studied in relationship to the position of the pronuclei—peripheral, sub-central or central. There was no significant association between the position of the pronuclei and the presence or condition of the reticulum ($\chi^2 = 0{\cdot}87$; $n = 2$; $P < 0{\cdot}70 > 0{\cdot}50$). The Chi Square method was also applied to test association between the condition of the reticulum and the number of hours after insemination. To avoid fallacies due to fixative and staining, only specimens fixed in Zenker and stained by Heidenhain's iron-haematoxylin were used. There was

found to be no association between the time and the reticulum ($\chi^2 = 1{\cdot}8$; $n = 1$; $P > 0{\cdot}10 < 0{\cdot}20$).

The Chi Square revealed at first an apparent association between the fixative and the reticulum. The data upon which the tests were made is shown in Table VI. In order to obtain sufficient numbers of instances in the various

Table VI. *Relationship of reticulum to fixatives and stains.*

(The numbers represent individual pronuclei.)

Fixative and staining	Reticulum indefinite or absent	Reticulum fragmentary or granular	Reticulum fine	Reticulum of medium thickness	Reticulum coarse	Totals
Zenker's fluid; Heidenhain's iron-haematoxylin	4	20	6	6	6	42
Zenker's fluid; Mayer's haemalum	4	2	10	0	0	16
Perenyi's fluid; Heidenhain's iron-haematoxylin	3	9	3	10	6	31
Mann's fluid; Heidenhain's iron-haematoxylin	1	0	0	0	0	1
Mann's fluid; Mayer's haemalum	6	0	0	0	0	6
Flemming's fluid; Heidenhain's iron-haematoxylin	4	2	0	0	0	6
Flemming's fluid; eosin tinted only	11	4	4	0	0	19
Totals	33	37	23	16	12	121

cells of the Chi Square, the groups of Table VI had to be merged into larger groups. Thus the fixatives were first classified as "Zenker" and "Non-Zenker," and the reticulum was classified according to its absence, granularity or presence as continuous strands, not broken up into granules. The results of the first test were: $\chi^2 = 10{\cdot}4$; $n = 2$; $P < 0{\cdot}01$. When, however, all the specimens fixed in Flemmings fluid were eliminated, and the test was carried out with the specimens fixed some in Zenker's, some in Perenyi's and some in Mann's fluids (including Mann's fluid with formol), there was found to be no significant association of reticulum and fixative ($\chi^2 = 3{\cdot}3$; $n = 2$; $P < 0{\cdot}20 > 0{\cdot}10$). The Flemming specimens contributed heavily to the groups containing absent or indefinite reticulum and granular reticulum. Since most of these Flemming specimens were merely tinted with eosin, and not otherwise stained, the reticulum was less likely to be demonstrated than in deeply stained specimens. It appeared therefore possible that if these eosin-tinted specimens alone were excluded, no association would be found between reticulum and fixative. The Chi Square test showed such to be the case ($\chi^2 = 5{\cdot}2$; $n = 2$; $P < 0{\cdot}10 > 0{\cdot}05$). The apparent high association between fixative and reticulum can therefore be sufficiently accounted for by the faint staining of the majority of the specimens that had been fixed in Flemming's fluid.

A comparison of the reticular conditions under Zenker fixation with those

under Perenyi fixation was made by the Chi Square method, only the central pronuclei stained with Heidenhain's iron-haematoxylin being chosen. There was no significant dependence of the reticular conditions on differences between those fixatives ($\chi^2 = 2{\cdot}7$; $n = 2$; $P < 0{\cdot}30 > 0{\cdot}20$). Even when the specimens fixed in Zenker's fluid and stained by Mayer's haemalum and eosin were included, the Chi Square test did not reveal any significant result due to this type of staining ($\chi^2 = 1{\cdot}4$; $n = 2$; $P < 0{\cdot}50 > 0{\cdot}30$). This last result also indicates that the specimens fixed in Mann's fluid did not produce any significant preponderance of pronuclei with reticula belonging to any of the classes shown in Table VI.

The influence of the different methods of staining was further investigated by the application of the Chi Square test to the Zenker-fixed specimens, some of which were stained with Heidenhain's iron-haematoxylin and others with Mayer's haemalum. The condition of the reticulum according to this direct test was independent of the staining method ($\chi^2 = 1{\cdot}8$; $n = 1$; $P < 0{\cdot}20 > 0{\cdot}10$). On the other hand, indirect evidence of the influence of staining was obtained in the following way. The pronuclei fixed in Zenker's fluid, Mann's fluid, and Perenyi's fluid were considered. When the pronuclei stained with Mayer's haemalum were all excluded, the result of the Chi Square test showed no association between fixative and reticulum ($\chi^2 = 2{\cdot}9$; $n = 2$; $P < 0{\cdot}30 > 0{\cdot}20$). When the pronuclei fixed in Mann's fluid and stained with Mayer's haemalum were alone excluded, and those fixed in Zenker's fluid and stained with Mayer's haemalum were still retained, there was still no significantly positive result ($\chi^2 = 1{\cdot}1$; $n = 2$; $P < 0{\cdot}70 > 0{\cdot}50$). When, however, the pronuclei fixed in Zenker's fluid and stained by Mayer's haemalum were excluded, and all those fixed in Mann's fluid were retained, there was a significantly positive result ($\chi^2 = 6{\cdot}5$; $n = 2$; $P < 0{\cdot}05 > 0{\cdot}02$). In brief, the position may be stated thus. When all the pronuclei fixed in Zenker's, Mann's or Perenyi's fluids are considered, irrespective of staining, there is no association between the reticulum and the fixative. When all the Mayer-stained pronuclei are excluded, or when only the Mann-fixed Mayer-stained pronuclei are excluded, the reticulum appears still to be independent of the fixative; but when only the Zenker-fixed Mayer-stained pronuclei are excluded, there is a distinct relationship between the fixative and the reticulum. Examination of Table VI reveals the fact that many of the Zenker-fixed Mayer-stained specimens had fine reticula. The positive Chi Square result indicates that this type of staining had a statistically appreciable tendency to produce finely granular reticula.

THE PRONUCLEAR MEMBRANE

(*a*) *Wrinkling of the membrane*

Before the first segmentation of the fertilised ovum, the pronuclear membrane must disappear. It is natural to suppose that, as described by Sobotta (1895) in the mouse, a preliminary to this disappearance is the wrinkling of

the membrane, noted in the present series of specimens in many instances. The Chi Square test showed that there was no significant association between the time after insemination and the degree to which the membrane was wrinkled —definitely, moderately or slightly—($\chi^2 = 2{\cdot}61$; $n = 1$; $P < 0{\cdot}20 > 0{\cdot}10$). The wrinkling of the membrane was also independent of the fixatives used ($\chi^2 = 2{\cdot}211$; $n = 2$; $P < 0{\cdot}50 > 0{\cdot}30$).

In some pronuclei the wrinkling of the membrane was general; in others it was most marked at the sides where the two pronuclei were adjacent (fig. 2). This difference in location was independent of the time after insemination ($\chi^2 = 2{\cdot}52$; $n = 1$; $P < 0{\cdot}20 > 0{\cdot}10$).

(*b*) *Completeness of the membrane*

To obtain evidence of the commencing disappearance of the pronuclear membrane and possible fusion of the pronuclei, notes were taken of the completeness or incompleteness in the various specimens. In determining the condition of the membrane all the specimens which were fixed in Flemming's fluid were excluded, since in these, most of which were tinted with eosin, the membrane was frequently indistinct. Out of 26 pairs of central pronuclei, seven pairs had membranes which were of doubtful completeness, and in one pair the membrane seemed clearly to be common to the two pronuclei. With one exception these eight pairs were from a ferret killed 64¼ hours after insemination. The remaining specimen, in which the completeness of the pronuclear membranes was doubtful, was from a ferret killed 76½ hours after insemination. In all the other specimens, taken from ferrets killed 41½, 47½, 51¾, 64¼ and 76½ hours after insemination, the membranes were complete. It was thought possible that where the two pronuclei overlapped in the section doubt might be thrown on the completeness of the membrane—a doubt simply due to difficulties of observation. The Chi Square test showed that the recorded completeness of the membrane was independent of the overlapping of the two pronuclei ($\chi^2 = 0{\cdot}72$; $n = 1$; $P < 0{\cdot}50 > 0{\cdot}30$).

So far, then, it has merely been demonstrated that the only specimens showing doubtful completeness of the pronuclear membrane were obtained comparatively late after insemination. There is very little evidence to indicate whether or not actual fusion of the pronuclei occurs, as it has been stated to do in the rabbit (van Beneden, 1875), or whether the two pronuclei remain separate up to the stage of the first segmentation, as is the general rule (Sobotta, 1895: mouse; Rubaschkin, 1905: guinea-pig; O. van der Stricht, 1923: various Mammals).

THE COMPARATIVE METHOD

Statistical treatment of the data has so far proved chiefly negative relationships between phenomena, and it may be asked whether it is necessary to confine oneself to such statements as can be proved statistically. The usual method with material such as is now being studied is to arrange the different

specimens in as reasonable an order as possible so as to indicate a series of steps from the state of peripheral pronuclei, completely basiphilic, to the spireme of the first segmentation spindle. It may be asked whether it is not possible to fit the specimens at present under consideration into the schemes described by other authors for other animals. Such a method should be used with caution. In the first place, the schemes so described are simply the interpretations placed by the authors upon the specimens studied. In the second place, minor differences are present in different animals, and it entails considerable assumptions to transfer information from a rodent's ovum, poorly supplied with lipoid, to the ferret's ovum, which is very richly supplied with lipoid.

One of the most thorough descriptions of early developmental stages was that of Sobotta (1895) on the mouse. In this animal the chromatin of the pronuclei was stated to be at an early stage in the form of large masses, one in the male pronucleus, several in the female. Then followed a subdivision of the chromatin and a spreading of it over the rest of the pronucleus as fine particles. These then condensed to form thread-like fragments, which later became a single much wound thread. Thus arose the spireme of the first segmentation spindle.

According to Kremer (1924) in the mouse pronucleus there is at a very early stage no typical reticulum. The chromatin granules, after being arranged around a single nucleolus and near the pronuclear membrane, unite to form large masses. Later still there is a separation of the chromatin substance and particles are laid down on the reticulum. Subsequently much of the chromatin disappears.

Both Sobotta and Kremer described pale globules, probably formed by a loss of basiphilic material, or of the staining quality of the chromatin.

Lams and Doorme (1908), also working on the mouse, described the fragmentation of the chromatin into small masses, arranged at the pronuclear membrane or along fine filaments which were placed radially about the large plasmosome. Later this plasmosome was found to disappear, the chromatin granules were noted to unite and form with the network rodlets—the chromosomes at the spireme stage of the first segmentation spindle.

In the rat Sobotta and Burckhard (1911) described for the early stages a fine linin framework with large chromatin nucleoli; for the later stages a more thread-like spreading of the chromatin on the framework.

To Kremer (1924) it appeared as if at a certain stage in the rat the smaller chromatin particles arose by a kind of budding from the chief nucleolus. This chromatin then spread out as finer or coarser divisions on the reticulum, and later much of it disappeared.

Huber (1915) gave little detail regarding the rat at this stage of development.

For the guinea-pig Lams and Doorme (1908) stated that a process occurred similar to that already described for the mouse.

Among Carnivores, to which class the ferret belongs, a fairly full description

of the very early development of the cat was given by R. van der Stricht (1911), but he did not give very much detail of the minute processes undergone by the pronuclei. He wrote of the nuclein granules, and masses occurring in the pronuclei as they increased in volume, and numerous chromatin globules in the pronuclei when large.

Longley (1911) and Hill and Tribe (1924) gave descriptions of a few specimens of pronuclear stages obtained from the cat.

O. van der Stricht (1923) gave information concerning the developing ova of the dog; but was more concerned with the cytoplasm than with pronuclear transformations.

With the aid of these data concerning other animals, an attempt may be made to arrange a possible sequence of phenomena in the pronuclei of the ferret.

At the outset several assumptions based on the results obtained in the preceding sections may be made. It may perhaps be assumed that in the early stages the chromatin particles tend to be smaller than in somewhat later stages, that the pronuclei in the early as well as in the later stages possess a reticulum, and that the presence of a globule with a non-basiphilic centre is a mark of an intermediate or late stage of development. An attempt may then be made to select a number of specimens illustrating a possible developmental process.

In three specimens (nos. 2, 75 and 77), all with central pronuclei, there were no large chromatin particles. Two of the specimens were fixed in Flemming's fluid and tinted with eosin, so that the basiphilic reaction of the chromatin was not shown. In all three the reticulum was granular. In no. 2 (fig. 5), a Zenker specimen stained with Heidenhain's iron-haematoxylin, the small globules of chromatin formed definite varicosities on the reticulum. Some of the chromatin was partly decolorised.

These three specimens may perhaps be taken to illustrate a stage occurring early after the stages of peripheral pronuclei described above. Three other ova may serve to illustrate a possible subsequent stage. In no. 69, a Flemming-fixed specimen tinted with eosin, the pronuclei showed granules of varying sizes, none very large, and a reticulum which appeared to be made up of rows of granules. Ova nos. 93 (fig. 1) and 99, fixed in Perenyi's and Zenker's fluids respectively, and both stained with Heidenhain's iron-haematoxylin, showed globules of moderate size. The reticulum was rather coarse and showed basiphilic granules. In one of the pronuclei in no. 99 there was one moderately large pale globule. Each of the pronuclei of no. 93 possessed a moderately large pale purple sphere with a dark purple membrane.

As possibly illustrating much later stages, five specimens with large chromatin masses in the pronuclei may be discussed. All the pronuclei were central and were stained with Heidenhain's iron-haematoxylin. In ovum no. 85 the pronuclear particles were stained a deep purple and the reticulum was formed of strands of granules and globules. In no. 46 all the particles were stained a deep purple; the reticulum was discontinuous, formed of strands and

rods. In ovum no. 86 a few of the large globules were light purple in contrast to the deep purple of the rest; the reticulum was moderately fine with varicosities. In no. 88 all the particles were a deep blue-black; the reticulum was coarse and similarly stained. In no. 89 there was a light purple mass in one pronucleus; the rest of the particles were deep purple; the small chromatin globules and granules were spread in strands. This description gives, it will be seen, no very coherent picture. If it be assumed that it represents a late stage of development, as contrasted with the peripheral pronuclei described above, and possibly with the central pronuclei described earlier in this section, then it may be supposed that intermediate stages will be represented by pronuclei containing granules of varying sizes, some large, some intermediate, some small. On the other hand, some of these last-mentioned specimens may represent a stage in which the chromatin is again divided after being aggregated into large masses. A review of these specimens showed that it was impossible to arrange them in any satisfactory scheme. In some of them the reticulum was fine, in others moderately fine, in others coarse. The chromatin particles in some were all stained a deep purple with Heidenhain's iron-haematoxylin; in others there were one or more pink or pale purple or colourless globules.

In the series of central pronuclei as a whole, the pink or pale purple or colourless globules were large or moderately large. In one specimen (no. 16) there was noted a reticulum with small swellings, mostly stained pink with eosin. It was further interesting to note that specimens fixed in Zenker's fluid and stained with Mayer's haemalum and eosin, presented pronuclei that were less deeply stained by the basic stain than were the nuclei of other tissues in the same section. This was not the case with specimens fixed in Zenker and stained by Heidenhain's iron-haematoxylin; nor was it the case with specimens fixed in Mann's fluid and stained with Mayer's haemalum.

The question raised by Kremer (1924) as to the elimination of chromatin and the production of chromidial substance in the cytoplasm, it is intended, shall be discussed in a subsequent communication. So far as investigations have proceeded at present, there appears to be no evidence of a diminution of chromatin from the pronuclei.

DIFFERENCES BETWEEN THE PRONUCLEI

The possibility of distinguishing the male from the female pronucleus is naturally one of very great interest. It is intended that size-differences, which are frequently claimed as a characteristic, should form part of the subject of a future communication. Differences in position, also, are more adequately treated in connection with the cytoplasm and polar bodies.

The most conclusive proof of the derivation of a pronucleus is the presence of a spermatozoon tail near it; but it should not be forgotten that a tail might follow the head of a spermatozoon into the ovum, and then be severed from the head or male pronucleus and come to lie nearer to the female than to the

male pronucleus. It would therefore be unsound to argue from the proximity of a sperm tail that either the male or the female pronucleus was the larger, or that they had certain differences of structure, unless these characteristics were found in several instances.

The occurrence of the sperm tail in the fertilised ovum was demonstrated by O. van der Stricht (1902) in ova of the bat. Sperm tails are also found in the ova of the mouse (Lams and Doorme, 1908), guinea-pig (Lams and Doorme, 1908), rat (Sobotta and Burckhard, 1911). O. van der Stricht (1923) stated that sperm tails were only exceptionally seen in the ova of the dog, and further pointed out that their occurrence had not been observed during the investigations conducted by R. van der Stricht (1911) on the ova of the cat.

In the present collection, out of 54 ferret ova at the pronuclear stage, apparently suitably fixed and stained, only one showed definitely the presence of a sperm tail within the ovum. In another specimen a structure which was very probably a sperm tail was observed. In the uterine tube sperm tails are observed seldom, relative to the frequency with which deeply stained sperm heads are seen. The failure to demonstrate tails inside the ovum is therefore at least as likely to be due to technique as to absence of the tails. It was to technical causes that O. van der Stricht (1923) attributed the apparent absence of tails from the ooplasm.

The ovum in which the definite sperm tail was observed was no. 88 (fig. 7). In this ovum the two pronuclei were centrally placed, in contact. One of them, B, was more truly central in the section than the other, A, for pronucleus A was placed a little more towards 9 o'clock. Both were spherical but flattened against each other. The membranes were moderately fine, darkly stained, somewhat wrinkled and possibly incomplete. The chromatin was in the form of deeply blue-black globules, large irregular lobulated masses and granules of various sizes. The reticulum was in parts coarse and deeply stained. Passing from pronucleus A towards 7.30 o'clock was a definite dark greyish stained fine strand actually in the ovum. This was separated by a small clear space from the pronucleus. The ratio of the volumes of the pronuclei calculated from the formula for the volume of an ellipsoid was roughly: A : B as 45 : 60. The pronucleus near which the sperm tail was found was therefore the smaller. No structural difference between the pronuclei was detected.

If the direction of the tail indicated the path of the sperm pronucleus, the latter must have entered near the present position of a polar body.

In ovum no. 97 the pronuclei were both peripheral, of approximately equal size and of similar structure, consisting of medium-sized and small globules and granules, all stained a deep purple, a reticulum and a fine unwrinkled membrane. Near pronucleus A on the surface of the ovum was a sperm tail. A fine thread-like structure in the ovum was possibly a continuation of the sperm tail to the pronucleus. Pronucleus B was nearer to the polar bodies.

The presence of a centrosome was used by Sobotta and Burckhard (1911) as an indication of the male pronucleus in the rat. These authors held that the

sperm-centrosome divided later to give the two centrosomes of the first segmentation stage—a view held also by Sobotta (1895) for the mouse. Summarising his own work and that of his colleagues, O. van der Stricht (1923) stated that sperm-centrosomes and sperm-asters and traces of ovular centrosomes were visible at certain stages of fertilisation in the ova of the bat, guinea-pig and dog.

Among the pronuclear specimens of the ferret at present under consideration, no aster or centrosome was detected.

Among structural differences that have been described between the two pronuclei is the presence of a greater number of chromatin particles in the female pronucleus of the mouse (Sobotta, 1895). This difference was not observed as a constant feature in the ova of the ferret, and even when there was an apparent difference in the quantity of chromatin it was impossible to state which pronucleus was male and which female.

Out of ten ova in which both pronuclei were peripheral, eight showed differences in structure to a greater or less degree. Reference may be made for details of some of these to the section, above, in which the structure of the early stages of the pronuclei is discussed. These differences, in some instances at least, appear to be concerned with the rate of development of the two pronuclei. Of greater interest is the difference which existed in some ova between the pronuclei when these were at the centre of the ovum. Out of 49 ova in which the two pronuclei were central, nine showed pronuclei which differed in structure or staining, apart from size, shape, position or the proximity of sperm tails. These differences varied considerably from ovum to ovum. Thus in one ovum (no. 5) the chromatin was more uniformly distributed in one pronucleus than in the other; in one (no. 14) the chromatin of one pronucleus was more indefinite and more pink stained, while the reticulum was more abundant; in a third (no. 85) there were larger globules of chromatin and probably actually more chromatin in one pronucleus than in the other. In ovum no. 93 (fig. 1) one pronucleus differed from the other by possessing a more continuous reticulum, a chromatin globule that was paler than the rest and a central area clear of chromatin. It may be reasonably suggested that such differences as were observed in the structure of the two pronuclei might be attributed to:

(1) Differences in rate of development, notable in the early stages, but less notable later, because in the long resting period, when the pronuclei are together in the centre of the ovum, there is time for them each to arrive at a similar condition preparatory to the formation of the first segmentation spindle.

(2) Temporary differences in the physico-chemical state of the pronuclei, of no great significance.

(3) Sexual differences.

To proceed further than a mere record of these varied differences and possible causes would obviously be to indulge in profitless speculation.

A TRINUCLEATED OVUM

R. van der Stricht (1911) described the arrangement of three pronuclei in a fertilised ovum of the cat. He interpreted this as due to the penetration of the ovum by two sperms. Hill and Tribe (1924) also gave instances of trinucleated ova from the cat, which they also presumed to have arisen by double fertilisation. Kremer (1924), mentioning a trinucleated ovum of the mouse, pointed out that such a condition might be due to the fertilisation of a binucleated mature ovum. In a recent investigation of the pluriovular follicle the author (1928) paid attention also to the plurinucleated ovum. The literature seems to indicate that binucleated ova are comparatively rare. In the ferret not one of the sections of 496 adult ovaries examined was found to contain such an ovum. On the other hand, it is known that polyspermy in other animals is not uncommon (Wilson, 1925, p. 416), and therefore it seems more reasonable to attribute a trinucleated fertilised ovum to this phenomenon than to the previous existence of a mature binucleated ovum.

In ovum no. 45 of the present collection there were three pronuclei. Two (A and B) were centrally placed, almost in contact. They possessed chromatin globules and granules of various sizes, fairly uniformly scattered through the pronuclei. One globule in each pronucleus had a colourless centre; the remainder were stained blue-black. The reticulum was moderately fine. There were no structural differences between these two pronuclei. The third pronucleus, C, was placed in a section subsequent to the lowest section containing the nearer of the other two pronuclei, so that it was not in contact with the others. It was situated about half-way between the centre of the section and 7 o'clock. It resembled the other two in structure, except that it contained no pale globule. The ratio of the volumes as calculated from the formula for the volume of an ellipsoid was: A : B : C as 1·13 : 1·14 : 1·00. Pronuclei A and B were therefore of similar volume; while pronucleus C was distinctly smaller.

ACKNOWLEDGMENTS

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SUMMARY

The purpose of this investigation has been partly to obtain detailed information upon the earliest stages of development of the tubal ova of the ferret, and partly to demonstrate the type of results ensuing from statistical methods not hitherto applied in this way to such specimens.

Serial paraffin sections, 10 microns thick, of 101 ova of the ferret were examined, from 30 different ovaries and 19 different animals. Out of these ova, 71 were at the pronuclear stage of development, and in them the pronuclei were examined in detail.

Statistical treatment was applied to the data, and the statistical method chiefly employed—the Chi Square method—has been explained (p. 264).

Since the actual number of hours that elapsed after fertilisation cannot be determined, a measure of the progress of development is obtained from the position of the pronuclei at the different stages of their migration towards the centre of the ovum. With the aid of this it has been proved that in this collection of specimens, treated as a whole, the number of hours that elapsed between the insemination and the death of the animal afford a measure of the progress of development of the ovum (p. 265). The position of the pronuclei, arranged according to the number of hours after insemination, is given in Table I (p. 266).

Allowance having been made for the possible dominating influence of abnormal specimens in so small a collection of peripheral pronuclei, the probable early transformations of the pronuclei are indicated (p. 267).

There was a highly significant association between the size of the chromatin particles in the pronucleus and the time after insemination (larger and medium-sized particles being found in the later stages). This association existed only in specimens fixed in Perenyi's fluid. Owing to the smallness of the numbers of the specimens it was impossible to prove that this association between time and the size of the particles really indicated an association with the development of the ovum or pronucleus. There was a highly significant association between the size of the particle and the fixative, Perenyi's fluid tending to give larger particles. There was, however, no significant association between the time after insemination and the fixative, and therefore the association between the time after insemination and the size of the particles could not be accounted for by the fixation.

Eosinophilic, pale or colourless particles were found in 31 out of 49 central pronuclei. No possible cause of these could be demonstrated. They occurred independently of the fixative and independently of the depth to which the rest of the section was stained with haematoxylin, and independently also of the time that had elapsed between insemination and death. It was shown (p. 271) by the theory of probability that the factors responsible for the production of these particles were not acting to anything like the same extent on the peripheral pronuclei as on the central.

The position of the chromatin particles—peripheral, uniformly scattered

or central—in the pronuclei was independent of the time after insemination and also of the size of the particles predominating in the pronuclei. Among the pronuclei where the chromatin was peripheral there was a much greater proportion showing only purple particles.

A reticulum occurred in pronuclei fixed either early or later in their development. Different varieties of reticulum were found—fragmentary or granular, fine, medium, and coarse. The presence or condition of the reticulum was independent of the position of the pronuclei, and of the number of hours after insemination. There was no association between the fixative and the condition of the reticulum; but specimens fixed in Zenker's fluid and stained with Mayer's haemalum and eosin produced a definite preponderance of pronuclei containing a fine reticulum.

There was no indication that the wrinkling of the pronuclear membrane was a preliminary to its disappearance. The wrinkling itself and the location of the wrinkling on the membrane were independent of the time after insemination. This wrinkling was also independent of the fixative used.

The only specimens showing doubtful completeness of the membrane were obtained comparatively late after insemination. There is very little evidence to indicate whether or not actual fusion of the pronuclei occurs.

An attempt is made (pp. 276–9) to indicate the possible developmental processes in the pronuclei with the help of information obtained from the researches of other authors on other Mammals. Few results are obtained from this substitution of the older and commoner method for the newer and less common statistical one.

A description is given of two specimens (p. 280) in one of which a definite spermatozoon tail was found in the ovum; while in the other a probable tail was observed. It is probably to technical factors (fixation and staining) that one must attribute the scarcity of such specimens.

No centrosome or aster was seen in any of the pronuclear ova.

Various inconstant differences in structure between the pronuclei are described (pp. 279–81).

A trinucleated ovum is described (p. 282). The condition is attributed to double fertilisation.

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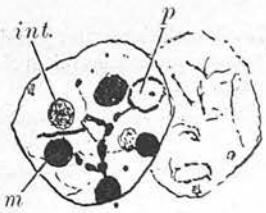

Fig. 1

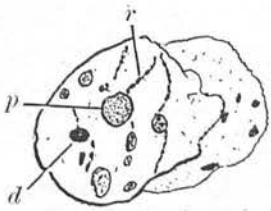

Fig. 2

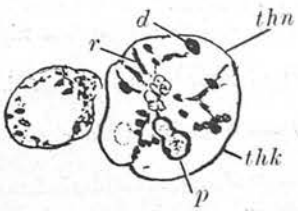

Fig. 3

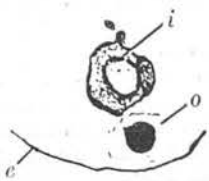

Fig. 4

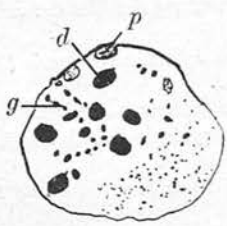

Fig. 5

Fig. 6

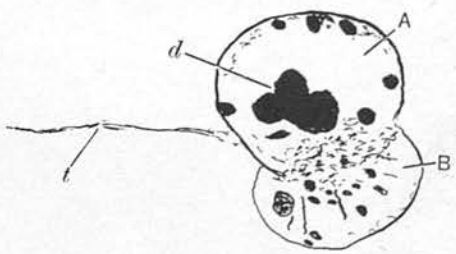

Fig. 7

EXPLANATION OF FIGURES

All the figures are pronuclei of the ferret, drawn with the aid of a camera lucida and are at a magnification of 1050 diameters.

Fig. 1. Ovum no. 93. Both pronuclei. Fixed in Perenyi's fluid; stained with Heidenhain's iron-haematoxylin. The reticulum is rather coarse, and shows basiphilic globules. (See pp. 278–81.)
int. Globule intermediate in staining between the dark and the pale globules.
m. Medium-sized globule of chromatin, darkly stained.
p. Pale purple sphere with dark purple periphery.

Fig. 2. Ovum no. 4. Both pronuclei. Fixed in Zenker's fluid; stained with Heidenhain's iron-haematoxylin and eosin. The pronucleus to the right is at a lower level in the section and is less sharply outlined. Deeply stained chromatin particles form varicosities on the reticulum. The membrane of the pronucleus on the left is most wrinkled at the side adjacent to the other pronucleus. (See pp. 273–6.)
d. A darkly stained globule of chromatin.
p. Pale globule of chromatin.
r. Reticulum with varicosities.

Fig. 3. Ovum no. 14. Both pronuclei. Fixed in Perenyi's fluid; stained with Heidenhain's iron-haematoxylin and eosin. The reticulum in the one to the right is very coarse. (See p. 273.)
d. Darkly stained globule.
p. Pale double globule.
r. Reticulum.
thk. Thick part of membrane.
thn. Thin part of membrane.

Fig. 4. Ovum no. 7. The two pronuclei, both peripheral. Fixed in Zenker's fluid; stained with Heidenhain's iron-haematoxylin and eosin. (See p. 268.)
e. Edge of ovum.
i. Inner pronucleus, larger, a ring with a pale centre and faintly purple lobulated periphery.
o. Outer pronucleus, smaller, a spherical homogeneous purple body.

Fig. 5. Ovum no. 2. One of the pronuclei. Fixed in Zenker's fluid, stained with Heidenhain's iron-haematoxylin and eosin. The reticulum appears merely as rows of granules. (See pp. 273–8.)
d. Darkly stained globule of chromatin.
g. Granules arranged in a row, indicating a reticulum.
p. Pale globule.

Fig. 6. Ovum no. 15. One of the pronuclei. Fixed in Perenyi's fluid; stained with Heidenhain's iron-haematoxylin and eosin. Both the pronuclei in this ovum had chromatin in globular form; but while one was central in the ovum, the other was peripherally placed. The chromatin is chiefly located in the outer parts of the pronucleus. (See pp. 268–73.)
l. Moderately large globule of chromatin.
r. Faint reticulum.
s. Small globule of chromatin.

Fig. 7. Ovum no. 88. Both pronuclei. Fixed in Zenker's fluid; stained with Heidenhain's iron-haematoxylin. Both the pronuclei were central in the ovum. One (A) is seen more nearly at its centre; the other (B) is seen nearer to one of its ends. (See p. 280.)
d. Darkly stained trilobar mass of chromatin.
t. Tail of spermatozoon.

THE MEASUREMENT OF FERRET PRONUCLEI

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The Measurement of Ferret Pronuclei

BY

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The Measurement of Ferret Pronuclei

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Presented by A. T. CAMERON, F.R.S.C.

INTRODUCTION

Nuclear measurement and cell measurement appear to be merely semi-mathematical academic exercises. That this is not so is illustrated, for example, by the recent work of Savage (1927, 1930) and his collaborators on the head (*i.e.* nuclear part) of spermatazoa. There is a growing tendency to see the histology of the future as a quantitative science, and during the past two decades much interest has been taken in nuclear measurement, largely owing to the influence of Hertwig's and Heidenhain's work on nucleoplasmic ratios. The degree of accuracy of the investigation has varied considerably, some workers* being content with less close approximations than others, for it seems not to be clearly realized that, only if they are precise and accurate, can histological measurements and calculations form a connecting link between structure and biochemical phenomena.

Jacobj (1925, 1926 *a* and *b*) investigated various tissues, such as the liver, pancreas and testis, and used animals of different groups, including Amphibians and Mammals. He exercised great care in selecting suitable formulae. Godlewski (1908, 1918, 1921) carried out similar research on embryonic stages of Echinoderms, and (1910) on amphibian epithelium, but did not apply statistical tests to his results as did Jacobj. In any such investigation proper evidence of the variability displayed by the readings is essential, especially when the conclusions are founded on small groups of individuals. Thus, in the

*No attempt has been made to treat the subject historically, but rather to indicate the type of work that has been carried on Further historical information may be obtained in the articles by A. F. Shull (1922, Relative Nuclear volume .... *Journ. Exp. Zool.* vol. 35, pp. 283-322), R. Erdmann (1911, Quantitative Analyse der Zellbestandtiele .... *Ergebn. Anat. u. Entwgesch.* Bd. 20, II Hälfte, S. 471-566), O. Koehler (1912, Uber die Abhängigkeit der Kernplasmarelation von der Temperatur .... *Arch. f. Zellforsch.* Bd. 8, S. 272-351).

comparison of 8 unripe ova of Asterias with 10 ripe ova of the same animal (Godlewski, 1918), it is very desirable to show whether the differences between the individuals in the groups would account sufficiently for the difference between the groups, even although it appears fairly obvious that this latter difference is so large as to be significant. A similar criticism might be levelled against the work of Berezowski (1910) on the sizes of nuclei and cell bodies of the liver, of cartilage and Purkinje cells, and of the epithelium of the tongue, stomach, intestine and renal tubules. It should be observed that the criticism of this kind of work is not a denial of the conclusions, but a statement of lack of confidence in untested results—an attitude that has been engendered by the application of statistical tests to histological material. Statistics in such work, it should be noted, is not so often a means of proving something that is otherwise doubtful, but more usually a means of casting doubt upon results by showing that they can be due to chance.

There is not only a lack of statistical criticism of results in much of the work on nuclear measurement, but also a failure to test the value of different methods of measurement. It was partly this failure that prompted the author to set forth the results contained herein, a further justification being the absence of systematic investigations on the sizes of nuclei at the earliest stages of mammalian development. This absence is observable in the classical work upon early mammalian development of Sobotta (1895), in the detailed investigations of O. van der Stricht (1923, etc.) and his co-workers, and also in the more finely quantitative work of Long and Mark (1911), as well as in numerous lesser publications. In connection with recent investigations of the pronuclei of the ferret (Mainland, 1930), some measurements of the pronuclei were made, and one purpose of the present article is to convey an idea of the results likely to be obtained from such measurements.

Material

For tests of the variation to which the results of nuclear measurement might be liable, measurements were made of the pronuclei of a typical tubal ferret ovum in which the pronuclei were centrally placed and in contact, the plane of contact being roughly at right angles to the plane of section. The ovum had been fixed, in the uterine tube, in Zenker's fluid and cut into paraffin sections at 10 microns. The tissue was tinted with eosin and stained with Heidenhain's iron-alum haematoxylin.

Methods of Measurement

The rotating stage of the microscope (a Zeiss research model) was not graduated, but marks were made on its periphery at intervals of 90 degrees, so that the stage could be rotated until any one of these marks coincided with a mark that was made on a small metal strip fastened to and projecting from the fixed stage, below the rotating stage. Thus when the long axis of the pronucleus had been measured with the eyepiece micrometer, the object was rotated through a right angle and the transverse axis measured. The only condition necessary to facilitate the second measurement was the accurate centering of the moveable stage before the introduction of the specimen, so that, even when the stage was rotating, the centre of the field remained approximately in the centre of the eyepiece. This method was introduced

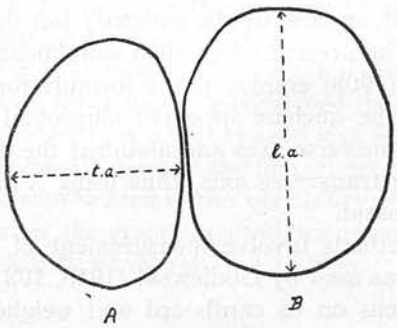

Diagram of Pronuclei, A and B.
l.a., long axis; t.a., transverse axis.

because of the unsatisfactory nature of the ordinary method of trusting to the eye for a determination of an axis at right angles to the long axis first measured. The rotation of the stage seems preferable to the rotation of the micrometer eyepiece for two reasons. The first is that an error in the adjustment of the eyepiece, of which the diameter is small, would have much greater effect in the centre of the field than an error of the same magnitude in the rotation of the stage, where the diameter is so much larger. In the second place, if the stage is rotated, the graduated screw of the micrometer can be retained in a position most convenient for rapid reading.

The micrometer eyepiece used was a Spencer filar micrometer with compensating lenses, and the objective was a Zeiss apochromatic lens (n.a. 1.30). The immersion oil, which was used in all the measurements of long axis, transverse axis and depth, was the appropriate

Zeiss cedar oil. For depth, measurements were made by the graduated fine adjustment of the microscope, the smallest interval representing 2 microns. For pronucleus *A*, depth involved the measurement of three sections, for pronucleus *B*, only of two sections.

Methods of Calculation

Calculations of the volume and area of a nucleus may be carried out as described by Jacobj (1925), who measured the longest axis of the nucleus, and, in the case of slightly oval nuclei, took the average of the longest axis and the transverse axis. He then used the formulae for the volume and area of a sphere. This method was shown to be as accurate as the more difficult method employing the formula of an ellipsoid, provided the difference between the axes was not great. In Jacobj's work unsuitable nuclei could be excluded, and the measurement of depth could, apparently, be omitted; but there must be many occasions such as the present when such simplification is not permissible. Godlewski (1908) employed the formula for the volume of a sphere, but where the nucleus appeared ellipsoidal he measured the longitudinal and transverse axes and assumed the depth or third axis to be equal to the transverse axis, thus using a modification of the formula for an ellipsoid.

These two methods involve measurement of the nuclear axes. Another method was used by Godlewski (1918, 1921), who traced the image of the nucleus on to cardboard and weighed the cardboard. Assuming that the image was roughly circular, and knowing the specific gravity of the cardboard, he calculated the radius and from that the volume and surface area of the nucleus.

In the present investigation the volume formula employed was that of an ellipsoid; $\frac{4}{3}\pi \cdot \frac{a}{2} \cdot \frac{b}{2} \cdot \frac{c}{2}$, where a, b, c, are the axes, a being the depth in μ, b the long axis in μ, c the transverse axis in μ, *i.e.*, the longest measurement at right angles to the long axis. The figures obtained by the eyepiece micrometer were each multiplied by 2.925, since each division of the micrometer scale was found by use of a stage micrometer to be equal to $2.925\mu \pm 0.002$.

For the computation of a formula for the surface of an ellipsoid of the shape of the pronuclei, I am indebted to Professor N. R. Wilson of the University of Manitoba. This formula is $\frac{\pi}{3}(ab+bc+ca)$, where a, b, c, are the axes as above. It should be noted that this formula is only applicable to bodies of this approximate ellipsoidal shape, in which

the axes do not differ very widely from each other. For a long, thin body the formula would probably give results with an error greater than that inherent in the observations.

For each pronucleus 25 observations were made of each of the three dimensions.

The measurement of depth by the fine adjustment of the microscope appears to be avoided by many of the investigators without a statement of their objections to it. These objections might take two forms, the one with relation to optical principles, the other to mechanical construction. In the first place, where dry objectives are used, serious errors are introduced owing to differences in the refractive index of the air, lenses and mounted specimen. Where immersion lenses and suitable oil are used, however this objection is nullified. Secondly, the construction of the fine adjustment is such that the manufacturers do not guarantee the uniformity of the readings obtained when different parts of the mechanism are in use. In spite of this, it was decided in the present case to employ the depth as the third axis of the pronucleus, for it should be observed that so many readings of the depth were taken that a fair chance was assured of obtaining readings from the different parts of the fine adjustment, and therefore the errors would be represented in the variability of the results. It is this variability that is the main object of the present study, and the question of depth will be again referred to later.

RESULTS

(A difference between quantities is considered significant only if it is three or more times its probable error.)

Volumes

Mean volume of *pronucleus A*: 5512.76 cubic μ
Probable error of mean: $\pm$47.9
Standard deviation of series: $\pm$355.7$\pm$33.5
Coefficient of variation of volume: 6.5 per cent.$\pm$0.623
Mean volume of *pronucleus B*: 6266.20 cubic μ
Probable error of mean: $\pm$40.5
Standard deviation of series: $\pm$2.2$\pm$28.49
Coefficient of variation of volume 4.8 per cent.$\pm$0.458
Difference between volumes of pronuclei: 753.44 cubic μ
Probable error of difference: $\pm$62.73.

There is, therefore, a definite difference between the volumes.

Difference between the coefficients of variation: 1.7
Probable error of difference: $\pm$0.60.

The variation between the different results of volume calculation is, therefore, not demonstrably different for the two pronuclei.

Areas

Mean area of surface of *pronucleus A*: 1538.64 square μ
Probable error of mean: ± 8.66
Standard deviation of series: $\pm 64.92 \pm 5.69$
Coefficient of variation of area: 4.2 per cent. ± 0.396
Mean area of surface of *pronucleus B*: 1652.16 square μ
Probable error of mean: ± 7.29
Standard deviation of series: $\pm 54.70 \pm 5.16$
Coefficient of variation of area: 3.3 per cent. ± 0.31.

The difference between the coefficients of variation is again obviously insignificant.

Comparison of Variability (Coefficients of variation with probable errors)

	Pronucleus A	*Pronucleus B*
Volume	6.5 ± 0.62	4.8 ± 0.46
Area	4.2 ± 0.396	3.3 ± 0.31
Depth	4.1 ± 0.39	4.3 ± 0.40
Long axis	1.14 ± 0.11	0.38 ± 0.036
Transverse axis	4.4 ± 0.41	1.02 ± 0.10

In both pronuclei it is the variability in the measurement of depth that accounts for a large part of the variability of the volume and area. In both pronuclei the long axes show less variability than the other dimensions.

Difference between coefficient of variation for depth and coefficient of variation for long axis:

In *pronucleus A*: 2.96 ± 0.41
In *pronucleus B*: 3.92 ± 0.40.

In both pronuclei, therefore, the readings of depth are considerably more variable than the readings of long axis.

Difference between coefficient of variation for depth and coefficient of variation for transverse axis:

In *pronucleus A*: 0.3 ± 0.57
In *pronucleus B*: 3.28 ± 0.41.

In the first pronucleus the readings of the transverse axis vary as much as the readings of depth; in the second pronucleus the readings

of the transverse axis are much less variable than those of depth. The variability of the volume in pronucleus *A* is therefore caused to a large extent by variability in the transverse axis readings as well as by variability in the readings of depth.

Comparison of Depth and Long Axis

If it is desired to dispense with depth measurement, it is necessary to know with what accuracy one can substitute for it the readings of the other axes. In the present instance one may from one's general knowledge of pronuclear fusion assume that each pronucleus is originally a sphere, and that when the two come in contact each will be shortened at right angles to the plane of contact, but will retain the other two axes approximately equal, *i.e.*, in the specimen examined the long axis and depth should be equal since the plane of contact was, roughly, at right angles to the plane of section.

Pronucleus A

Mean long axis:	$24.33\mu \pm 0.038$
Mean depth:	$26.12\mu \pm 0.14$
Difference:	$1.79\mu \pm 0.14$

Pronucleus B

Mean long axis:	$25.09\mu \pm 0.013$
Mean depth:	$24.36\mu \pm 0.14$
Difference:	$0.63\mu \pm 0.14$

In the one pronucleus (*A*), the longitudinal axis was less than the depth; in the other (*B*), the longitudinal axis was greater than the depth. This result appears very instructive, for if the measurement of depth always caused a serious bias in a certain direction, that is, by its very nature gave too high or too low a reading then one would expect it always to differ in the same way from the measurement of the longitudinal axis, on the assumption, which is apparently legitimate, that the depth and long axis in this case are approximately equal. It has just been shown that the measurement by fine adjustment (depth measurement) may give either a greater or smaller reading than the micrometer measurement (long axis). Hence one feels justified in placing reliance on the depth measurement. Moreover, the fact that a difference of reading between the long axis and depth exists in both pronuclei shows that it is unwise, without preliminary tests, to substitute an eyepiece micrometer reading of long axis for a depth reading.

Comparison between the Two Pronuclei

Long axes:

Pronucleus A:	$24.33\mu \pm 0.038$	
Pronucleus B:	$25.09\mu \pm 0.013$	
Difference:	$0.76\mu \pm 0.04$	; significant

Transverse axes

Pronucleus A:	$16.55\mu \pm 0.097$	
Pronucleus B:	$19.57\mu \pm 0.026$	
Difference:	$3.02\mu \pm 0.10$	; significant

Depth

Pronucleus A:	$26.12\mu \pm 0.14$	
Pronucleus B:	$24.36\mu \pm 0.14$	
Difference:	$1.76\mu \pm 0.20$	; significant.

The greatest difference is between the transverse axes, that is, although there is so much difference between the volumes of the two pronuclei, the difference in axes is chiefly between the transverse axes, perpendicular to the plane of contact. In other words, when two pronuclei come into contact the forces acting are such as to cause them to be of similar dimensions in planes parallel to the plane of contact and therefore of very different dimensions at right angles to this plane.

Further light can be thrown on this fact as follows:

Difference between long axis of pronucleus A and depth of pronucleus B: $0.03\mu \pm 0.14$; not significant.

Difference between depth of pronucleus A and long axis of pronucleus B: $1.03\mu \pm 0.14$; significant.

There is, therefore, no significant difference in the one case between the depth of one pronucleus and the long axis of the other; in the other case, the difference, though significant, is not great.

Comparison with the Methods that do not introduce Depth Measurements

The results already shown have made it obvious that it is not wise to neglect depth measurements without a well-established reason, depending on the nature of the case under investigation. The case of the pronuclei seems to be a special one, but in reality there are many other instances in which nuclei are elongated in one axis, *e.g.*, in some columnar epithelial cells, and, unless one has proved that the other two axes are equal to one another, the three axes must be used in calculating the volume and area. It is of interest to note the difference between the volume of pronucleus A obtained above by use of the three dimensions and the result obtained by taking the mean of the long and transverse axes and using the formula for a sphere.

Volume calculated by use of three axes: 5512.76 cubic$\mu \pm 47.9$.
Volume calculated by use of mean of long and transverse axes: 4478.87 cubic$\mu \pm 35.6$.
The difference ($966.11\mu \pm 59.7$) is large and significant.

SUMMARY

The present investigations have been undertaken because of the need for critical tests both of nuclear measurement and of the calculation of volume and area of nuclei, and because, also, of the absence of such measurements of mammalian pronuclei.

Twenty-five sets of measurements were taken of each of the two pronunclei of a tubal ferret ovum, in mounted and stained paraffin sections. Each set of measurements consisted of: (1) long axis; (2) transverse axis; (3) depth. (1) and (2) were obtained by an eyepiece micrometer. (2) was determined after the measurement of (1) by rotating the microscope stage bearing the specimen through exactly one right angle. (3) was obtained by the fine adjustment of the microscope. The oil immersion method was used throughout. Allowance was made for the possible inaccuracy of the fine adjustment by taking readings at various parts of this mechanism.

The volumes and areas were calculated by the formulae for an ellipsoid.

The results were subjected to statistical analysis, the variability of the volumes, areas, and of the measurements themselves being specially examined.

The measurement of the long axis was the least variable. The depth measurement (coefficient of variation between 4 and 7) accounts in a large part for the variation in volume and area, but the variability of the depth is, in one of the pronuclei, no greater than that of the transverse axis.

The forces acting between two pronuclei that are in contact are such as to make the two of similar dimensions in planes parallel to the plane of contact, and therefore very different at right angles to this plane.

Here, as in many other investigations, the differences in dimensions of the three axes will not allow omission of the depth measurement, and with the precautions here taken, as much dependence can be placed on the results of depth measurement as upon the measurement of transverse axis.

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THE SIZES OF FERRET PRONUCLEI

V Pron- uclei Size

VI Polar Body

VII Cyte- plasm

VIIi Zona Gran- ulosa

IX Cyum Vol- umes

X Ovar- ian Stroma

Appen- dix

THE SIZES OF FERRET PRONUCLEI

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INTRODUCTION

Almost everywhere throughout the literature on pronuclear development are found general references to size. The two pronuclei are stated to be of different size, and their changes in size are mentioned, but beyond this, with regard to mammalian pronuclei, very little exact work has been carried out. In those records where measurements have been recorded, they have not been analysed so as to show their correlation with other quantities or with structural phenomena, and moreover little indication is given of the dependence that can be placed on the data, for the variability due to technique in preparation and measurement is neglected. The present article is written to indicate how these defects may be made good, to show what kind of results may be obtained, and to demonstrate, if possible, how large the collection ought to be to give satisfactory results. The number of prepared specimens of ferret pronuclei in existence is very limited, and in such a case the determination of the number of specimens desired is one of the most fundamental investigations.

For historical information on nuclear and cytoplasmic measurements, reference may be made to the work mentioned in a recent article by the author (Mainland, 1931 a), and also to the articles by Shull (1922), Erdmann (1911) and Koehler (1912). Much of this kind of work has been done on Invertebrate material, some of it being on embryonic stages, but even in such cases the stages are mostly post-pronuclear.

MATERIAL AND METHODS

The observations discussed here were made at the same time as the structure of the ferret pronuclei was studied (Mainland, 1930), and the series of specimens was the same in both cases. Measurements were made on the pronuclei of 52 ova, some fixed in Zenker's fluid, some in Perenyi's, and a few in Mann's fluid, the staining methods being Heidenhain's iron-haematoxylin and Mayer's haemalum. The ova were in paraffin sections cut at 10 microns.

Details of the method of measurement and calculation have been given in a recent paper (Mainland, 1931a) in which tests were recorded showing the variability due to experimental error. It is sufficient here to state that the volumes and areas were calculated from three dimensions of each pronucleus:

(1) The long axis in the largest section of each pronucleus,

(2) The transverse axis, i.e., at right angles to (1), the accuracy of the position of this second axis being insured by rotation of the microscope stage from one fixed point to another 90° distant,

(3) Depth, determined by the graduated fine adjustment of the microscope. In the article referred to, the use of depth measurement is discussed and justified, and its importance emphasized.

The volumes were calculated from the formula for the volume of an ellipsoid: $\frac{4}{3}\pi \cdot \frac{a}{2} \cdot \frac{b}{2} \cdot \frac{c}{2}$, where a,b,c are the axes.

The areas of the surfaces of the pronuclei were calculated from the formula $\frac{\pi}{3}(ab + bc + ca)$, kindly computed for the purpose by Professor N.R. Wilson of the University of Manitoba.

RESULTS

The accompanying table (I) shows the result of the calculation of volume and area. All the results obtained are given in the table , but in subsequent discussions only those pronuclei are considered that occurred in pairs. Where only one promucleus could be satisfactorily measured the results were not taken into account.

Comparison of Volumes and Areas

Central pronuclei (35 pairs):

Mean volume of larger pronuclei: 4628.2 cubic microns.

Standard deviation of series: ± 1584.0.

Coefficient of variation: 34.2 per cent.

Probable error of coefficient of variation: ± 3.06

Mean area of larger pronuclei: 1332.6 square microns.

Standard deviation of series: ± 307.4.

Coefficient of variation: 23.1 per cent.

Probable error of coefficient of variation: ± 1.96.

Difference between coefficients of variation of volume and area: 11.1

Probable error of difference: ± 3.63.

The difference is therefore significant, for it is more than three times its probable error.

Mean volume of smaller pronuclei: 3516.2 cubic microns.

Standard deviation of series: ± 1371.0.

Coefficient of variation: 39.0 per cent.

Probable error of coefficient of variation: ± 3.58.

TABLE I. Volumes and areas of Pronuclei

A. Pronuclei Central in Ovum

No. of Ovum	Pro-nucleus	Volume (μ^3)	Ratio of Vols. (Greater)/(Lesser)	Area (μ^2)	Ratio of Areas (Greater)/(Lesser)
2	A	4171.0	1.55	1253.0	1.33
	B	2697.0		942.0	
3	A	2908.0	1.45	987.4	1.28
	B	4206.0		1261.0	
4	A	5399.0	1.93	1488.0	1.55
	B	2794.0		965.2	
5	A	5746.0	1.19	1553.0	1.12
	B	4828.0		1386.0	
6	A	3782.0	1.20	1174.0	1.14
	B	4546.0		1338.0	
14	A	4019.0	2.04	1233.0	1.60
	B	1975.0		768.6	
16	A	2291.0	1.18	837.1	1.11
	B	1936.0		757.1	
17	A	5161.0	1.47	1458.0	1.28
	B	3513.0		1138.0	
18	A	5492.0	1.25	1571.0	1.10
	B	4393.0		1424.0	
19	A	2977.0	1.24	1012.0	1.15
	B	3689.0		1168.0	
20	A	5451.0	1.02	1509.0	1.03
	B	5586.0		1550.0	
22	A	6042.0	1.24	1632.0	1.16
	B	4874.0		1410.0	
23	A	7837.0	1.78	1909.0	1.46
	B	4393.0		1304.0	
47	A	5439.0	1.74	1504.0	1.44
	B	3123.0		1041.0	
55	A	3387.0	1.35	1093.0	1.21
	B	2509.0		901.4	
57	A	2609.0	1.23	918.4	1.15
	B	3217.0		1055.0	
58	A	4111.0	1.37	1242.0	1.23
	B	3010.0		1013.0	
59	A	2764.0	1.56	953.2	1.34
	B	4290.0		1277.0	
60	A	4568.0	1.31	1372.0	1.20
	B	3481.0		1142.0	

TABLE I (continued). Volumes and areas of pronuclei

A. Pronuclei Central in Ovum

No. of Ovum	Pronucleus	Volume (μ^3)	Ratio of Vols. (Greater)/(Lesser)	Area (μ^2)	Ratio of Areas Greater/Lesser
62	A	2174.0	1.13	784.1	1.13
	B	2445.0	~~1.13~~	893.4	
63	A	1912.0	1.14	683.4	1.02
	B	1680.0	~~1.14~~	699.3	
64	A	2266.0	1.63	848.5	1.22
	B	1579.0		697.5	
85	A	4597.0	1.14	1340.0	1.09
	B	5227.0		1458.0	
86	A	5532.0	1.23	1515.0	1.14
	B	4501.0		1324.0	
87	A	2038.0	1.27	779.9	1.18
	B	2580.0		910.4	
88	A	4506.0	1.37	1326.0	1.23
	B	6177.0		1636.0	
89	A	5195.0	1.56	1470.0	1.34
	B	3336.0		1101.0	
92	A	2966.0	1.31	1001.0	1.19
	B	3870.0		1194.0	
93	A	1782.0	1.88	724.3	1.50
	B	3345.0		1087.0	
94	A	2086.0	1.12	761.1	1.26
	B	2336.0		960.6	
95	A	3826.0	1.08	1158.0	1.03
	B	3542.0		1128.0	
96	A	5191.0	1.14	1486.0	1.07
	B	5896.0		1586.0	
98	A	6790.0	1.08	1733.0	1.04
	B	6287.0		1661.0	
99	A	7005.0	1.21	1698.0	1.09
	B	5772.0		1561.0	
100	A	7012.0	1.20	1782.0	1.12
	B	8389.0		2001.0	
45	A	2827.0	1.09	971.0	1.06
	B	2894.0	1.12	985.4	1.08
	C (subcent.)	2584.0		912.2	

B. Pronuclei Subcentral in Ovum

8	A	205.3	1.203	168.3	1.137
	B	244.9		189.7	
44	A	3315.0	1.32	1093.0	1.22
	B	2513.0		893.2	

C. Pronuclei Subcentral and Peripheral

43	A (periph.)	479.2	1.31	301.6	1.18
	B (subcent.)	626.2		353.2	

TABLE I (continued). Volumes and areas of pronuclei

D. Pronuclei Central and Peripheral

No. of Ovum	Pro-nucleus	Volume (μ^3)	Ratio of Vols. Greater / Lesser	Area (μ^2)	Ratio of Areas Greater / Lesser
15	A(central)	2310.0	1.624	849.1	1.38
	B(periph.)	1422.0		613.7	

E. Pronuclei Peripheral

7	A	358.4	11.86	244.9	5.07
	B	30.20		48.3	
9	A	22.8		39.2	
10	A	24.11	31.32	40.5	10.02
	B	754.9		405.2	
11	A	24.02	1.68	40.4	1.35
	B	14.3		30.0	
28	A	313.9		223.5	
29	A	139.8	1.10	130.5	1.07
	B	126.3		122.1	
30	A	551.0	3.26	325.4	2.15
	B	168.8		151.6	
36	A	150.4		138.7	
41	A	117.5		118.5	
48	A	60.0		77.2	
91	A	356.2	3.88	246.8	2.43
	B	1383.0		600.8	
97	A	600.8	1.08	351.1	1.07
	B	556.2		327.4	

Mean area of smaller pronuclei: 1111.1 square microns.

Standard deviation of series: ± 292.8.

Coefficient of variation: 26.4 per cent.

Probable error of coefficient of variation: ± 2.27.

Difference between coefficients of variation of volume and area: 12.6.

Probable error of difference: ± 4.24.

The difference here is almost significant.

The reason for the enlargement of the pronuclei after insemination is not known in detail. It is not clear whether the effect, and perhaps the main object, is the establishment of a certain volume, or the establishment of a certain area of surface. The results just given suggest, at first sight, an answer to the question. The differences of size of the ova probably influence the differences in size of the pronuclei, but in these data the ova were the same, thirty-five specimens, and the comparison was made between the differences of volume on the one hand and the differences of area on the other. The areas are much less variable than the volumes, and from this it might be supposed that a certain area rather than a certain volume was the aim or effect, at least, of the enlargement. When reference is made, however, to the measurements of a single pair of test pronuclei (Mainland, 1931a) it will be seen that this explanation does not hold. In these tests the coefficient of variation of volume in twenty-five measurements of the ~~larger~~ smaller pronucleus was 6.5 per cent ± 0.623 and the coefficient of variation of area calculated from the same measurements was 4.2 per cent ± 0.396. The difference was 2.3 and its probable error was ± 0.72.

The difference is therefore significant. When the measurements of volume and area of the other pronucleus were compared with each other, there was found to be a difference in the variation that was almost significant, as in the case of the smaller pronuclei in the present series of thirty-five pairs. Although the variability was much greater in the present series than in the series of measurements made on a single pair, yet the area measurements varied less in both cases and one can account in both cases for this by the methods of measurement and calculation, for it is obvious that such an explanation accounts for the difference when the same pronucleus was used for the calculations. The object of the enlargement of the pronuclei is not, apparently, simply the establishment of a definite area for metabolic interchange between pronuclei and cytoplasm.

Comparison between Central and Peripheral Pronuclei

The numbers of observations in this series are unfortunately small (7 pairs), but the results are nevertheless of some interest.

Mean volume of larger pronuclei: 544.6 cubic microns.

Standard deviation of series: ± 417.5

Coefficient of variation: 76.7 per cent.

Mean volume of smaller pronuclei: 182.3 cubic microns.

Standard deviation of series: ± 188.9.

Coefficient of variation: 103.6 per cent.

Mean area of larger pronuclei: 299.7 square microns.

Standard deviation of series: ± 170.6.

Coefficient of variation: ± 56.9 per cent.

Mean area of smaller pronuclei: 138.1 square microns.

Standard deviation of series: ± 104.9.

Coefficient of variation: 76.1 per cent.

In each case the variation between peripheral pronuclei is greater than the corresponding variation between central pronuclei. In spite of the smallness of the numbers, it is evident that when the pronuclei are peripheral they undergo greater changes of size than after they have become established at the centre of the ovum. This view is confirmed by comparison of specimens such as number 8 and number 44 (table I, B). In both of these the pronuclei are subcentral and yet there are very great differences in pronuclear size.

Combined Areas of Central Pronuclei

Since the areas were less variable than the volumes, it was decided to consider only the former as a measure of size. When the areas of the two pronuclei are added together it may be assumed that to some extent the sum is an index of development - that the greater sums will be found at the later stages, but, on the other hand, this might be counterbalanced by a secondary diminution in size, perhaps preparatory to segmentation. If there is a natural tendency for the central pronuclei to reach a maximum area and to maintain it, the frequency distribution curve of combined pronuclear areas should be skewed to the right, that is, most of the specimens should be found to possess areas greater than the average. The 35 pairs of central pronuclei here recorded are insufficient to prove this conclusively, but the figures suggest that the hypothesis is correct.

Combined areas of pairs of central pronuclei:

1001 - 1500	square microns	. . . . .	1
1501 - 2000	" "	. . . . .	8
2001 - 2500	" "	. . . . .	9
2501 - 3000	" "	. . . . .	10
3001 - 3500	" "	. . . . .	7
			35

The only external measure of developmental stages available for these ova was the length of the period between insemination and the killing of the animal, and the limitations of this measure ~~of this measure~~ have been shown elsewhere (Robinson, 1918; Mainland, 1930). The mean combined area of central pronuclei at the different post-inseminal periods is shown below:

At 41½ hours after insemination:	1797.9 square microns	(2 observations)
At 47½ " " " :	2664.6 " "	(9 ")
At 51¾ " " " :	2725.5 " "	(2 ")
At 64¼ " " " :	2549.8 " "	(12 ")
At 76½ " " " :	2545.0 " "	(1 ")
At116½ " " " :	2097.3 " "	(4 ")

There is no suggestion of regular changes in the means as the time advances. The number of observations is small and the variation in each time class was found to be great. The ova at 47½ hours and at 64¼ hours are most numerous. The difference in the means between these is 114.8 square microns, but the probable errors are ± 87.11 and ± 110.79 respectively, and therefore the difference is not significant. Even if time, as here measured, has some influence, there must be other factors responsible for the great amount of variation in size, apart from the time, and apart also from errors of measurement, for the errors of measure-

ment (Mainland, 1931a) do not produce such great variation as that found here (see above, pp.4-5). Other factors responsible for the variation might be the size of the ovum (upon which investigations are at present being carried out); temporary metabolic changes; and differences in preparation, e.g. of fixative. The influence of fixatives was studied as follows:

Mean combined area of pairs of central pronuclei.

Fixative	Area	Observations
Perenyi	2227.3	9
Zenker	2646.8	23
Mann	1535.4	3

Since the numbers of observations were small, Fisher's t-test was applied (Fisher, 1930 p.107) in the comparison between Zenker and Perenyi specimens and the result ($t = 0.65$; $n = 30$) showed that the difference in fixative did not account for size differences.

Ratio of Greater Area to Smaller.

The ratio is an expression of the relative areas of the two pronuclei. The greater the difference in size, the greater the ratio. Inspection of table I suggests that the ratio is greater in the ova with peripheral pronuclei than in those with central pronuclei. The results were tested as follows:

Mean ratio of areas for 35 pairs of central pronuclei: 1.210.

Probable error of mean: ± 0.0169 Standard deviation of series: ± 0.149
Coefficient of variation of series: 12.3 per cent.
Mean ratio for 7 pairs of peripheral pronuclei: 3.308.

Probable error of mean: ± 0.77. Standard deviation of series: ± 3.03

Coefficient of variation of series: 91 per cent.

Difference between means: 2.098 ± 0.77;not significant.

The difference between the means of the central and peripheral pronuclei is not significant,owing to the smallness of the numbers of peripheral pronuclei and to the great variation in size of these, but the difference between the ratios is made clear by taking the extreme possible ratio for the central pronuclei, which can be estimated from the standard deviation of the series (0.149). Three times this (i.e. 0.45), added to the mean gives the highest possible ratio to be expected in the series,i.e. 1.66. Now 5 out of the 7 ratios of peripheral pronuclei are above this limit(one being 5.075 and another 10.020). Thus the peripheral pronuclei tend to have very much higher ratios than the central pronuclei, that is, the members of a pair of peripheral are very much more apt to differ widely in size than are the members of a pair of central pronuclei.

Several questions might be raised with regard to the ratio of areas in the case of the central pronuclei. It might be asked whether, in an ovum that was going to give rise to a female the ratio was different from that in an ovum destined to become a male, that is, whether the chromatin constitution of the pronucleus influenced the ratio. Table ~~2~~ II shows the ratios arranged according to the animal from which the ova came. It will be seen that the ratios do not tend to group themselves in two classes, although certain of the ova must be males and certain must be females. If there is any influence of chromatin constitution, its effect is obliterated by variation due to other causes.

The totals given in the second-last row of table II show that the frequency-distribution curve would be skewed to the left. The numbers are small, but there seems to be a tendency for the majority of the central pronuclei to be nearly equal in area rather than very different. It may next be asked whether the central pronuclei do not tend more and more to arrive at a ratio near unity as their development proceeds. This was tested by taking a mean combined area for each class of ratio (table II). There appeared to be some tendency for the greater combined area to be associated with the smaller ratio. A straight line regression equation was calculated by Fisher's method (Fisher, 1930), to show whether the mean combined areas could be arranged in a straight line descending as the ratios increased. The variations in the individual items was so great, however, that the t-test showed that the slope of the straight line was not significantly different from the horizontal, that is, the areas remained, so far as could be shown, constant with reference to the ratios.

TABLE II. Ratios between Areas of Central Pronuclei --- Numbers of Ova arranged according to Ratios

Designation of Animal	Ratios of Areas						
	1.01-1.10	1.11-1.20	1.21-1.30	1.31-1.40	1.41-1.50	1.51-1.60	Total
26.7	5	3	2	1	1		12
G 9	1	2	1				4
DA 5 R					1		1
GB 4	3	3			1	1	8
DA 38		2					2
DA 33			1	1		1	3
FZ 1.1		2	2	1			5
Total	9	12	6	3	3	2	35
Mean Combined Area of Pairs of Pronuclei (μ^2)	2760.2	2411.6	2121.3	2332.1	2523.1	2227.4	

Relative Sizes of Central Pronuclei

The size difference between the two members of a pair of central pronuclei is chiefly of interest because of the sexual difference between the two. It becomes desirable to differentiate in as many ways as possible the two pronuclei, and if relative size can be taken as an index of sexual difference, it may be of great importance in ova where it is not possible to distinguish the male pronucleus from the female by the obvious characteristic - the possession of a sperm tale.

There was one ovum (no. 88) in this series that contained an undoubted sperm tale in its cytoplasm (Mainland, 1930), and the pronucleus near which it was found was the smaller (see table I). Unless the pronuclei has been displaced, this appears to show that the male pronucleus is the smaller. In the guinea-pig the ~~larger~~ male pronucleus is stated by Lams (1913) to be the larger, and the figures of guinea-pig ova shown by O. van der Stricht (1923) reveal the fact that the numbers upon which this statement was based are quite adequate to exclude pathological or freak specimens. Hill and Tribe (1924) describe two ova of the cat in each of which there was a supernumerary pronucleus. In these specimens there was a large and a small central pronucleus, while the extra pronucleus was comparable in size to the larger central pronucleus. The authors concluded that in the cat, as in the guinea-pig, the larger pronucleus was the male. The argument has a certain weakness, perhaps, because it is based on abnormal specimens. One specimen of the series of ferret ova was comparable with these of Hill and Tribe (no. 45, table I, at end of section A). In this the two central pronuclei were of nearly equal size. The extra pronucleus (pronucleus C), presumably the male, was smaller than either ~~than~~ of them. In the ferret,

therefore, there are two specimens, both in agreement, which suggest that the smaller pronucleus is the male.

In the description of the pronuclei refferred to above (Mainland, 1930) there was a discussion of various structural differences between the two pronuclei, e.g., the presence in certain of the central pronuclei of eosinophilic or pale globules, differences in shape and size of the chromatin particles, differences in the reticulum of the pronuclei. The two pronuclei of one ovum differed so seldom from each other in these respects that it was impossible to distinguish the male from the female or the larger from the smaller by any of these structural peculiarities. The peculiarities were due to factors influencing both pronuclei, in some cases technique, in others the developmental stage.

Relation of Central Pronuclei and Polar Bodies

Table III shows the arrangement of pronuclei with reference to polar bodies. The chi square test showed that the actual arrangement differed from the theoretical random arrangement, and even when the three ova with equidistant pronuclei were omitted the chi square test result was still significant. The conclusion is that there is a definite tendency for the smaller of the two central pronuclei to be nearer the polar body.

In the guinea-pig(Lams, 1913) the larger pronucleus is nearer the point of emission of the polar bodies, and, as mentioned above, in that animal the larger pronucleus is probably the male. It has already been shown that the evidence points to the male pronucleus as the smaller in the ferret, and it has now been shown that the smaller pronucleus is nearer the polar bodies. This appears to confirm the previous suggestion that the male is the smaller pronucleus, for the phenomena suggest the following

TABLE III Arrangement of Central Pronuclei with reference to Polar Bodies

Number of Ova	Larger Pronucleus Nearer	Smaller Pronucleus Nearer	Pronuclei Equidistant	Total
Actual	5	16	3	24
Theoretical (Random Distribution)	8	8	8	24

$\chi^2 = 12.25$; $n = 2$; P is less than 0.01

statement: In some animals the male, and in others the female, is ~~there~~ the smaller of the central pronuclei, but in either case the male pronucleus tends to be nearer the point at which the polar bodies are found.

The significance of this relationship to the polar bodies requires a little further discussion. It has been shown (Mainland, 1931 b) that the ferret ovum has a polar distribution of its cytoplasm, i.e., there is a granular pole and a pole at which more deutoplasm or lipoid material is assembled. It has been further shown that polar bodies tend to be located nearer the granular pole rather than the deutoplasmic pole. The polar bodies therefore, although severed quickly from the ovum, tend to form a landmark with reference to the granular pole, which is therefore the "animal" pole of the ovum. One may therefore suggest that the pronuclear arrangement is such that the smaller tends to be turned towards the "animal" pole. A direct test was made of this suggestion, and the result was at first sight in disagreement with it, for, out of 17 ova, 8 showed the larger central pronucleus near the granular zone, 7 showed the smaller pronucleus nearer, and in 2 ova the pronuclei were equidistant. The arrangement therefore appeared a random one. It was shown, however, by the chi square test that the data just recorded could also agree with those obtained above for the distribution of the pronuclei with reference to the polar bodies. The disagreement, therefore, was only apparent.

Absolute Size of Central Pronuclei

The size (area of surface) of the central pronucleus may be taken as an index of development, for it is known in general that the pronucleus enlarges in the course of its development, and it is in particular indicated here that the pronucleus worked up

towards a maximum, and did not thereafter change greatly in size (see section on combined areas of pronuclei with reference to their frequency-distribution). It might be expected, therefore, that some of the features of pronuclear structure previously described (Mainland, 1930) ought to be correlated with the development of the pronucleus in this way.

(i) Sizes of Particles of Chromatin.

No. of Pronuclei	Size of particle most Notable in Pronucleus	Mean Area of Pronucleus (μ^2)
4	small	1363.5
6	medium	1089.4
10	large	1341.8
32	various	1273.9

Without much statistical analysis it was clear that there was no association between pronuclear and size of chromatin particle.

(ii) Fineness of Reticulum.

No. of Pronuclei	Reticulum	Mean Area of Pronucleus (μ^2)
17	fine	1256.2
9	medium	1298.6
16	coarse	1290.5

Again, inspection of the variation among the data from which these averages were obtained showed, without calculation of probable errors, that there was no significant relationship demonstrable between the reticulum and the size of the pronuclei.

(iii) Presence of Eosinophilic, Pale or Colourless Globules.

No. of Pronuclei	Globules eosinophilic, etc.	Mean Area of Pronucleus (μ^2)
18	none	1388.6 ± 12.53
32	one or more	1426.7 ± 39.56

Here also there was no significant difference. It is easily observed that the very small peripheral pronucleus is basiphilic, but the data just recorded show that in the central pronuclei

no association between size (area) and structure. If there is any association, it is too small to show through the great variation present in the pronuclear sizes.

The facts thus elicited by measurement of the pronuclei appear to be largely negative, and one must endeavor to ascertain what positive contribution they have made to our knowledge of pronuclear development. It has been already shown (Mainland, 1930) that, even in a collection of 43 ferret ova, obtained, some early and some late in the stage of central pronuclei, it is impossible to demonstrate a definite sequence of pronuclear transformations. It is now shown that the sizes of the pronuclei at this stage are very variable. They are much more variable, indeed, than was suspected by the author when making the observations. This fact in itself affords justification for the investigation. Not only are the sizes very variable, but are, so far as can be shown, not associated with structural differences. This fact is bound to modify our conception of pronuclear development. One may conceive of the pronuclei enlarging and coincidentally proceeding through a series of structural changes; but the evidence so far obtained does not support this conception.

It is quite probable that the negative character of the results obtained is largely due to the great variability of pronuclear size. When such variability has been demonstrated, the further object of the investigator is to show how large the series of specimens should be to compensate for the variability. A test of this kind was made on the series quoted above in which there was a comparison of areas of pronuclei with regard to the presence or absence of eosinophilic globules. It was assumed that the means and the variability remained the same as the numbers of observations increased. For the difference to be

significant, the probable error would need to be at most $\frac{38}{3}$, say 12. 'x' was assumed to be the number of times the observations, i.e. number of specimens, required to be increased . The probable errors would then be $\frac{1}{\sqrt{x}}$ of the present ones. It was then shown by a solution of a simple equation that the value of 'x' would require to be about 12. Therefore the observations would have to be increased nearly 12 times to make the difference between the means significant. On the other hand, of course, as the observations increased, the variability might also increase, or the means might become less different.

Further investigation is therefore indicated. This investigation may proceed in two directions, both by greatly increasing the numbers of specimens, and by determining the relation of the ovum size to the pronuclear size.

SUMMARY

Measurements have been made of the pronuclei in the paraffin sections of 52 ferret ova. The volumes and areas have been calculated by the use of the formulae for an ellipsoid, and the results have been analysed statistically. There is great variation between the areas of the central pronuclei of 35 different ova. There is even greater greater variation between the corresponding volumes, but this does not prove that a definite area of external surface is the object or result of pronuclear enlargement, for in a previous investigation (Mainland, 1931 a) a series of measurements of the same pair of pronuclei was made and the resulting volumes varied more than the corresponding areas.

The peripheral pronuclei were few (7 pairs), but these pronuclei vary from ovum to ovum much more than the central pronuclei.

Owing to the smaller variation in areas, these were used, instead of the volumes, in the subsequent investigations. The two areas of each pair of central pronuclei were added together and arranged in a frequency-distribution series. Although the series is too small to give definite proof, it shows that as the size increases the numbers increase and then more rapidly decline. This suggests that the pronuclei enlarge up to a maximum and do not subsequently diminish.

There is no indication that the size of central pronuclei increases as the time after insemination increases.

The difference in fixative had no apparent influence on pronuclear size.

The ratio between the areas of the two members of each pair of pronuclei was found by dividing the area of the greater by the area of the smaller.

There is a tendency for the central pronuclei to be nearly equal in area, rather than very different, for there is no ratio above 1.60.

The pronuclear ratio is not demonstrably influenced by the fact that some of the ova were destined to give rise to males and others to females.

It appears as if the pronuclei with the greater combined area (i.e., presumably, those more advanced in development) tend to have a ratio nearer unity than the rest, but this cannot be conclusively proved from this material.

The pairs of peripheral pronuclei tend to have much greater ratios (e.g. over 10.0) than the central pronuclei, that is, the two peripheral pronuclei differ more from each other in size than do the central pronuclei.

One ovum containing a sperm tail and one ovum with a supernumerary pronucleus are in agreement in suggesting that the male pronucleus is in the ferret the smaller.

The smaller of the central pronuclei is in a significant majority of cases nearer the polar bodies, that is, nearer the "animal pole" of the ovum, than is the larger pronucleus.

There is no significant association between the size (area) of a pronucleus and (i) the sizes of its chromatin particles, (ii) the fineness of its reticulum, (iii) the presence in it of eosinophilic, pale or colourless globules.

A test has been carried out with the data on the eosinophilic or pale globules, to show that, if the variability of the data and the difference between the average pronuclear areas remained the same, the number of observations would have to be multiplied by 12 to render this difference ~~significance~~ significant.

Further investigations should involve increasing the number of specimens and studying the relationship of ovum size to pronuclear size.

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Donald Mainland: The Sizes of Ferret Pronuclei.

ABSTRACT

Statistical analysis has been made of the volumes and surface areas of ferret pronuclei in paraffin sections(central pronuclei:35 pairs; peripheral pronuclei: 7 pairs). There is more variation between volumes (e.g. coefficiant of variation for 35 central pronuclei: 34.2 per cent) than between areas(e.g.,coefficient of variation for same 35 pronuclei: 23.1 per cent),but a similar difference was found previously(Mainland) in a series of 25 test measurements on one pair of pronuclei.

The peripheral pronuclei vary more than the central pronuclei from ovum to ovum.

The central pronuclei appear to emlarge to a maximum and not subsequently to diminish.

The lapse of time after insemination and the difference of fixative have no apparent influence on the size(area) of the central pronuclei.

For the central pronuclei the ratio $\frac{\text{area of greater}}{\text{area of smaller}}$ is not more than 1.50,but for the peripheral pronuclei may be over 10.0.

The sex of the ovum has no apparent influence on this ratio.

The presence of a sperm tail in one ovum and an extra pronucleus in another indicatesthat the male pronucleus is the smaller.

The smaller central pronucleus tends to be nearer the polar bodies, i.e.,nearer the animal pole of the ovum.

There is no significant association between size(area) and structure of a pronucleus(size of chromatin particle,fineness of reticulum or presence of non-basiphilic globules)

To secure significant results with some of these data,it would be necessary,on account of their variability,to multiply the number of observations by 12. Further investigations should also involve the study of the relationship of size of ovum to size of pronucleus.

A QUANTITATIVE STUDY OF THE POLAR BODY OF THE FERRET, WITH A NOTE ON THE SECOND POLAR SPINDLE

VI Polar Body

VII Cyte-plasm

VIII Zona Gran-ulosa

IX Ovum Vol-umes

X Ovar-ian Stroma

Appen-dix

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A QUANTITATIVE STUDY OF THE POLAR BODY OF THE FERRET, WITH A NOTE ON THE SECOND POLAR SPINDLE

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FIVE FIGURES

INTRODUCTION

In a recent article on the pronuclei of the ferret ovum (Mainland, '30) an attempt was made to justify the addition of another to the already numerous descriptions of mammalian pronuclei. To justify an article on the relatively unimportant polar bodies might seem even more difficult, but in the first place it may be pointed out that the polar bodies of carnivores have never been systematically studied in detail, and the structure of the ova of the ferret, dog, and cat differs so much from that of the ova of the mouse and rat that one is not justified in supposing that the polar bodies would resemble each other in these two classes. Further, the minute regard paid by such investigators as O. van der Stricht to the pronuclei and cytoplasm of tubal ova has not been paralleled by the treatment of the polar bodies of mammals even outside the carnivore group. The reason for this lack of interest has possibly been the feeling that the polar bodies, once liberated, become of no further consequence in the development of the ovum. It should not be overlooked, however, that a knowledge of the number of polar bodies produced is of very great importance to the understanding of the maturation process of the ovum. The position of the polar bodies, also, in cases where they remain attached by a spindle, is of very great value in a study of the position of entrance of the sperm and even of subsequent segmentation.

Considerations such as these have seemed to warrant the present investigations. The method has been quantitative and statistical—a method that entails perhaps five times the amount of labor involved in simple observation. The advisability of such a method might be questioned in the light of the few positive results obtained by the application of this novel treatment to the pronuclei in the article mentioned above. The answer to such a questioning would be that, where there is variability, there is no logical alternative to the statistical method. Simple observation leads to the formation of impressions without actual proof. One can, indeed, by single instances, indicate possibilities and on some occasions prove a negative. For example, if the two polar bodies that have arisen from the division of the first one are of different structure, it can be concluded that structural difference does not indicate difference of origin. If a positive generalization is required, however, statistical methods are necessary.

One of the chief results of the use of statistical methods is the demonstration that collections which would be adequate for simple observation are only partly adequate for this treatment. Thus, one is led to appreciate how large a collection may have to be. One is further led to hesitate before accepting some of the non-statistical generalizations based on collections of similar size to, or smaller than, one's own. On the positive side, in this present article, certain results will be recorded which would not have been achieved without the use of quantitative and statistical methods. Beyond this, the methods that have been developed are applicable to much larger collections of specimens, either of mammalian or non-mammalian ova, of invertebrate ova, or even, in some instances, to cells and other structures bearing relationships similar to those existing between polar bodies and ova. It is a consideration of these facts that has prompted the author to give some possibly tedious detail and to conduct the reader through what may be at first sight rather complicated processes of mathematical reasoning.

STATISTICAL METHODS

The statistical method chiefly employed here is the chi-square method. One use of this method was briefly explained in a previous paper (Mainland, '30). More complete and detailed descriptions are to be found in Fisher ('28) and Yule ('27). It is not intended to repeat here what was said in the article above mentioned, but one or two further remarks may be made. Briefly, the chi-square method is a test of agreement or divergence between actual occurrences and expected occurrences. In the paper just referred to, the calculations of expected occurrences were based upon the actual occurrences in the specimens examined. The method has been used in the present paper in a case where the calculated numbers were based, not upon the actual occurrences, which are subject to errors of sampling, but upon a pure hypothesis in which there could be no error of sampling. Details of this variety of the chi-square test are exemplified in table 2 (p. 218). The difference between the actual and the expected occurrences may be so small that it could be accounted for by errors of sampling in the observation of the actual occurrences; that is, if one could observe more specimens, the theoretical number would be approached more nearly. On the other hand, some other factor or factors may be responsible for the difference, and if the value of chi square is above a certain amount, the difference is considered significant, and the presence of one or more of these other factors is proved. To determine if the value of chi square is significant, Fisher's ('28) table is employed. This table involves the use of the terms n and P, n being based on the number of groups into which the material is divided and P representing the probability of the occurrence, from 'chance' errors of sampling, of a chi square greater than the one found. Values of P from 0.05 downward are significant. The term 'significant,' applied to a value of chi square or to a difference between averages, is frequently met in the following pages. It should always be recalled that in this, as in all similar simple statistical treatments of data, the 'significant' point is the point

at which the chances are roughly 20 to 1 that the quantity has real meaning. In other words, the odds are roughly 20 to 1 against the results under discussion being due to chance or to the insufficiency of the data.

The other statistical method used here is the calculation of the standard deviation and probable error. This calculation was recently given in some detail (Mainland, '29). In that paper references may be found to some of the works useful to those who have not previously employed the method.

One of the greatest disadvantages in the present investigation and in all similar investigations is the smallness of the available samples. To overcome this disadvantage, it has been possible to employ Fisher's t test (Fisher, '28, p. 107), to obtain more accurate results than would be provided by the ordinary method of comparison of means by probable errors.

PREVIOUS INFORMATION UPON FERRET POLAR BODIES

It is to the observations made by Robinson ('18), while investigating another problem, that we are indebted for the knowledge we possess of polar-body formation in the ferret, namely, that the first polar body and second maturation spindle are formed normally after insemination, while the ovum is still in the ovary. Beyond this, there have been published, so far as the author is aware, no investigations upon the ferret polar body.

MATERIAL

The present investigations were carried out on the same specimens as were used for investigation of the ferret pronuclei (Mainland, '30), namely, 101 ova of the ferret, some from the uterine tube and some from the periovarian space of nineteen animals, the ovaries that contributed being thirty in number. Twelve of the ova were fixed in Flemming's fluid and merely tinted with eosin, and, as this method failed to demonstrate polar bodies, these ova, along with three others rejected on account of imperfect data, were excluded from the main part of the research. The eighty-six ova that remained had been fixed, some in Zenker's fluid, others in

Perenyi's, others in Flemming's, others still in Mann's fluid, with in some instances the addition of formol. Serial paraffin sections had been cut at 10 μ and stained, some with Heidenhain's iron-alum haematoxylin, others with Mayer's haemalum, and all had been counterstained with eosin. The stages of development included the second polar spindle stage, the stages of peripheral, subcentral, and central pronuclei, and the stage of commencing segmentation.

FALLACIES

The only structures that might, in a very few instances, have been confused with polar bodies are the rounded cell-like bodies found either in the oolemma or penetrating the ovum. These have been made the subject of a special investigation, and it has been concluded that they are products of the zona granulosa. Actually, there was no difficulty in distinguishing these from polar bodies. The greater depth of staining of the oolemma bodies, the larger size of their nucleus-like central mass, when that was present, and other peculiarities served to make differentiation easy.

NUMBERS OF POLAR BODIES

A general discussion of polar-body formation in animals is to be found in Wilson ('25, p. 493, etc.), where it is pointed out that typically three polar bodies are formed, the first dividing while the second is being produced. The frequent failure of the division of the first polar body is, however, noted, and it is further recorded that in some cases, especially in eggs heavily laden with yolk, the polar bodies are not actually extruded from the egg, their nuclei remaining in the protoplasm near the periphery. The conformity of mammalian ova to the common type has been doubted in respect of the mouse by Sobotta ('08), who was of the opinion that in only about one-fifth of the ova were two polar bodies produced. He explained this by supposing that the first polar spindle was directly converted into the second, while the chromosomes typically destined for the first polar body

degenerated. The same author, along with Burckhard (Sobotta and Burckhard, '11), noted that in tubal ova of the rat there was generally only one polar body, which they supposed was the second, the first having degenerated during ovulation. The frequent absence of polar bodies in tubal ova of the mouse was confirmed by Long and Mark ('11), who examined a large number of ova and had a definite numerical basis for their statements. They recorded that out of 507 eggs with complete second polar spindles, 189 had no polar body. They further stated that more of the older eggs had no polar bodies, as compared with younger ones. Kirkham ('07) explained the loss of the first polar body in the mouse by migration through a weak part of the oolemma.

Compared with the series of mouse ova examined by Long and Mark, the present collection of ferret eggs is small, and yet it may be justifiably considered a large collection of carnivore ova. The collection of cat ova observed by R. van der Stricht ('11) has been up to the present the most extensive series of early tubal carnivore ova recorded in detail. The specimens in that collection corresponding to these of the ferret were forty-four in number.

In the present collection of ferret ova there were, as has been stated, twelve specimens fixed in Flemming's fluid and merely tinted with eosin. This method of preparation was totally inadequate to show polar bodies, and in this investigation only such ova were considered as would not be liable, on account of lack of staining, to vitiate the results. There was, therefore, a total of eighty-six ova, each liable, so far as staining was concerned, to show the presence of a polar body. The distribution in respect of polar bodies was as follows:

Fourteen showed no polar body.
In fifteen the polar bodies were doubtful.
In thirty-one there was only one definite polar body.
In seventeen there were only two definite polar bodies.
In eight there were only three definite polar bodies.
One ovum had four polar bodies.
The total number of polar bodies is therefore ninety-three.

The absence of a polar body at this early stage of development might be due to one of the causes suggested by the authors referred to above—failure of development of a polar body, early degeneration, or migration. Explanations which have not been stressed, and which for the ferret appear to be important, are, first, the loss of a section or a part of a section in the preparation of the material, and, secondly, the obscuring of a polar body by the thickness of the oolemma. In respect of the care taken to preserve the entire ova, the collection examined may be assumed to be as satisfactory as any collection of mammalian ova; but the shape, thickness, and hardness of the oolemma were largely responsible for the two sources of error mentioned above. The final section of the ovum on this account was liable to be lost, either because of its rounded shape or because of its thickness. In other cases, where the final section was present, the density of the oolemma was liable to obscure such objects as polar bodies. It is reasonable to suppose that such factors should in part be responsible for the absence of polar bodies from the fourteen ova recorded above.

Effect of loss of sections on number of polar bodies

To prove whether there was any demonstrable association between loss of sections and absence of polar bodies, the ova were arranged according to the presence or absence of polar bodies and according to whether the series of sections of the various ova were complete or not. To this classification the chi-square test was applied, and the result ($\chi^2 = 2.55$; $n = 1$; $P < 0.20 > 0.10$) showed that the absence of polar bodies in the series as a whole was not demonstrably dependent on loss of sections.

Further investigations were carried out to show in how many of the fourteen ova the absence of polar bodies might have been due to loss of sections. The assumption underlying this investigation was that every ovum, except those with the second polar spindle still present, should have three polar bodies. In those with the second polar spindle the first polar

body might not have divided, and these ova were treated separately in the calculations.

For each incomplete ovum the numbers of sections lost were estimated, and the average number of sections per ovum was calculated, including both the sections present and those assumed to be lost. For eighty-six ova the average number of sections per ovum was 10.48 ± 0.19.

At the outset it has to be noted that the liability to loss of polar bodies will vary according to the sections in which they are present. Those in the last sections will be more liable to loss than those higher up, and if there are two polar bodies, one high up in the series of sections and the other in the last section, the ovum will be very unlikely to come into the class of fourteen ova with absent polar bodies. Out of nine ova with three (in one ovum, four) polar bodies, two showed all the bodies in the same section, five showed them in successive sections, while in two ova one of the bodies was two sections or more distant from its nearest neighbor. It was assumed that those proportions held throughout the series, and upon that basis an estimate was made of the number of ova that might be expected to have no polar bodies owing to loss of sections.

Since the calculations varied according to the number of sections lost, the ova were grouped according to this number. In all except one or two ova, the sections lost were terminal sections, with or without adjacent sections. The summary of the results of the calculations is as follows:

Estimated number of ova with polar bodies entirely absent	
1) In ova with one section missing,	0.80 ova
2) In ova with only two sections missing,	0.39 ova
3) In ova with more than two sections missing,	3.04 ova
Total,	4.23 ova

Table 1 shows the comparison between, 1) the actual number of ova with absent polar bodies and missing sections, and, 2) this theoretical number. The chi-square test applied to these data showed that the difference between the actual

numbers and the theoretical numbers was not significant. ($\chi^2 = 2.07$, $n = 1$, $P > 0.10 < 0.20$). Therefore the absence of polar bodies from ova with missing sections can be sufficiently accounted for by loss of sections.

In the list of polar bodies given above, however, fifteen ova are recorded as having 'doubtful' polar bodies. These structures were objects that suggested polar bodies in appearance, for example, by their rounded contour or by the presence of basophil granules. They might be degenerate polar bodies or stained albuminoid material in the perivitelline space, or, possibly, in one or two ova, some of the products of the zona

TABLE 1

Comparison between the actual number of ova without polar bodies and the theoretical number of such ova arrived at by a consideration of lost sections

	NUMBER OF OVA WITHOUT POLAR BODY	NUMBER OF OVA WITH ONE OR MORE FOLAR BODIES	TOTAL
Actual number of ova (A)	9 (23 per cent)	30 (77 per cent)	39
Theoretical number of ova (T)	4.23 (11 per cent)	34.77 (89 per cent)	39

NOTE: The 'doubtful' polar bodies (see text) are included as if they were definite polar bodies, and contribute to column 3.

granulosa that have been mentioned above in the discussion on fallacies. In the investigations recorded up to this point they have been considered as polar bodies, that is, the ova containing them have been excluded from the class of nine with absent polar bodies and missing sections. Under these circumstances, it has been possible to account for definite absence of polar bodies by loss of sections. It may, however, be assumed that the 'doubtful' bodies were not polar bodies, and then the class of ova with missing polar bodies will be increased. Of the fifteen ova containing these bodies, eight had missing sections. Hence, if they were not polar bodies, the total number of ova with absent polar bodies and missing sections would be seventeen instead of nine. There would, in

that case, be a discrepancy of $17 - 4.23 = 12.77$ between the actual number of ova with missing polar bodies and the theoretical number. The chi-square test showed that this difference was significant, and therefore loss of sections cannot account for absence of polar bodies unless the 'doubtful' polar bodies are interpreted as degenerate polar bodies.

It will be recalled that the assumptions upon which all the calculations have been based is that three polar bodies were present in all ova after fertilization. Similar calculations were made on the assumption that only two polar bodies were present after fertilization. In that case the expected number of ova without polar bodies owing to loss of sections would be 5.46. If nine were the actual number of such ova, there would, as shown by the chi-square test, be no significant difference between the actual and the theoretical number. Even if the 'doubtful' polar bodies were not degenerate polar bodies, there would still be no real difference between these two numbers. Hence, loss of sections can account for all the absent polar bodies if only two were present in each ovum after fertilization. Either, therefore, there is degeneration of polar bodies at this stage or lack of formation, or some other factors are at work. What these other factors may possibly be will be considered later.

Effect of thickness or density of terminal section of ovum on number of polar bodies

In ova that were complete, loss of sections naturally could not account for absence of polar bodies. The technical factor that might be responsible here is thickness or density of the oolemma of the last section of the ovum.

Out of the eighty-six ova under consideration, forty-seven were judged to be complete. By calculations similar to those made above in connection with lost sections, it was shown that if three polar bodies were expected after fertilization, the number of ova without polar bodies owing to thickness or density of the terminal section should be 1.82. The actual number was five, and the chi-square test showed that it was

impossible to detect any real difference between the actual and the theoretical number. When, however, it was assumed that the 'doubtful' polar bodies were not degenerate polar bodies, then the number of ova with absent polar bodies was increased from five to twelve, and there was a real difference between the actual and the theoretical number.

Moreover, if there were only two polar bodies in all the stages after fertilization (i.e., if the first polar body failed to divide), the theoretical number of ova with absent polar bodies would be 3.28, and the difference between this and the actual number (twelve) is still significant. Hence, missing polar bodies cannot sufficiently be accounted for by obscuring of the polar bodies by density of the last section of the ovum, even in conjunction with the failure of the first polar body to divide.

Thus, the conclusion is similar to that of the last subsection, namely, that thickness of oolemma can only account for the ova with missing polar bodies if the 'doubtful' polar bodies are counted as degenerate polar bodies. Actual observation showed that in two out of the five complete ova with absent polar bodies, the terminal section was not thick or dense enough to obscure polar bodies if these had been present and not degenerate.

One may summarize the investigations so far recorded on the numbers of the polar bodies as follows:

Provided that degeneration of polar bodies occurs at these early stages and is indicated by the 'doubtful' polar bodies observed in the ova, entire absence or apparent absence of polar bodies can be accounted for, 1) in ova with missing sections, by loss of sections, and, 2) in ova that are complete, by thickness or density of the oolemma of the terminal sections.

The question of degeneration requires further attention.

Degeneration as a cause of missing polar bodies

If the polar bodies degenerated (or if they migrated), it would be expected that there would be more ova without

polar bodies in the later stages after ovulation. Long and Mark ('11) stated that this was so in the mouse. To prove such a statement, statistical tests are necessary. Such tests were applied to the ova of the ferret, and at the same time the 'doubtful' polar bodies were taken into consideration, since if these were degenerate polar bodies it would be expected that they would be more frequent in the later stages after fertilization. Chi-square tests showed that the presence of these 'doubtful' polar bodies and the entire absence of polar bodies were independent of the number of hours after insemination and of the stage of development of the ovum as indicated by its nuclear material. This does not, however, prove that the 'doubtful' bodies are not polar bodies, for the time after insemination and even the nuclear development of the ovum are not direct indications of the age of the polar body. Some polar bodies, for example, may be so old at the time of fertilization that they have started to degenerate, whereas others may persist much longer. These and other factors suggest that a much larger series would be necessary to show connection between degeneration of the polar bodies and the stage of ovular development. It has been shown above that, if the 'doubtful' polar bodies are degenerate polar bodies, the missing polar bodies can be accounted for, and this suggestion must be held out until all the other methods of accounting for missing polar bodies are exhausted.

Polar-body migration

No evidence was obtained to suggest that migration of the polar body through the oolemma could account for absence of polar bodies.

Failure of polar-body formation

If a polar body failed to separate from the ovum, there would nevertheless be, as a result of the maturation process, a number of chromosomes, which would, presumably, persist for some time in the ovular cytoplasm. There was in the ferret no evidence of such persistence of unseparated polar-body chromosomes.

The lack of formation of the polar bodies may, however, influence the polar-body number in another way. If the first polar body fails to divide, and then the section containing it is lost or obscured, the only possible polar body to be found will be the second one, whereas if the first polar body had divided, there would be greater chance of at least one of its daughter cells being left. It will be recalled that reference was made to this subject in the subsection on "Thickness or density of the terminal section," and it was discovered that failure of the first polar body to divide did not sufficiently account for missing polar bodies, unless degeneration also had an influence.

In connection with the division of the first polar body, it is of interest to note the relationship between the number of the polar bodies and the stage of development of the ovum as indicated by its nuclear material. It was noted that out of thirteen ova at the second polar spindle stage, not one had more than two polar bodies. This is in conformity with the ordinary course of polar-body development. The chi-square test showed, however, that there was no significant difference between the number of polar bodies present after fertilization and those present before fertilization. This seems at first glance contrary to expectation, but the result may be due to the smallness of the numbers examined.

Although the numbers of specimens may be insufficient to elucidate this last-mentioned point, they have been sufficient to enable certain definite conclusions to be drawn regarding polar-body number. These conclusions are:

1. Migration of the polar body through the oolemma is not the cause of missing polar bodies.
2. Failure of separation of polar bodies from their ova is not the cause of missing polar bodies.
3. Loss of sections can partly, but only partly, account for absence of polar bodies from ova that have missing sections.
4. In ova that are complete, thickness or density of the oolemma of the terminal sections can partly, but only partly, account for polar-body absence.

5. Failure of division of the first polar body cannot account for all the missing polar bodies that cannot be accounted for by loss or thickness of sections.

6. Degeneration is very strongly indicated as a cause of absence of polar bodies.

It may be pointed out here that no attempt can be made to account for the occurrence of the ovum with four polar bodies. The excess may be due to division of either the first or the second polar body, and indicates a tendency to persistence and independent life beyond what is usually seen in the polar cells of mammals.

RELATION OF POLAR BODIES TO OVA

Physical connection with ovum

The mere observation of the polar bodies in the ova of a mammal not previously so studied cannot be expected to elucidate much further the status of the polar body and its relationship to the ovum. On the other hand, an adequate conception of the polar bodies in a mammal can hardly be obtained without some knowledge of certain facts and theories relating to polar bodies in general, including those of some non-mammalian animals.

When the maturation of the ovum is compared with that of the sperm, the polar bodies may be interpreted as rudimentary ova corresponding to three of the four spermatozoa which are the end products of maturation in the male (Mark, Bütschli, quoted from Wilson, '25). According to this view, the polar body and the ovum are completely independent entities. Strasburger (quoted from Wilson, '25, p. 619, etc.) argued from plants to animals, and interpreted the stage of polar body and mature ovum as the gamete-producing generation—the generation of the haploid number of chromosomes. This theory involves, in some sense, a close interrelationship of polar bodies and ovum.

In some animals phenomena occur that suggest that the polar bodies are independent organisms, capable on occasion of further development. Such phenomena are, 1) the divi-

sion of the fused polar nuclei of the parasitic hymenopteran insect Litomastix to form a mass of cells that almost surrounds the embryo (Silvestre, quoted from Wilson, '25); and, 2) the production of dwarf larvae in a platode worm, Prostheceraeus, by fertilization of a polar body by a sperm (Francotte, quoted from Wilson, '25). In a mammal such as the ferret the chief criterion of independence of the polar bodies is the rapidity of their physical separation. The records and illustrations of mammalian ova indicate a definite separation of the polar body from the ovum by a distinct cell membrane; but the persistence of spindle fibers between the polar bodies and the ovum for a greater or lesser period has been noted (Longley, '11, cat; O. van der Stricht, '23, guinea-pig, dog, bat, mouse). Long and Mark ('11) pointed out that the first polar body quickly lost its connection with the egg in the mouse, but for the most part little evidence has been presented to show how long such a connection may persist. It is open to question whether the persistence of the spindle fibers would indicate a vital connection of the cells; but if they remained as far as the first segmentation stage, they would be of considerable value in the study of this process.

It has been noted that out of eighty-six ova in the present series of ferret specimens, fifty-nine contained one or more definite polar bodies. The total number of polar bodies was ninety-three. In only one of these (in ovum no. 9) was there seen any structure definitely suggestive of fibers leading from the ovum to the polar body. In this ovum one pronucleus was peripherally placed, and a few strands led from it toward one of the polar bodies. In another ovum (no. 51) there was a possible spindle between the ovum and the polar body. The nuclear material in the ovum was at the second polar spindle stage. It is difficult in this series to say whether a long period has elapsed between the liberation of the polar bodies and the death of the ovum, and therefore one cannot estimate the rapidity with which the spindles disappear. One can, however, point out that in all the ova examined the polar spindles had disappeared by the time the pronuclei were centrally placed.

In one ovum (no. 53) there was a doubtful polar body and a structure like an intermediate body or midbody between it and the ovum, that is, a granule stained with basic dye. In another ovum (no. 51), besides the possible spindle, there was a possible intermediate body between the ovum and the polar body.

The conclusions to be drawn from this part of the investigation are:

1. The polar body is in the vast majority of specimens physically separate from the ovum.
2. The polar body is at liberty to migrate.
3. The polar body cannot therefore be presumed to mark any definite part of the ovum.

Distances between polar bodies and nuclear material of the ovum

Polar bodies are found at very variable distances from the peripheral pronuclei and the second polar spindle. Van Beneden and Julin ('80) wrote in reference to the bat that two of the three polar bodies were almost always together at the germinative pole of the ovum, while the third was removed from them by a quarter of a great circle or even more. If such a simple arrangement were general, there would be little difficulty in determining the generation to which a polar body belonged; but this is not so. If it were possible to determine the normal position of the polar bodies relative to the polar spindles and peripheral pronuclei, an indication of the sex of the pronucleus might be obtained, and also the generation to which the polar bodies belonged. In the first place, the first generation of polar bodies is known to be the one seen when the second polar spindle is present. Immediately after the liberation of the second polar body and formation of the female pronucleus, the second polar body would be expected nearer the female pronucleus than the first polar body, unless very rapid movement of the polar bodies or pronuclei had occurred. Secondly, if one pronucleus were always found nearer to the polar bodies than the other, it might reasonably be assumed that this was the female pronucleus.

For a determination of this kind a calculation of the position of the polar bodies in actual figures becomes necessary—a method that has not, apparently, been previously used in the study of mammalian ova. The positions of the peripheral pronuclei, polar spindles, and polar bodies were expressed according to the number of the section and according to the figures on the face of a clock, the clock face being represented

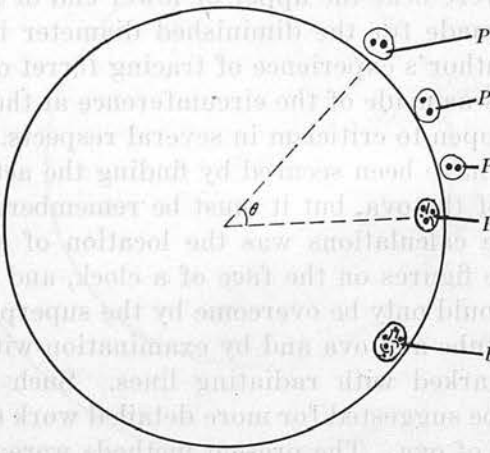


Fig. 1 Estimation of distances between pronuclei and polar bodies on the same plane. The large circle represents a section of the ovum. *P.B.(1)* is a polar body at about 1.30 o'clock. *P.B.(2)* is about 2 o'clock, and *P.B.(3)* about 2.30. *Prn.A* is a pronucleus at about 3 o'clock. *Prn.B* is at about 4.30. *Prn.A* is the nearest pronucleus to any polar body. *Prn.B* is the more remote pronucleus. The distance between *P.B.(1)* and *Prn.A* is about one and one-half hours, and therefore the angle θ is 45°. From the circumference of the section the approximate distance between the pronucleus and the polar body can be determined.

by the ovum (fig. 1). The liability to error became notable only when the polar bodies and the nuclear material were on different sections, but this error was minimized because the sections, which were cut mostly in continuous ribbons, had the same inclination throughout the slide. This could be shown, for example, where half of a polar body was in one section and half in another, and these were found at the same hour on the clock face. The sections were cut at 10 μ, and the average number of sections per ovum (see above, p. 202) could be

taken as very roughly ten. Hence the total depth of the ovum was roughly 100 μ. There was no indication that the ova were cut more frequently along the long axis than along the short or along some intermediate axis, and therefore 100 μ was assumed to be the average diameter of the middle section. The circumference would be, therefore, very roughly 300 μ. Where the sections containing the polar bodies, pronuclei, or polar spindles were near the upper or lower end of the ovum, allowance was made for the diminished diameter in accordance with the author's experience of tracing ferret ova, and a rough estimate was made of the circumference at these levels. This method is open to criticism in several respects. Greater accuracy would have been secured by finding the actual average diameters of the ova, but it must be remembered that at the basis of the calculations was the location of structures according to the figures on the face of a clock, and the error inherent here would only be overcome by the superposition of tracings of the tube and ova and by examination with the aid of an ocular marked with radiating lines. Such laborious methods are to be suggested for more detailed work on a more extensive series of ova. The present methods were employed merely to obtain an indication of the nature of the results to be expected, and certainly gave more trustworthy and finer determinations than would have been obtained by merely recording whether the polar bodies were near to pronuclei or spindles, or moderately remote from them, or very remote.

The difference in vertical depth between nuclear material and polar bodies was obtained by allowing 10 μ for each section, and the distance between nuclear material and polar bodies on the horizontal plane was recorded first in hours. From the number of hours, the fraction of the total circumference of the section was obtained in microns. By the theorem of Pythagoras, an estimation was made of the rectilinear distance between the objects examined. The distance on the vertical plane and the distance on the horizontal plane were assumed to be sides of a right-angled triangle, and the actual distance was assumed to be the hypotenuse. Figure 2

illustrates the method of estimation as applied to distances between polar bodies. The observer will note several fallacies in this simplified method, such as the assumption that

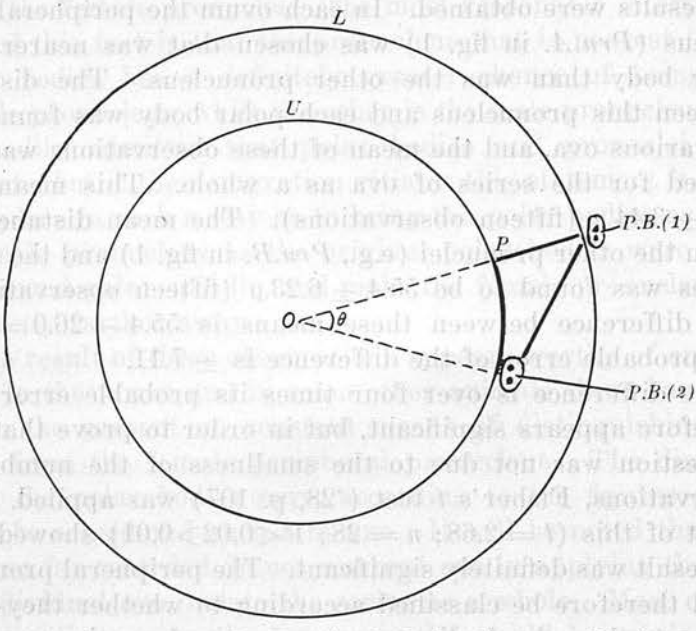


Fig. 2 An example of estimation of polar-body distances. The inner circle, *U*, represents an upper section of an ovum; the outer circle, *L*, represents the lower and larger section. *P.B.(1)* is a polar body at about 2.30 o'clock on section *L*, and *P.B.(2)* a polar body at about 3.30 o'clock on section *U*. The vertical distance from *P* on section *U* to *P.B.(1)* is determined by the number and thickness of the intervening sections. The arc between *P* and *P.B.(2)* is determined by the angle θ on the horizontal plane, and this angle is dependent on the number of hours (one) from *P.B.(1)* to *PB(2)* on the horizontal plane. The distance from *P.B(1)* to *P.B.(2)* is found by assuming that the lines from *P* to *P.B.(1)* and from *P* to *P.B.(2)* are at right angles to each other. This method was also used for estimating the distances between pronuclei and polar bodies. In such a case *P.B.(1)* or *P.B.(2)* would be replaced by a pronucleus in the adjacent part of the ovum.

the distance on the horizontal plane was the chord and not the arc of the circle between the points *P* and *P.B.(2)*.

From the ova with peripheral pronuclei treated as a whole, the mean distance was found between the various pronuclei and the polar bodies. Similarly, in the ova with second polar

spindles, the mean distance was found between the polar spindles and the polar bodies. For each mean the probable error was found. By comparison of these means the following results were obtained. In each ovum the peripheral pronucleus (*Prn.A.* in fig. 1) was chosen that was nearer to a polar body than was the other pronucleus. The distance between this pronucleus and each polar body was found for the various ova, and the mean of these observations was calculated for the series of ova as a whole. This mean was $26.0 \pm 3.44\,\mu$ (fifteen observations). The mean distance between the other pronuclei (e.g., *Prn.B.* in fig. 1) and the polar bodies was found to be $55.4 \pm 6.23\,\mu$ (fifteen observations). The difference between these means is $55.4 - 26.0 = 29.4$. The probable error of the difference is ± 7.11.

The difference is over four times its probable error, and therefore appears significant, but in order to prove that this suggestion was not due to the smallness of the number of observations, Fisher's t test ('28, p. 107) was applied. The result of this ($t = 2.68$; $n = 28$; $P < 0.02 > 0.01$) showed that the result was definitely significant. The peripheral pronuclei could therefore be classified according to whether they were nearer to the polar bodies or more remote from them.

The mean distance between the second polar spindles and the polar bodies was $28.5 \pm 2.84\,\mu$ (eleven observations), and the mean distance, given above, between the peripheral pronuclei nearest to the polar bodies and the various polar bodies was 26.0 ± 3.44 (fifteen observations). There was obviously no significant difference between these means. In this connection a further comparison is of interest. In the ova at the pronuclear stages, the peripheral pronuclei were chosen that were nearest to polar bodies (e.g., *Prn.A.* of fig. 1). There was found the mean distance between these pronuclei and the polar bodies more remote from them in the various ova (e.g., *P.B*(*1*) and *P.B.*(*2*) in fig. 1). There was no significant difference between this mean and the mean distance between polar spindles and polar bodies in the ova at the earlier stages of development. Therefore, the polar bodies at the stage of

peripheral pronuclei were situated in relation to one of the pronuclei at the same distance as the polar bodies were situated with reference to the polar spindles that were going to form the female pronuclei. The most reasonable interpretation of this fact is that the pronucleus that is nearest to the polar bodies has a definitely greater chance of being the female pronucleus, while the other is the male pronucleus. It is readily admitted that polar bodies and pronuclei may migrate, but if their migration vitiates this statement it must take place in such a way as to bring the polar bodies as near the male pronucleus as the original polar bodies were near the second polar spindle, and make the female pronucleus lie distinctly farther away.

The result of these observations appears worthy of emphasis. In the ferret and in some other animals there has been up to the present no constant means of distinguishing the male from the female peripheral pronucleus. The distance from the polar bodies may, according to the figures given here, be considered such a means. It will be noted that the data so far recorded do not warrant one in applying the test to individual ova, but to the series as a whole. Most of the investigations upon the two pronuclei would be carried out on series of specimens, and the difference between the pronuclei in respect of their distances from the polar bodies would form a very valuable basis for such investigations.

These computations have further shown that the polar bodies could not be separated into two classes according to whether they were near to or remote from the nearer of the two pronuclei. Moreover, the mean distance between the first polar bodies and the second polar spindle was the same as the mean distance between the 'nearer' pronucleus (presumably the female) and the polar bodies—both those near to this pronucleus and those more remote from it. Hence, there had been no appreciable migration of the polar bodies or nuclear material since the liberation of the first polar body.

Relation to central pronuclei

The relationship of the polar bodies to the central pronuclei is of some importance in connection with the polarity of the ovum and its subsequent segmentation. O. van der Stricht ('23) stated the conclusions based on the investigations carried out in his laboratory at Ghent, namely, that at the end of the fertilization period the pronuclei were so placed that the straight line determined by their centers passed through the animal and vegetative poles of the ovum, and was the line that indicated the plane of the first segmentation of the ovum. The workers at Ghent have established on satisfactory grounds the existence of a polarity in the ovular cytoplasm in various mammals. Investigations which the author has now almost completed prove the existence of a similar, if less marked, polarity in the ovum of the ferret. If the relationship stated by van der Stricht to exist between the pronuclear plane and the polar axis of the ovum were based on this cytoplasmic polarity, the question would not be relevant here; but in some of the observations upon which the generalization is based, the polar bodies have been used to indicate polarity, e.g., in parts of the work of Lams and Doorme ('08) on the mouse and in O. van der Stricht's observations on the dog ('23, fig. 136). The question at issue is whether the polar bodies after their liberation mark any characteristic or constant part of the ovum. Where a part of the polar spindle persists, as is probably the case in O. van der Stricht's ('23) figure 138, the polar body can be taken to mark a definite area on the ovum. This would, as has been seen, be a useless criterion in the ferret, and it is by no means a constant one in the animals examined by the Ghent workers. It might be argued by the latter that it has been established in ova where cytoplasmic polarity is present, that it has a constant relationship to the position of the polar bodies; but there has been no adequate statistical proof of this, and the author's experience leads him to consider a statistical proof imperative. This is especially necessary where the numbers of specimens observed are small. The work of Lams and Doorme ('08)

referred to above was concerned with twenty-one tubal ova at the pronuclear stage, the other ova examined by them being at later or earlier stages of development. R. van der Stricht ('11) examined thirty-six cat ova at the period of fertilization. These collections should be compared with the hundreds of ova examined by Long and Mark ('11).

It should further be noted that the Ghent workers claim a change of polarity in the ova of the bat and the rabbit during fertilization, and that R. van der Stricht ('11) found the polar bodies at various positions relative to the cytoplasmic poles of the ovum. On a-priori grounds, therefore, even if it be accepted that the pronuclei come to have a fixed relation to the cytoplasmic poles of the ovum, as O. van der Stricht stated, one does not expect to find any constant relationship between the polar bodies and the plane between the pronuclei. The investigation of this relationship in the ferret was carried out by quantitative and statistical methods.

In the ova with central pronuclei the direction of the plane between the pronuclei was noted, first in the plane of the section, according to the hours on the face of a clock, and secondly on a vertical plane, according to the direction in which the internuclear plane sloped as the different parts of the section were brought into view by raising and lowering the tube of the microscope. On a subsequent examination, the position of the polar bodies was noted according to the number of the sections containing them and according to their location with reference to the hands of a clock. From these data it was possible to calculate in each case the angle between the internuclear plane and the plane that was tangent to the surface of the ovum at the point where the polar body was found. Where pronuclei and polar bodies were not in the same or adjacent sections, difficulties arose, but, even in such ova, a more definite value could be given to the relationship than if one merely trusted to one's impressions. Figure 3 illustrates the method of estimation of the angles, and table 2 shows the polar bodies arranged according to the angles. In cases where an angle was calculated as 45°, the polar body

was counted as half in the class 60° and half in the class 30°; and similarly in the other cases where the calculated angle was halfway between two of the selected classes. To prove

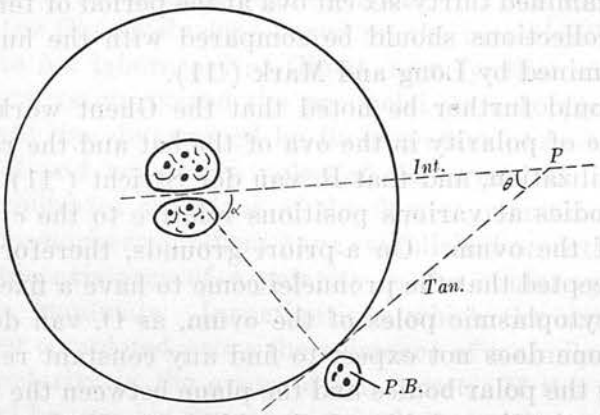


Fig. 3 Determination of the angle between the internuclear plane, *Int.*, and the tangent, *Tan.*, to the ovum at the site of a polar body, *P.B.* The internuclear plane meets the surface of the ovum about 3 o'clock, and the line between the ovular center and the polar body (i.e., the vertical to the tangent) meets the ovular surface at about 5 o'clock. The angle α is therefore one-sixth of $360° = 60°$, and therefore the angle $\theta = 30°$.

TABLE 2

SIZE OF ANGLE	ANGLE BETWEEN INTERNUCLEAR PLANE AND THE PLANE TANGENT TO THE OVULAR SURFACE AT THE SITE OF THE POLAR BODY				TOTAL
	90°	60°	30°	0°	
Actual number of polar bodies (A)	10.5	11	15.5	6	43
Number of polar bodies if distributed at random (T)	10.75	10.75	10.75	10.75	43
A — T	—0.25	0.25	4.75	—4.75	
$\frac{(A-T)^2}{T}$	0.0002	0.0002	2.1	2.1	4.2

whether the distribution of the polar bodies differed from the random distribution, the chi-square test was applied. There were forty-three polar bodies, and if these were distributed at random in the four classes, there would be 10.75 in each

class. The rest of the calculations are shown in table 2. The resulting value of χ^2 (4.2) was insignificant, and therefore the polar bodies were not distributed in any arrangement that appreciably differed from a random distribution.[1]

It will be noted that the first and second polar bodies must have been counted indiscriminately in the calculation, because there was no universal criterion for distinguishing the one from the other. The detailed records, however, revealed no tendency for the first and second polar bodies to differ in location in respect of the internuclear plane.

Owing to the smallness of the number of polar bodies, it was not possible to correlate the time after insemination with the size of the angles, and it has been shown (Mainland, '30) that the changes in the structure of the central pronuclei in this collection cannot be arranged in any order so as to give a criterion of development. Hence, it is impossible to show that the polar bodies have anything other than a random arrangement with reference to the internuclear plane, and the position of the polar bodies gives, so far, no clue to the plane of future segmentation of the ovum.

RELATION OF POLAR BODIES TO EACH OTHER

Physical connection

There were two ova in each of which two definite first polar bodies were present, that is, both ova were at the stage of the second polar spindle. In neither specimen was there a connection by spindle between the two polar bodies, nor was there a midbody between them. The division of the first polar body can therefore be completed before the fertilization of the ovum.

Out of the nine ova with three (in one case, four) polar bodies, four showed connection between two of the polar bodies in each ovum. These connections were either a darkly stained thread or a midbody. Hence, in four out of the nine ova, division of a polar body is marked in this way.

[1] It will be noted that there is a certain amount of significance in the last two columns of table 2, when these are considered by themselves, but the data are too few to justify an attempt to fit a curve to them.

In none of the sixteen ova that possessed two polar bodies and were at the pronuclear or first segmentation stages was there any such connection between the two polar bodies. The chi-square test was used as a method of comparing this with the ratio of 4 to 5 in the group of nine ova with three polar bodies just mentioned. The result showed that the difference was not demonstrably significant. It seems probable, however, that this lack of significance may be due to insufficient numbers of specimens, and it is recommended that this test be used wherever larger numbers of ova can be obtained. The difference might then be proved significant, and probably interpreted as due to lack of division of the first polar body.

Distances between polar bodies

In the ova where only two polar bodies were found, the distances between the polar bodies were estimated by the same methods as were used for the estimation of distances between polar bodies and pronuclei (pp. 211–213 and figs. 1 and 2). There were fifteen ova at the pronuclear stage possessing only two polar bodies each. The mean distance between the polar bodies in these ova was 20.1 μ, with a probable error of ± 4.1. The ova examined at the stage of second polar spindle and each possessing two polar bodies showed the polar bodies in each at 10 μ apart. These could obviously belong to the same series as the other fifteen ova, but the small numbers do not enable one to determine whether there was any real difference in the distance between the polar bodies at the second polar spindle stage and that between them after fertilization when the second polar body would be expected to be present.

In the ova with three polar bodies the distances were measured from each of the polar bodies to its two neighbors separately. The mean distance (for the whole collection) between the two polar bodies nearest to each other (e.g., *P.B's.* (*2*) and (*3*) in fig. 1) was 6.5 μ, with a probable error of ± 1.6 (eight observations). The mean distance between the remaining pairs of polar bodies, that is, those more

remote from each other, was 41.5 μ, with a probable error of ± 7.5 (sixteen observations).

A comparison was now made between the mean distances in the two sets of ova. The mean distance between the polar bodies in the ova with only two polar bodies appeared greater than that between the nearer of the polar bodies in the ova with three polar bodies (i.e., 20.1 — 6.5), but when Fisher's *t* test was applied, it was proved that the difference was not quite significant. The number of observations is so small that the difference might be due to errors in sampling.

There was no significant difference[2] between the mean distance of the polar bodies in the ova with two polar bodies and the mean distance of the more remote polar bodies in the ova in which all three were present.

Although these results do not prove any relationship between the various polar bodies, their failure to do so is probably on account of the scarcity of specimens. The question merits further investigation, for it appears to be suggested that the polar bodies where only two are present are as far apart as the more widely separated of the polar bodies where all three are present, and more widely than those that are closest together in these latter specimens. Those that are closest together in the three-polar-body ova are probably daughter cells of the first polar body. Those which are more remote are probably of different generations. It might therefore be suggested that the polar bodies of the two-polar-body ova were of different generations in a number of cases, and that to account for this it would probably be necessary in at least some specimens to postulate failure of the first polar body to divide.

[2] In the set of ova with three polar bodies, one polar body was estimated as being 155 μ distant from the other two. This polar body was largely responsible for the high value of the mean distance (41.5 μ) between the more remote polar bodies. When this polar body was omitted, the value of the mean was 25.3 μ (fourteen observations), and this, it will be noted, is much nearer the mean distance (20.1 μ) between the polar bodies of the two-polar-body ova.

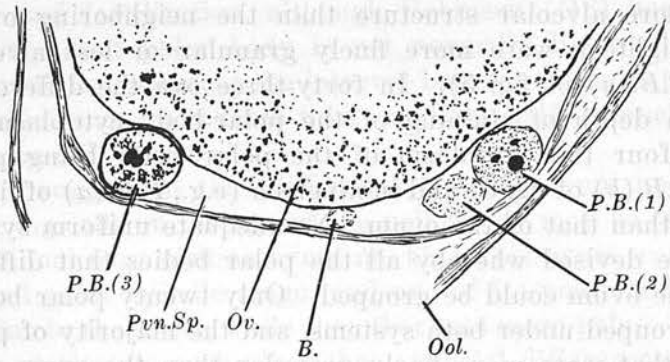
POLAR-BODY STRUCTURE

I. Cytoplasm

Comparison with ovum. No great attention has been paid to the structure of the polar-body cytoplasm in previous observations of mammalian ova. Even the records given by O. van der Stricht ('23) convey practically no information on this subject. The numerous and well-executed illustrations in his work indicate the absence of lipoid substance from the cytoplasm of the polar body even when in the ovum this substance is abundant. In the present collection the specimens fixed in Flemming's fluid indicated that there was little or no unsaturated lipoid material in the polar bodies.

Long and Mark ('11) noted that in the mouse the cytoplasm of the second polar body shortly after it was liberated had the clear appearance noted in the first polar body, but that later the second polar body assumed generally the aspect of the ovular cytoplasm. Longley ('11) recorded that in the cat the second polar body seemed not to have as dense cytoplasm as the first one. Both of these records involve a comparison between the polar bodies and the ovum. In comparing polar bodies of different ova with each other, apparent differences are apt to arise, due to different fixation, depth of staining, and so on. To eliminate this, the adoption of the cytoplasm as a standard of comparison is the most satisfactory course, although it would fail to disclose changes in polar-body structure if the ovular cytoplasm changed concurrently in the same way. Differences between the various polar bodies in one ovum can be recorded without much risk of fallacy due to technique, but even here such factors as thickness of the polar body have to be allowed for.

In the polar bodies observed in this series of ferret ova it was noted whether the cytoplasm of the polar body was similar to or different from that of the ovum (figs. 4 and 5). Out of ninety-three polar bodies, the cytoplasm in twenty-nine was of structure similar to that of the ovum near to it, while in sixty-four the structure was different. The type of difference varied. In thirty-five the differences were in granularity,



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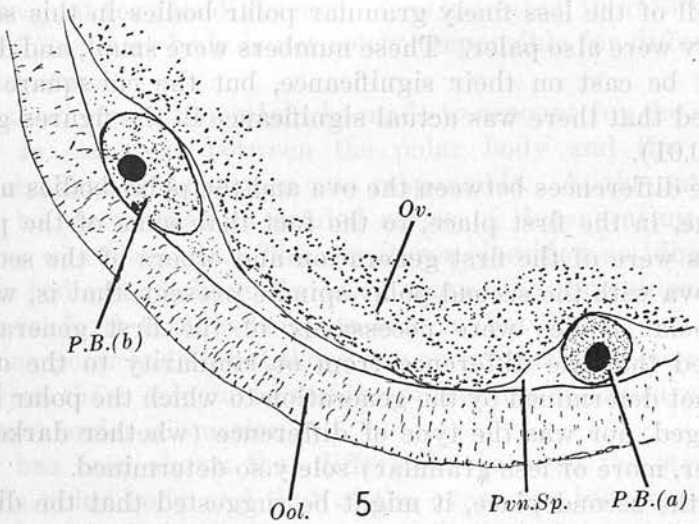

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Fig. 4 Polar bodies showing spatial relationship to each other and difference in structure. *Ool.*, oolemma, in fragments; *Ov.*, ovum; *P.B.*, polar body; *Pvn.*, perivitelline space. There is a body (*B*) that is not a polar body, also present in the perivitelline space. (Ovum no. 31, fixed in Perenyi's fluid, stained with Heidenhain's iron-haematoxylin and eosin. × 1200.)

Fig. 5 Polar bodies showing difference in structure from each other and from the ovum. *Ool.*, oolemma; *Ov.*, ovum; *P.B.*, polar body; *Pvn.*, perivitelline space. (Ovum no. 46, fixed in Perenyi's fluid, stained with Heidenhain's iron-haematoxylin and eosin. × 1200.)

seventeen being less uniformly and finely granular, that is, of a more alveolar structure than the neighboring ovum, while eighteen were more finely granular or less alveolar (e.g., *P.B.*(*a*) of fig. 5). In forty-three ova the differences were in depth of staining of the polar-body cytoplasm, in twenty-four the cytoplasm of the polar body being paler (e.g., *P.B.*(*b*) of fig. 5) and in nineteen (e.g., *P.B.*(*a*) of fig. 5) darker than that of the ovum. No adequate uniform system could be devised whereby all the polar bodies that differed from the ovum could be grouped. Only twenty polar bodies were grouped under both systems, and the majority of polar bodies that were more finely granular than the ovum were also darker (nine, as compared with two, which were paler) and all of the less finely granular polar bodies in this set of twenty were also paler. These numbers were small, and doubt might be cast on their significance, but the chi-square test showed that there was actual significance in the figures given ($P<0.01$).

The differences between the ova and the polar bodies might be due, in the first place, to the fact that some of the polar bodies were of the first generation and others of the second. The ova with the second polar spindle present, that is, where the polar bodies were necessarily of the first generation, showed that the difference from or similarity to the ovum was not determined by the generation to which the polar body belonged, nor was the type of difference (whether darker or lighter, more or less granular) solely so determined.

In the second place, it might be suggested that the differences between polar body and ovum were dependent on the time that had elapsed since the polar body was produced. In the series as a whole, the chi-square test revealed no association between the nuclear development of the ovum and the resemblance of the polar body to the ovum or difference from it. The nuclear development of the ovum is, however, an imperfect indication of the age of the polar body, and a larger collection of specimens would be required before it could be used with confidence, for it is unknown what the

exact time relationship is between liberation of the first polar body and fertilization, although Robinson ('18) has shown that the first polar body is not normally liberated before insemination, and that the interval between insemination and ovulation may vary from thirty and one-half to ninety-three and one-half hours.

The suggestion that difference between ovum and polar body depends on the age of the polar body may be tested by individual ova, such as ovum no. 65. This ovum was at the second polar spindle stage, and one of its polar bodies was similar to the ovum, while the other was more pale. The two were so close to each other that artificial differences in fixation and staining of different parts of the preparation could not account for this difference. It is indicated, therefore, that age of the polar body is not solely responsible for differences in structure.

A third suggestion might be made to account for the difference in structure between the polar body and the ovum, namely, that degeneration was responsible. At the outset, it may be stated that no graded series of degenerating polar bodies was observed. The structures classified as 'doubtful' polar bodies have been discussed above, and the evidence has been set forth that leads one to believe that these were degenerating polar bodies, but there was no evidence of a connection between those structures and the differences in appearance at present under discussion.

It has been shown that differences between the structure of the polar body and that of the ovum are independent of the development of the ovum as indicated by the ovular nuclear material. This might also be taken as a proof that, so far as this series is concerned, the differences in the polar-body structure were not associated with degeneration, because the more advanced the ovular development the more likely would the polar bodies be to degenerate. Again, this suggestion cannot be considered substantiated, because the ovular development is not a direct test of the time which a polar body has been in existence.

The fourth suggested cause for differences in structure between polar bodies and ova is the technique—fixation or staining. With the aid of the chi-square test it was shown that the differences in structure were independent of the fixative, independent of the method of staining, and independent also of the depth to which the sections were stained.

The results of these investigations on structure may be summarized as follows:

1. Structural differences or similarity between the polar body and the ovum are not due to the generation to which the polar body belongs (i.e., whether it is a first or a second polar body).

2. The differences are not due solely to the age of the polar body.

3. They are, so far as can be shown, independent of the fixative used, of the staining method, and of the depth of staining.

4. There is no evidence that they are due to degeneration of the polar body, but the possibility of such a cause has not been disproved. On the other hand, the differences may be merely representative of temporary metabolic changes.

Comparison with cytoplasm of other polar bodies of the same ovum. Among the ova with two or more polar bodies a comparison was made of the cytoplasmic structure of the various polar bodies present in each ovum. In ovum no. 50 the two polar bodies, both products of division of the first polar body, were similar in structure to each other, but in ovum no. 65 the two daughters of the first polar body differed from each other, one being darker than the other, and in ovum no. 31 all three polar bodies differed in structure. Therefore, difference in cytoplasmic structure is not an indication that polar bodies belong to different generations. In ovum no. 14 all three polar bodies were similar in cytoplasmic structure. Therefore, similarity in cytoplasmic structure does not necessarily indicate that the polar bodies are of the same generation.

In spite of these facts, there might be a tendency for similarity in structure to represent similarity in origin, and dif-

ference in structure to represent difference in origin. This question is discussed in the next subsection.

Relationship between structure and distance of polar bodies from each other. The distances between the polar bodies were calculated as recorded above, and it appeared possible that the polar bodies that were more remote from each other were more commonly different in structure. When the ova with only two polar bodies were used, the data were:

	Polar bodies similar to each other (pairs)	*Polar bodies different from each other (pairs)*	*Total*
Polar bodies 10 μ or less apart,	5	1	6
Polar bodies more than 10 μ apart,	1	6	7

On account of the smallness[3] of the numbers, the chi-square test is apt here to give results which appear significant but are not really so. The value of χ^2 (roughly 8) for this series, is, however, so much in excess of the significant value for a table of this size (namely, $\chi^2 = 3.8$) that one may hold with fair confidence that the difference is significant.

When the ova with three polar bodies were included, making a total of twenty-seven pairs, the significance of the result became even greater.

In the ova with three polar bodies, those nearest to each other might be presumed to be the daughter cells of the first polar body. In the ova with two polar bodies it has been indicated that the polar bodies that are nearest to each other are more apt to resemble each other than those that are remote. Moreover, this tendency seems to be exaggerated when the ova with three polar bodies are included in the calculations. These facts can be interpreted best by supposing that in the ova with two polar bodies, also, the polar bodies that are nearest to each other belong to the first generation, and that

[3] The influence of the smallness of the numbers of individuals in a chi-square test becomes of importance when the theoretical or calculated number in a cell or square is less than 5. There is no necessity for the excessive caution that prompted the author (Mainland, '30, p. 270) to doubt the significance of a chi square in which the actual (as opposed to the theoretical) number was less than 5. The actual number can be zero, provided the calculated number is not less than 5.

the polar bodies at a greater distance from each other belong to different generations.

There are shown above six instances in which there were differences between polar bodies and the polar bodies were remote from each other. It is hardly likely that in each of these ova the polar body lower in the series of sections was one of the daughter cells of the first polar body, and that its sister cell had been lost by loss of sections. Hence, if one or more of the lower polar bodies were second polar bodies, the existence of only two polar bodies in the ovum must be accounted for by the failure of the first (i.e., the upper) polar body to divide.

It has, therefore, been shown that there are strong indications that the polar bodies nearest to each other tend to resemble each other in cytoplasmic structure. This has been explained by supposing that they are daughter cells of the first polar body. It has further been indicated that, when structure of polar bodies and distance between them are taken into account, it is probable that one must postulate in some ova a failure of the first polar body to divide.

II. Nuclear material

Appearance. As in the case of the cytoplasm, no great interest has been displayed in the nuclear structure of polar bodies. In the mouse, Kremer ('24) recognized a similarity of structure between the nucleus of the polar body and the pronuclei of the ovum in the earlier and also in the later stages of development; but he did not give much detail regarding the nuclear structure of the polar bodies. Long and Mark ('11), also working on the mouse, found that no nucleus was formed in the first polar body, unless, perhaps, after it had divided into two during the cleavage of the ovum; whereas in the second polar body the chromosomes were quickly transformed into a nucleus. Long and Mark (loc.cit.) described also in the first polar cells chromosomal bodies less deeply stained than the rest and in some instances associated with vacuolating parts. These bodies and vacuolating parts

were interpreted as indicating degeneration of the chromatin. In the rat, according to Sobotta and Burckhard ('11), the chromosomes (at least of the second polar body) never formed a resting nucleus. The appearance of the figures in O. van der Stricht's ('23) records suggests that this statement is true also of the animals that he and his collaborators investigated, namely, the mouse, rat, bat, dog, cat, and guinea-pig.

The investigations of R. van der Stricht ('11) and of Hill and Tribe ('24) on the cat similarly bear out the statement made by Longley ('11) that in neither polar body of that animal was a definite nucleus formed. Longley found that in the first polar body the chromatin might exist as a number of threads or granules, or as a single thread or a compact mass. It was claimed that it was possible to distinguish sometimes the first polar body from the second by the fact that in the second polar body the chromatin was in small granules or even in the form of a single granule.

In the ferret ova examined the chromatin material was in the vast majority of polar bodies in the form of globules. The number of these was recorded, but their size was not actually measured. It was noted as small, medium, or large.

The question of the actual existence of a nucleus in the polar body merits some attention, because it serves to throw light on the status of the body as an independent cell. In one polar body (the only one present in ovum no. 27) there was a suggestion of a nuclear membrane, but this was noted in no other polar body. It may be pointed out that in the ovum of this specimen the nuclear material was at the second polar spindle stage, and therefore the polar body was of the first generation. In another specimen (ovum no. 29) there were many scattered chromatin granules forming an imperfect reticulum in the polar body. This ovum was at the stage of peripheral pronuclei. Apart from these instances, there was no suggestion of nuclear formation in the ninety-three polar bodies examined.

The occurrence of structures differing from the typical globular chromatin was investigated. Out of eleven polar bodies of the first generation (that is, associated with ova at the second polar spindle stage), five had chromatin that was angular or suggestive of rod-like bodies. With these polar bodies were compared seventy-one polar bodies associated with ova later than the stage of the second polar spindle. Of these seventy-one, only fifteen contained angular or rod-shaped chromatin. The chi-square test showed that this difference in proportion was not sufficiently great to be significant ($\chi^2 = 3.1$; $n = 1$; $P < 0.10 > 0.05$). The polar bodies with the two types of chromatin are therefore scattered fairly evenly throughout the series.

The interpretation of these differences in structure of the chromatin is not easy. The irregularity of the particles might indicate a degeneration or a retention of the chromosomal structure. The presence of regular spherical particles might indicate degeneration, but it will be recalled that the pronuclei of the ferret present chromatin globules in the greater part of their career (Mainland, '30), and these pronuclei are not degenerate. The difference was not indicative of a difference between the first and second polar bodies.

In order to determine whether the angular particles were chromosomal remnants, it would be necessary to obtain a larger collection of polar bodies, so that various stages might be represented from the true chromosomes to the globules. One ovum (no. 67) showed what were apparently definite chromosomes. This ovum had been fixed immediately after the production of the second polar body. In one of the two polar bodies present were eleven chromatin globules, and in the ovum near this polar body was a chromatin mass like a short, rather coarse spireme. This was probably a stage in the development of the female pronucleus from the daughter chromosomes of the second polar division, and the eleven chromatin globules of the polar body were probably the other daughter chromosomes of the same division. There was no definite connection between the polar body and the ovum and

no remnants of a spindle. The only other polar body found in connection with this ovum had no definite chromosomal material, but was coarsely granular and deeply stained, so that the chromatin may have disintegrated into the cytoplasm. This was the only ovum with a polar body at this stage of development, and no generalization can be founded upon it. None of the other ova with angular or rod-like particles could be definitely stated to show chromosomes.

The globules of chromatin present in the polar bodies varied in number from one to eight, but when, as occasionally happened, the chromatin was in the form of fine granules, the number of granules was greater. This might suggest that the smaller the size of globules or granules, the greater the number. This suggestion was not borne out by the results of further examination, for there were some polar bodies with one medium-sized globule each and others with one large globule each.

The difference in size of the chromatin particles was definitely not indicative of difference between the first polar body and the second.

Comparison with nuclear material of other polar bodies of the same ovum. Differences in nuclear material between polar bodies of the same ovum consisted of differences of size and differences of shape of the chromatin particles (figs. 4 and 5). Out of the seventeen ova with two polar bodies examined for this purpose, fifteen had polar bodies that differed from each other in respect of nuclear material. In these fifteen were included the only two ova in which one could be reasonably certain that both polar bodies were of the first generation. Out of six ova with three polar bodies each, four showed polar bodies that were all different from each other in respect of the nuclear material, while two of them showed two polar bodies similar to each other and one polar body that was different from each of the others in this respect. In the ovum with four polar bodies all the bodies differed in this respect.

Thus, difference in nuclear material does not indicate that the polar bodies belong to different generations.

So far as can be ascertained at present, the peculiarities in nuclear structure of the polar bodies must, like those of the pronuclei themselves, probably be interpreted in terms of physicochemical phenomena that are yet beyond our knowledge.

Relationship of cytoplasmic and nuclear structure. Some association between cytoplasmic and nuclear differences might have been expected, for two reasons. First, if cytoplasmic differences denoted that one of the polar bodies was a second and the other a first, then nuclear differences might also be expected. Secondly, the nuclear material in a polar body might be disintegrated and so influence the cytoplasmic staining. Thus a deeply stained cytoplasm would be present where the nuclear material was sparse. To find out whether the differences between nuclear material were associated with those between the cytoplasm, twenty-seven pairs of polar bodies were studied. The result of the chi-square test ($\chi^2 = 0.6$; $n = 1$; $P < 0.50 > 0.30$) showed that the nuclear material and the cytoplasm varied independently.

SECOND POLAR SPINDLES

These specimens of the ferret ova were not fixed or stained with a view to the study of the polar spindle, nor were they sufficiently numerous to justify a final determination of the chromosome number. It may, however, not be without interest to discuss here some of the points raised in connection with the thirteen ova examined at the stage of the second polar spindle.

Spindle fibers and centrosomes

In ten of the thirteen specimens the fibers of the spindle were visible to a greater or less degree. In none was a definite centrosome seen either at the central or peripheral pole of the spindle; in only three was there a possible indication of such a body. It may be pointed out in this connection that O. van der Stricht and his collaborators have observed the centrosomes or central bodies at the apices of both matura-

tion spindles in the bat, mouse, cat, and dog (O. van der Stricht, '23).

Stages of mitosis observed

In six (possibly seven) of the ova the chromosomes were at the end of the prophase of mitosis, that is, the chromatin particles, in the form sometimes almost of spherical bodies, were arranged so as to form an equatorial plate. In five (possibly six) ova the metaphase was seen. In this stage the chromosomes were more rod-like and had become arranged in the long axis of the spindle preparatory to the anaphase.

Chromosome number

Among the spindles in the prophase, the chromosomes were counted as eight in three ova and ten in the other three ova. The differences are presumably due to imperfections of the preparation or to observational errors, and the number ten is necessarily nearer the correct one than is eight. The series is very small, but since it is unique it appears desirable to obtain at least an indication of the maximum possible chromosome number in the ferret. The series of six observations was assumed to be part of a larger series, in which the same errors of observation and technique were present throughout. The mean of the six observations was found to be nine. The standard deviation of the series is—

$$\sqrt{\frac{\text{Sum of the squares of the deviations from the mean}}{\text{One less than the number of observations}}}$$

(On account of the small number of observations, it is desirable to allow for error by dividing by one less than the number of observations, instead of the actual number, which is sufficiently accurate in a large series.) The standard deviation therefore is $\pm$ 1.095. Roughly, three times the standard deviation includes all the possible variates, and therefore, if more ova were examined, one might expect to find haploid chromosome numbers up to nearly thirteen (i.e., the mean plus 3 times 1.095), but not beyond thirteen.

Now, in one of the metaphase ova there were nine or ten rods apparently double at the ends; in another two, twelve

rods; in a fourth, fifteen or sixteen, of which some seemed double. If it is assumed that the prophase could show at most thirteen chromosomes, one must conclude that in this last ovum some of the original dyads have split, whereas in the other metaphase specimens this need not be assumed.

It was not found possible to distinguish the different chromosomes by shape or size.

Direction of long axis of spindle relative to ovular surface

According to Long and Mark ('11), the second polar spindle in the mouse does not become perpendicular to the surface until the abstriction of the second polar body has begun. Sobotta ('08), however, held that in this animal both spindles are situated tangentially in the monaster stage, and turn into a radial position during the transformation into the dyaster stage. Similarly, in the rat (Sobotta and Burckhard, '11) this order of events has been noted. Again, O. van der Stricht ('23) stated that in the mouse the second polar spindle in metakinesis undergoes a rotation of 90° and becomes perpendicular to the surface of the ovum. In the specimens of cat ova described by Longley ('11) the second polar spindle was always perpendicular to the surface.

In the present collection of ferret ova the specimens had been cut in such a way that a variety of views of the spindles were obtained in the different ova. In each specimen there was noted by the eye the relation between the long axis of the polar spindle and the ovular surface. In one ovum (no. 33) this axis was about 45° to the tangent drawn at the surface of the ovum, but this particular specimen was at a doubtful stage and its chromosomes could not be counted, so that it may probably be neglected without bias. In one of the others (ovum no. 6), the most advanced metaphase of the collection, the long axis of the spindle was not perpendicular to the surface, but was less than 30° from the perpendicular. This difference might be accounted for by the presence of a body projecting inward from the oolemma. In the other eleven ova, both prophase and metaphase specimens, the long

axes were all within 30° of the perpendicular to the ovular surface both in the plane of the section and in the plane at right angles to this.

DISCUSSION OF CHIEF RESULTS

When these investigations are surveyed as a whole, it will be seen that, after due allowance has been made for technical causes of polar-body absence, some other responsible factors still remain. Degeneration is strongly indicated as one of these. Another possible factor is failure of the first polar body to divide. Although there is nowhere an adequate proof of this, it will be observed that it is suggested as the conclusion of several separate sets of investigations.

The polar body is a definite cellular entity, separating quickly from the ovum and existing apart from it. It is imperfect in the sense of having no definite nucleus.

There is some indication that difference in structure of polar bodies represents difference of generation, but no other means has been discovered of distinguishing the first from the second polar body.

The polar body has been shown to be of great value in the identification of peripheral pronuclei in the collection as a whole. It is, so far as can be shown, of no value in localization either with reference to the ovular surface or with reference to the plane between the central pronuclei.

Throughout these investigations, suggestions have been obtained that demand a greater collection of specimens, both of polar bodies and polar spindles, for their elucidation.

SUMMARY

The investigations recorded here form the first extensive study of the polar bodies of a carnivore, and by them more detailed information has been acquired than by previous investigations on these bodies in non-carnivore mammals.

The quantitative and statistical methods employed have been discussed in some detail, for it is felt that they may profitably be used on the polar bodies of non-mammalian, even of invertebrate, animals.

Serial paraffin sections of eighty-six ova of the ferret were examined, representing all stages from the second polar spindle to the stage of commencing segmentation, inclusive. The ova contained ninety-three definite polar bodies.

Allowance was made for the sections lost from ova with missing sections, and for the thickness or density of the oolemma of the terminal sections, which was liable to obscure polar bodies. There was thus calculated the number of ova that should have missing polar bodies if these two factors were alone responsible. The conclusions of the investigations on polar-body number were that polar bodies were not absent because of migration through the oolemma or because of failure of separation of the polar bodies from the ovum. Loss of sections and thickness or density of the terminal oolemma, it was decided, could partly, but only partly, account for absence of polar bodies. It was improbable that failure of division of the first polar body could account for all those missing polar bodies that could not be accounted for by loss or thickness of sections. Degeneration was very strongly indicated as a cause of absent polar bodies.

The absence of spindle-fiber remnants indicates that the polar body is in the vast majority of specimens physically separate from the ovum. It is at liberty to migrate, and does not, therefore, necessarily mark any definite part of the ovum.

Quantitative determinations were made of the mean distances between polar bodies and peripheral pronuclei (or polar spindles), and it was proved that the peripheral pronuclei could be classified according to whether they were near to or more remote from the polar bodies of their ova. The pronuclei nearest to polar bodies were at the same distance from the polar bodies of their ova as were the second polar spindles (i.e., the precursors of the female pronuclei). These facts indicate (p. 215) that a valuable criterion has been discovered, whereby, in the collection as a whole, male and female pronuclei can be differentiated.

The polar bodies could not be distinguished from each other by their distance from the peripheral pronuclei, and this fact, along with their relationship to the polar spindles just mentioned, indicates that there had been no appreciable migration of polar bodies or nuclear material since the liberation of the first polar body.

The polar bodies were not distributed in any arrangement that differed appreciably from a random distribution with reference to the internuclear plane of the central pronuclei. Therefore, the position of the polar body gives, so far, no clue to the plane of future segmentation of the ovum.

The division of the first polar body can be completed, and the spindle fibers disappear, before fertilization.

The lack of spindle fibers and intermediate bodies between the polar cells of the ova with two polar bodies, as compared with the ova with three polar bodies, suggests that, in a larger collection, one might be able to conclude that the former ova showed only two polar bodies because the first had failed to divide (p. 220).

A comparison was made of the mean distances between the polar bodies in the ova with two such cells and those with three. The polar bodies in the ova of the former class were as far apart as the more widely separated pairs of polar bodies in the ova where three polar bodies were present. The data suggest that the polar bodies nearest together in the ova with three polar cells are nearer than the polar bodies of the two-polar-cell ova, but the difference is not quite significant. If this insignificance is due to smallness of the collection, it is suggested that the polar bodies of the two-polar-cell ova are commonly of different generations, just as are probably the more remote of the polar bodies in the three-polar-cell ova. Failure of division of the first polar body would again be postulated in some of the specimens (p. 221).

There is little or no unsaturated lipoid material in the polar bodies.

Structural differences or similarity between the cytoplasm of the polar body and that of the ovum are not due solely to

the generation to which the polar body belongs, nor solely to the age of the polar body, nor to the fixative or staining. There is no evidence that they are due to the degeneration of the polar bodies, but such a cause has not been disproved.

Polar bodies of the same ova were compared with each other in respect of their cytoplasm, and it was decided that differences in structure did not definitely indicate that the polar bodies were of different generations, and that similarity did not indicate similarity of origin. There was, however, a distinct tendency for polar bodies that were near to each other to resemble each other more than those that were remote. This was interpreted as indicating that like polar bodies were of the first generation and unlike polar bodies were of different generations. On these grounds, it was again argued that failure of the first polar body to divide must in some specimens be postulated.

In only two polar bodies were there suggestions of a nucleus. In the majority the chromatin was in globular form. In some it was angular or rod-like. This shape could not be proved to represent chromosomal remnants, and neither the rod-like nor the globular bodies could be shown to indicate either degeneration or a difference between the first and second polar bodies.

The size of the globules was not closely dependent on the number.

The differences between the cytoplasmic structure of polar bodies were independent of those between nuclear structure.

Thirteen ova were examined at the stage of second polar spindle—some in prophase, some in metaphase. In ten of these the spindle fibers were visible to a greater or less degree. In only three specimens was there a possible indication of a centrosome.

By statistical treatment of the six prophase specimens, the maximum haploid chromosome number was suggested as thirteen.

It was impossible to distinguish the different chromosomes by shape or size.

Except for two doubtful specimens, all the spindles, both in prophase and in metaphase, were, as judged by the eye, within 30° of the perpendicular to the ovular surface.

In a brief discussion (p. 235) reference is made to the various indications that failure of the first polar body to divide may be the explanation of different facts that have been noted in the course of the work. Emphasis is laid on the need for larger collections, so that suggestions obtained here may be further pursued.

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THE EARLY DEVELOPMENT OF THE FERRET:

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THE EARLY DEVELOPMENT OF THE FERRET: THE CYTOPLASM

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INTRODUCTION

One of the most fundamental problems of embryology is the structure of the unsegmented ovum, and yet, compared with the widespread interest displayed in the cytoplasm of non-mammalian ova, the attention paid to this aspect of mammalian ova has been very limited. It has been left almost entirely to the Belgian school of the Van der Strichts to carry on minute investigation of mammalian ooplasm, to emphasise its importance and to disclose the fact that each mammalian species has its characteristic ovum. It appears to be felt by some investigators that the cytoplasm of the mammalian ovum is outside the province of the embryologist, although why he should avoid cytology in this way, while investigating so thoroughly the nuclear structure of the ovum, is hard to understand. The one aspect is assuredly as important as the other, and it is on this account that the author presents these records of investigations carried out on the cytoplasm of the unsegmented tubal ovum of the ferret.

MATERIALS AND METHODS

There are three main types of treatment that may be used to disclose the structure of the ooplasm. The first is the common method, whereby, after fixation in, for example, formol, Zenker's or Perenyi's fluids, the ovum is carried through fat-solvents, e.g. xylol, into paraffin. By this method the lipoid material is not shown, nor are the mitochondria well displayed. The second type of method—fixation in a fluid containing osmic acid—enables one to preserve the lipoids that contain unsaturated fatty acids. The third type of method—employment of a special mitochondrial technique—may be combined with the second type just mentioned, but the treatment by Flemming's fluid, unless the acetic acid in it is very small in amount, does not preserve the mitochondria (Lee, 1928, p. 332).

The specimens investigated in the present study had not been treated by the third type of method, but some had been subjected to the first type and others to the second. The ova were the same as those used in a recent investigation of the pronuclei(Mainland, 1930)[1], being 101 ova liberated from 30 different

[1] The work referred to (Mainland, 1930) was a quantitative study of the structure of ferret pronuclei. Attention was paid in it to such matters as staining, position and relative size of chromatin particles, wrinkling of pronuclear membrane, fineness of reticulum; an endeavour being made to correlate all changes with the development of the pronuclei. Thus, nonbasiphilic globules were found only in pronuclei after they had left the periphery of the ovum. Statistical tests were applied to the data, and it was shown that when such precautions were taken it was not possible to describe an ordered series of transformations as has been done for the pronuclei of some other Mammals.

The influence of technique of preparation received special attention, e.g. Perenyi's fixative was shown to produce large particles of chromatin more commonly than did the other fixatives.

Descriptions were given of an ovum with a sperm tail in it and of a trinucleated ovum.

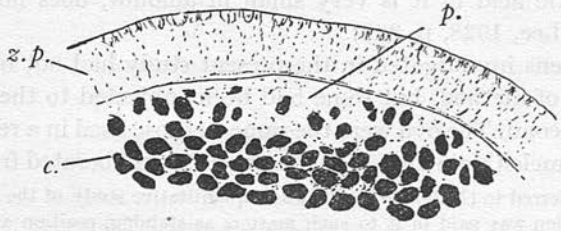
ovaries belonging to 19 different animals. All the ova were at stages of development found immediately after ovulation. Seventy-one out of the 101 were at the pronuclear stage; some were at the second polar spindle stage; and some at the stage of the first segmentation spindle. Paraffin sections cut at 10 μ were used throughout. For further details regarding the preparation, reference should be made to the article mentioned above (Mainland, 1930, p. 262).

As in the study of the pronuclei, statistical methods were applied to the data, for it is only by such tests that assurance can be obtained that one's statements are not based merely upon personal opinion, and that one's opinion is not formed upon inadequate data. As one reads the reports of the most careful embryological investigations, one realises how much greater would be one's feeling of conviction if only the data had been subjected to some simple quantitative tests, or at least set forth in such a way as to make these tests possible. The statistical test chiefly used here is the Chi Square test, discussed and exemplified in the paper already mentioned (Mainland, *l.c.*, p. 263). The probable error method, and the regression method are also used. At the appropriate place in the description the purpose and nature of the tests are indicated. At this point reference should be made to the frequent use of the term "significant." When this word is applied, for example, to a difference between quantities, it means that the odds are about 20 (or more) to 1 that the difference is not accidental or due to "chance." Similarly, if an association between two phenomena is said to be "significant," the meaning is that the odds are at least 20 to 1 in favour of such an association.

STRUCTURE OF OVUM

Histological appearance

The fundamental structure of the cytoplasm of the tubal ovum of the ferret has been indicated by Robinson (1925). The cytoplasm consists chiefly of



Text-fig. 1. A portion of a Flemming-fixed ovum, showing dark, lipoid-bearing "yolk bodies." (Specimen merely tinted with eosin; camera lucida drawing; magnification approx. 1150.) *c.*, central portion of ovum; *p.*, peripheral portion, containing less lipoid than central portion; *z.p.*, zona pellucida.

lipoid[1]-bearing material commonly called yolk or deutoplasm, which appears as black globules in the ova fixed in Flemming's fluid (text-fig. 1). After

[1] The term "lipoid" is used in this article in its widest sense, to signify a fat or fat-like body.

fixation in Zenker's, or Perenyi's fluids the lipoid is dissolved out during the processes of dehydration and clearing. The result of such processes is shown in all the figures of the accompanying plate, where the cytoplasm presents a mass of vacuole-like spaces, the mass being surrounded by a comparatively narrow granular zone. This granular zone here and there also contains in the Flemming-fixed ova some lipoid globules (text-fig. 1).

Comparative histology

As mentioned above, the importance of the structural differences between the ova of different Mammals has been emphasised chiefly by O. van der Stricht (1923, etc.) and his collaborators. Of the ova illustrated by O. van der Stricht (*l.c.*) the one most closely resembling the ferret in respect of lipoid material is that of the dog. The pig contains even more such material (Corner, 1928, p. 1121). The ova of the cat (R. van der Stricht, 1911; see also Hill and Tribe, 1924) vary in respect of the amount of lipoid. Some contain almost as much lipoid as do those of the ferret, while others contain less. Corner (*l.c.*) makes the interesting suggestion that the greater abundance of yolk in the ova of these animals as compared with those of the rat, guinea-pig and rabbit is correlated with the extent to which the ova are developed before they are attached to the uterus. One of the most serious objections to this suggestion is furnished by the ova of the cat. These, as has been mentioned, can be divided into two classes according to the amount of lipoid they contain. If Corner's theory is correct it would apparently be necessary to suppose that these two classes of ova became embedded in the uterus at different times. There seems to be no evidence of this. Further light is thrown on the theory by consideration of other animals. Summaries of information on the implantation of Mammals are to be found in Robinson (1904) and in Marshall (1922). The ova of the dog, cat, pig and also of the ferret are more advanced before implantation than those of, for example, the mouse, in which the lipoid of the ovum is very scanty compared with that of the other animals just mentioned. The ovum of the guinea-pig, however, may contain relatively as much lipoid as that of the cat (compare Pl. XV, fig. 40 of R. van der Stricht, 1911 with Pl. XII, fig. 93 of O. van der Stricht, 1923), and yet the guinea-pig ovum becomes implanted at an earlier stage than that of the cat (Marshall, 1922, pp. 472 and 444). Thus Corner's suggestion appears open to serious criticism.

Chemical structure

The evidence of the chemical nature of the yolk-bodies is obtained solely from the action of osmic acid in Flemming's fixative. The present state of knowledge of osmic fixation of fats is summarised by Parat (1927). Osmic acid is a specific reagent for unsaturated fatty acids, and practically the only interesting member of these in the animal organism is oleic acid.

On comparison of the Flemming-fixed ova with those fixed in Zenker or Perenyi, it is seen that the vacuoles in the latter specimens correspond to

the darkly stained lipoid globules of the former. This seems to suggest that the two elements in the cytoplasm are (*a*) the protoplasmic framework and (*b*) the lipoid, containing unsaturated fatty acid radicles, this lipoid being dissolved out after the non-osmic fixatives.

The blackening with osmic acid is very intense, and the question naturally arises whether the original lipoid contains only unsaturated fatty acids or whether part of it contains saturated fatty acids, and is therefore not preserved by osmic fixation. Analogies with non-mammalian ova are of course dangerous, but it is of interest to note, for example, that two of the lipoids in egg-yolk are lecithin and sphingomyelin. Of these the former contains unsaturated as well as saturated acid radicles (Maclean and Maclean, 1927, p. 26), and the lignoceric acid radicle present in sphingomyelin (*ibid.* p. 62) is a saturated acid (Leathes and Raper, 1925, p. 13). As is well known, the ordinary fats occur naturally as mixtures of saturated and unsaturated fats. One would therefore expect that the lipoids of the ferret ovum contained saturated as well as unsaturated fatty acid radicles, and one must harmonise that view with the fact that the blackening of the globules is intense, as if they were entirely composed of lipoids with unsaturated fatty acids. From the work summarised by Parat (1927), it appears that, when less than 50 per cent. unsaturated acid is present, the blackened material is very soluble, but, when more than 50 per cent. unsaturated acid is present, it is very stable after fixation by osmic acid. Since, therefore, the globules in these ferret ova persisted after being subjected to powerful fat solvents, one may conclude that the fatty material contained at least 50 per cent. of the unsaturated fatty acid. It should be observed that these statements imply that a considerable amount of saturated fatty acid might be present and yet the lipoid globules might be very darkly stained. It is conceivable that the particles containing the unsaturated fatty acid radicles are intimately mixed up with particles containing saturated fatty acid radicles. When the lipoid containing the saturated acids was dissolved out, the unsaturated acids, already made insoluble by the osmic acid, would remain and the blackened material would be so uniformly distributed as to give a very dark appearance. It may be remarked in passing that there is still a field open for carefully controlled histological experiments to determine the results of osmic acid fixation of various artificially prepared emulsions of different lipoids.

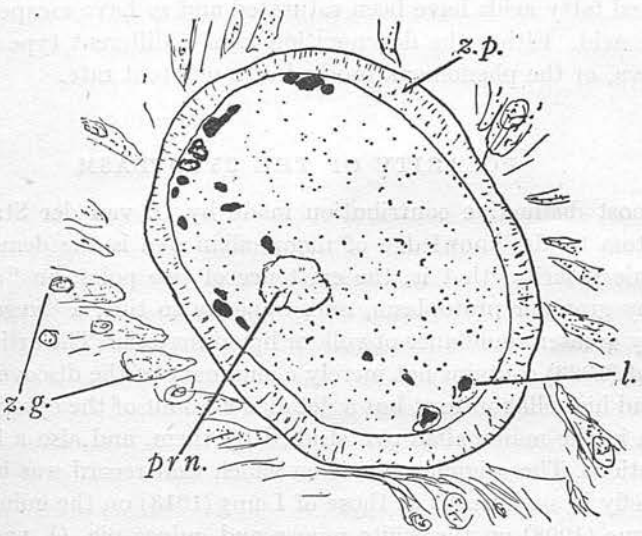
Minute structure of yolk globules

The question that next arises is whether the globule is a simple droplet or has a framework of protein or other material. When fixed in Zenker's or Perenyi's fluid and stained by Heidenhain's iron-haematoxylin and faintly tinted by eosin, the network between the vacuoles is grey or blue-black, and the vacuoles may be almost colourless. When fairly heavily stained with eosin the vacuoles take on a pink tinge. It may be asked whether this does not indicate some material, probably protein, in the vacuole. The size of the globule or vacuole affords the solution of this problem. As may be estimated

from fig. 4 of the accompanying plate, the vacuole is in the neighbourhood of 3 μ in diameter. The sections were cut at 10 μ, and the globules, as shown in the figure, are usually much less than 3 μ from their neighbours (see also text-fig. 1). Hence, in a section, the walls of two or even three globules intervene between the source of light and the eye, and the vacuolar appearance is due to the thinness of these interglobular walls, not to the absence of material. The pink tinge can therefore be explained as a stain of the interglobular material and does not indicate a framework in the globules.

Abnormal ova

Although the typical tubal ovum, like the typical large ovarian ovum, shows abundant lipoid, four ova were examined in which, after fixation in



Text-fig. 2. An abnormal ovum (No. 76), fixed in Flemming, but showing very little lipoid. (Specimen merely tinted with eosin; camera lucida drawing; magnification approx. 500.) *l.*, lipoid globule; *prn.*, pronucleus; *z.g.*, zona granulosa; *z.p.*, zona pellucida.

Flemming's fluid, the lipoid globules were almost entirely absent (e.g. text-fig. 2). These ova appear to merit some detailed discussion, to ascertain what light they throw on the technique of preparation and on the abnormal metabolism of ova. All of these ova were at the stage of central or subcentral pronuclei. Three were from the left uterine tube of one animal, and the same tube showed two other ova in which the lipoid substance was abundant. Hence the absence of lipoid was not due to a characteristic of one ovary. These two sets of ova from the same animal—one set with abundant lipoid and the other with practically none—were on different slides, and therefore differences in the technique might be responsible. The lipoid from the one set might have been removed during preparation, owing to their being subjected

to more intense solvent action. Such an argument could not, however, apply to the other ovum (no. 76), where the large ovarian ova present in the same sections, and therefore subjected to the same treatment, contained the usual amount of lipoid material. This abnormal ovum (text-fig. 2) was a fertilised ovum apparently held back mechanically in the periovarian space by a cyst-like body, the nature of which could not be determined. The other three ova were all in the uterine tube and there was no evidence of mechanical hindrance to development. One may therefore legitimately look on ovum no. 76 as degenerate, although it is unprofitable to speculate on the cause of degeneration. It is more valuable to compare the appearance of this degenerate ovum with that of degenerate ovarian ova. In the latter, degeneration is denoted by the presence of large masses of osmic-blackened fatty material, whereas in this fertilised ovum the lipoid has almost entirely disappeared, that is, the unsaturated fatty acids have been saturated and so have escaped fixation by the osmic acid. Either the degeneration is of a different type from that of ovarian ova, or the phenomena proceed at a different rate.

POLARITY OF THE CYTOPLASM

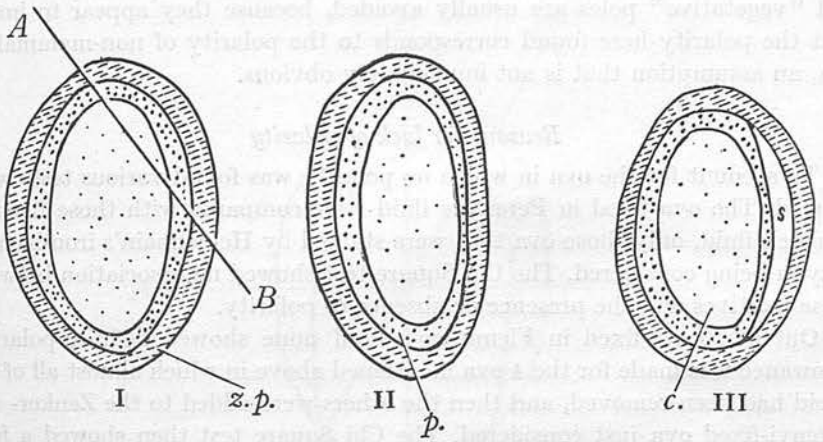
The most distinctive contribution made by O. van der Stricht and his collaborators to the knowledge of mammalian ova is the demonstration of cytoplasmic polarity, that is, the existence of two poles, an "animal" pole marked by granular protoplasm, and, opposite to this, a "vegetative" pole marked by greater abundance of yolk or lipoid material. The article by O. van der Stricht (1923) contains not merely a summary of the discoveries made by himself and his collaborators, but a detailed account of the cytoplasmic transformation in the mammalian ova studied by them, and also a large number of illustrations. The original reports on which that record was based may be found chiefly in such works as those of Lams (1913) on the guinea-pig, Lams and Doorme (1908) on the white mouse and guinea-pig, O. van der Stricht (1905) on the bat, and R. van der Stricht (1911) on the cat. From this body of work a polar arrangement of the ooplasm has been established as occurring in the bat, white mouse, guinea-pig and cat. With regard to the dog the chief information is contributed by O. van der Stricht in the article already mentioned (1923). Polarity is stated to be found in this animal, but a critical examination of the illustrations given there does not convince the present author of its presence in the tubal ova at the pronuclear stage.

Sources of error

In the ferret, owing to the large amount of lipoid material, it is not immediately easy to recognise a polar distribution of deutoplasm. When polarity appears to exist, therefore, one must eliminate all possible sources of error. In the investigation here recorded the sources of error considered were as follows:

(1) Position of section through ovum. Thus, in text-fig. 3 I, the breadth of the outer granular zone near the end *A* of the line *AB* would be less than near the end *B*. This error can be eliminated by careful focusing and by comparison with the breadth of the zona pellucida adjacent.

(2) Mode of cutting of ovum. The part first met by the microtome knife might be crushed, so that the size of the granular zone on two sides of the section might be artificially different (text-fig. 3 II and III). This source of error can be eliminated by observing whether the zona pellucida adjacent is flattened or whether the perivitelline space (*S* in text-fig. 3 III) between the ovum and the zona pellucida has been enlarged.



Text-fig. 3. Diagrams of ova. In I, the peripheral granular zone (closely dotted) is fairly uniform in thickness, and in the section of the drawing no polarity of the cytoplasm would appear. If the section were cut along the line *AB* the one part would show a broader granular area than the other. In II, the ovum has been flattened on the right, and the granular zone is narrower than on the left. In III, the ovum has been knocked away from the zona pellucida, and the granular zone is again artificially narrowed. *c.*, central portion, sparsely dotted, lipoid-bearing; *p.*, peripheral portion, closely dotted, granular; *z.p.*, *s.*, perivitelline space; zona pellucida.

(3) Fixation. Most of the ova were fixed in the uterine tube, one aspect of which faces the ovary and the other the abdominal cavity. It was possible that the fixing fluids reached one side of the ova earlier than the opposite side or in a different concentration. Allowance was made for this error, but it is improbable that it was important in the case of such a small body lying free in the uterine tube.

Ova in which polarity was demonstrated

After these sources of error had been eliminated it was found that out of 100 of the ova examined the proportion of ova classified according to polarity were:

Ova with definite polarity ...	...		29
Ova with possible polarity ...	...		21
Ova without polarity	...	...	50
			100

Illustrations of ova with polarity are shown in the accompanying plate.

It should be noted[1] that in the discussion that follows the terms "animal" and "vegetative" poles are usually avoided, because they appear to imply that the polarity here found corresponds to the polarity of non-mammalian ova, an assumption that is not immediately obvious.

Reasons for lack of polarity

To account for the ova in which no polarity was found various tests were applied. The ova fixed in Perenyi's fluid were compared with those fixed in Zenker's fluid, only those ova that were stained by Heidenhain's iron-haematoxylin being considered. The Chi Square test showed no association between these fixatives and the presence or absence of polarity.

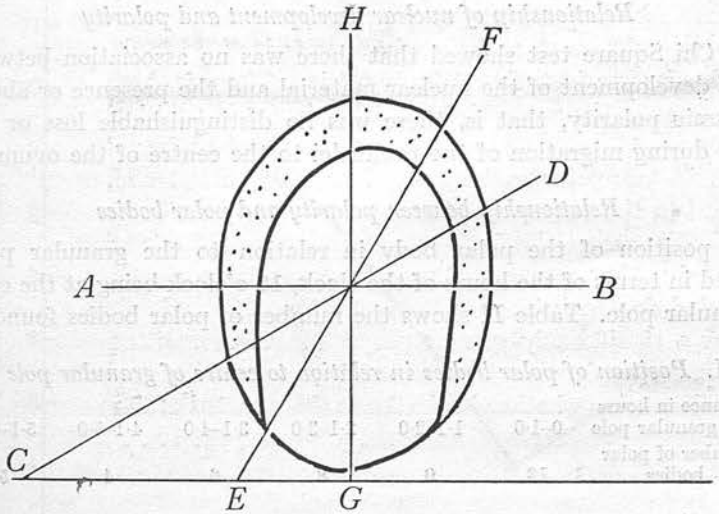
Out of 16 ova fixed in Flemming's fluid none showed definite polarity. Allowance was made for the 4 ova mentioned above in which almost all of the lipoid had been removed, and then the others were added to the Zenker- and Perenyi-fixed ova just considered. The Chi Square test then showed a high association between fixative and polarity, an association due to the introduction of the Flemming-fixed specimens. Most of these Flemming-fixed ova had been merely tinted with eosin and not stained further. Fixation in Flemming's fluid, therefore, without staining, does not clearly demonstrate polarity.

Among the ova fixed in Zenker's fluid a comparison was made between those stained by Heidenhain's iron-haematoxylin and eosin and those stained by Mayer's haemalum and eosin. The Chi Square test showed that there was a definite association between staining and the demonstration of polarity. None of the 14 ova stained by Mayer's haemalum and eosin showed definite polarity, while 13 out of 23 stained by Heidenhain's iron-haematoxylin and eosin showed definite polarity.

Since Zenker and Perenyi fixation do not differ essentially in regard to the demonstration of polarity, it may be asked why, when the staining is the same, some of the ova fixed by these methods show polarity and others do not. The explanation appears to depend upon the plane of section of the ova. Let it be assumed that every ovum has a polarity similar to the ova in fig. 1 or fig. 2 of the accompanying plate. It is reasonable to suppose that there are equal

[1] The lipoid pole is not devoid of granular material, nor is the granular pole devoid of lipoid.

chances for an ovum to be cut in any plane with reference to the interpolar axis. Text-fig. 4 represents an ovum with a peripheral granular region and two poles, granular and lipoidal. For simplicity only those sections passing through the centre of the ovum will be considered. Some sections will pass horizontally through the centre (plane *AB*), and in such sections no polarity will be detectable. Some will pass at an angle of 30° to the horizontal (plane *CD*), and then the polarity may not be definite, although actually the granular regions may not be of equal extent at the two ends of the section. The definiteness in this case depends on the exact plane of the section and the exact extent to which the granular matter encroaches on the lipoidal pole. Other sections will pass at an angle of 60° to the horizontal (plane *EF*), and so will demonstrate



Text-fig. 4. Diagram of ovum, showing the different effects of cutting along different planes. The granular zone is dotted. (For description see text.)

polarity. Others again will pass at right angles to the horizontal (plane *GH*), and then also the sections will show definite polarity. Any other planes might be chosen, but those selected are representative and are arranged at equal distances from each other. It should also be noticed that these planes (except *HG*) all commence to the left of *G*, and there will be as many planes commencing to its right. It is, however, sufficient to consider only the one set. On an average, therefore, out of every 4 sections there will be 2 showing polarity, 1 showing doubtful polarity and 1 not showing polarity. The comparison with the actual numbers is shown in Table I. The actual numbers agree very closely indeed with the theoretical. Hence the apparent absence of polarity can be very well accounted for by the plane at which the sections of the ova were cut.

Table I. *Comparison between the actual numbers of ova classified according to polarity and those expected according to the differences in planes of section. (The ova concerned are those fixed in Zenker or Perenyi and stained with Heidenhain's iron-haematoxylin.)*

No. of ova	Polarity definite	Polarity doubtful	Polarity absent	Total
Actual (a) ...	27	10	14	51
Theoretical (t) ...	25·5	12·75	12·75	51
$a-t$	1·5	−2·75	1·25	0
$\frac{(a-t)^2}{t}$	0·09	0·59	0·12	0·80

$\chi^2=0{\cdot}80$; $n=2$; P is between 0·90 and 0·80.

Relationship of nuclear development and polarity

The Chi Square test showed that there was no association between the stage of development of the nuclear material and the presence or absence of cytoplasmic polarity, that is, there was no distinguishable loss or gain of polarity during migration of the pronuclei to the centre of the ovum.

Relationship between polarity and polar bodies

The position of the polar body in relation to the granular pole was expressed in terms of the hours of the clock, 12 o'clock being at the centre of the granular pole. Table II shows the number of polar bodies found at the

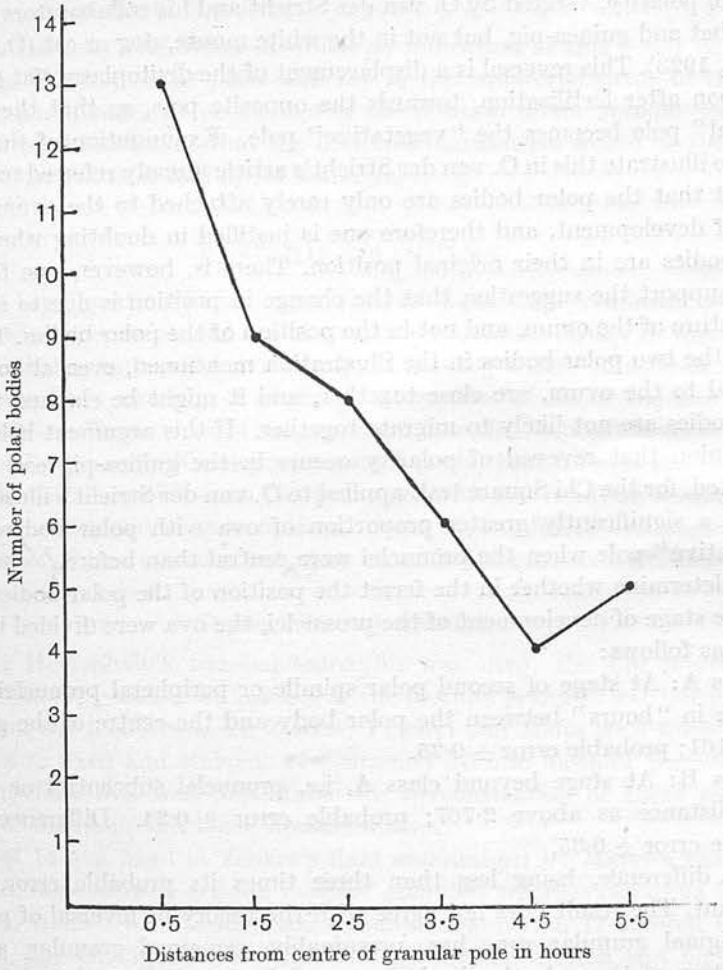
Table II. *Position of polar bodies in relation to centre of granular pole of ovum*

Distance in hours from granular pole	0–1·0	1·1–2·0	2·1–3·0	3·1–4·0	4·1–5·0	5·1–6·0
Number of polar bodies	13	9	8	6	4	5

different distances up to six hours from the centre of the granular pole. The coefficient of correlation between the distance from the centre of the granular pole and the number of polar bodies found is −0·931. There is therefore a very high negative correlation, that is, the farther away one goes from the centre of the granular pole, the fewer polar bodies one meets. To show whether this coefficient was significant or due to chance, Fisher's t-test was applied (Fisher, 1930, p. 159). $t = 15{\cdot}7$; $n = 4$, and therefore the coefficient of correlation was highly significant.

The graph in text-fig. 5 shows how closely the numbers correspond to a straight line. The straight line most closely fitting the actual numbers is expressed by the equation $Y = 12{\cdot}386 - 1{\cdot}629x$, where Y is the number of polar bodies calculated from the equation, and x is the number of hours from the centre of the granular pole. This equation is the regression equation of y (the number of polar bodies) on x (the number of hours), and indicates that by one can predict very closely the number of polar bodies found at the various distances from the granular pole.

The fact that most polar bodies are found near the granular pole suggests that this pole is probably the "animal" pole, that is the one at which the polar divisions occur. The reason for the almost rectilinear fall in frequency is not immediately evident, for it would be expected that most of the polar bodies should be near the "animal" pole, and that there would be a ~~very~~ sharp fall in numbers as one passed from the "animal" pole.



Text-fig. 5. Graph showing numbers of polar bodies found at different distances from the centre of the granular pole. Distances are in "hours."

In the interpretation of this phenomenon it is important to recall the experimental work on the frog's ovum referred to by Wilson (1925, p. 1021). This work showed that if the ovum be kept inverted the cytoplasmic materials undergo a rearrangement under the influence of gravity. This might be suggested as the cause of the peculiar arrangement of polar bodies with reference

to the poles in ferret ova, but it is obvious that it could not wholly account for the phenomena, since there would be roughly equal chances of the ova being inverted, lying on their sides, or being correctly orientated at the time of fixation, and therefore the polar bodies should be distributed at random with reference to the poles. This is not the case.

A possible explanation of the distribution of the polar bodies is the "reversal of polarity," stated by O. van der Stricht and his collaborators to occur in the bat and guinea-pig, but not in the white mouse, dog or cat (O. van der Stricht, 1923). This reversal is a displacement of the deutoplasm (fat globules, etc.) soon after fertilisation, towards the opposite pole, so that the original "animal" pole becomes the "vegetative" pole. Examination of the figures given to illustrate this in O. van der Stricht's article already referred to, reveals the fact that the polar bodies are only rarely attached to the ovum at this stage of development, and therefore one is justified in doubting whether the polar bodies are in their original position. There is, however, one fact that might support the suggestion that the change in position is due to a change in structure of the ovum, and not in the position of the polar bodies. This fact is that the two polar bodies in the illustration mentioned, even although not attached to the ovum, are close together, and it might be claimed that the polar bodies are not likely to migrate together. If this argument holds, then the opinion that reversal of polarity occurs in the guinea-pig can be substantiated, for the Chi Square test, applied to O. van der Stricht's illustrations, showed a significantly greater proportion of ova with polar bodies at the "vegetative" pole when the pronuclei were central than before that stage.

To determine whether in the ferret the position of the polar bodies varied with the stage of development of the pronuclei, the ova were divided into two classes as follows:

Class A: At stage of second polar spindle or peripheral pronuclei. Mean distance in "hours" between the polar body and the centre of the granular zone 2·161; probable error ± 0·25.

Class B: At stage beyond class A, i.e. pronuclei subcentral or central. Mean distance as above 2·767; probable error ± 0·24. Difference 0·606; probable error ± 0·35.

The difference, being less than three times its probable error, is not significant. The result does not agree with the theory of reversal of polarity. The original granular zone has, presumably, remained granular, and the distribution of the polar bodies is best explained as due to their migration, for, if the polar bodies migrate, there will naturally be fewer and fewer the farther one passes from the "animal" pole. The cause of the migration is obscure.

Relationship of polar axis to pronuclei

In the mammalian ova observed by O. van der Stricht and his collaborators there was ~~observed~~ detected a general law that, when the pronuclei were centrally placed in the ovum, the line between the centres of the pronuclei passed through

the two poles. In the ferret ova an estimate was made by the eye of the angle between the internuclear plane and the polar axis. In 12 ova the plane was at right angles to the axis, in 9 ova at about 45° to the axis, and in 5 ova it was parallel to the axis. This suggests agreement with the law just stated, but statistically these numbers were shown not to be significant.

Relationship of polar axis to plane of first segmentation

There was only one ovum in which an indication of this could be determined. In this ovum the polar axis lay in the equatorial plane of the first segmentation spindle. If no change in the position of the spindle occurred, the ovum would divide so that the first two blastomeres would be similar in respect of the granular and lipoid material.

BASIPHIL GRANULES

It has been mentioned above that the technique employed in the preparation of these specimens did not demonstrate mitochondria. It was noted, however, that many of the ova displayed small granules[1] that took on the stain of the basic dye (fig. 3 of plate). They were of about the size of the smallest chromatin granules of the pronuclei. The chief interest of these bodies lies in connection with Kremer's statement (1924) that there is in the mouse elimination of chromatin from the pronuclei with the resulting formation of chromidial substance in the ooplasm. Some of the ferret ova suggested a confirmation of this opinion, but more exact investigations were undertaken to prove or disprove it.

Association with technique

When Heidenhain's iron-haematoxylin was used, the Chi Square test showed that the presence or absence of the basiphil granules was independent of fixation, the fixatives being Zenker, Perenyi and Mann with formol. Out of 57 ova so fixed and stained, 44 contained definite basiphil granules. The Flemming-fixed ova were unsuitable for demonstration of these particles, because of the numerous black lipoidal masses.

Out of 14 ova fixed in Zenker's fluid and stained by Mayer's haemalum and eosin, none showed definite basiphil granules; whereas out of 24 Zenker-fixed ova, stained with Heidenhain's iron-haematoxylin, 17 showed basiphil granules. The Chi Square test showed that this association was highly significant.

Among the specimens stained by Heidenhain's iron-haematoxylin it appeared possible that the deeper stained ova were more liable to contain basiphil granules, but the Chi Square test showed no association between depth of staining and presence of these granules.

[1] In the cytoplasm of some of the ova bodies were met that were associated with similar bodies in the zona pellucida. A separate communication dealing with these is in preparation, and further mention of them has no bearing on the present investigations.

Association with pronuclei

If the basiphil granules were due to elimination of chromatin, it would b expected that such granules would be present in the ova with central pronucl more frequently than in the ova with non-central pronuclei. The Chi Squa test showed that this was not so.

It may be recalled (Mainland, 1930) that some of the central pronucl showed non-basiphil globules—colourless or eosin-stained. If the basiph granules in the cytoplasm were products of chromatin elimination, it wa reasonable to suppose that they would be more frequently met in the ov of which the pronuclei showed non-basiphil globules. The Chi Square tes demonstrated that this was not so.

Finally, if the basiphil granules were derived from the pronuclei, it woul be expected that there would be a preponderance of ova in which the granule were near the pronuclei. The Chi Square test showed, however, that far mor ova had granules scattered in the cytoplasm than had them near the pronuclei

There is, therefore, no reason whatever to suggest that these basiphi granules were in any way derived from the pronuclei. The necessary conclusion is that they are cytoplasmic. There is nothing to indicate that they are product of the imperfect fixation or breakdown of mitochondria, and therefore thei origin and function is still undetermined.

ACKNOWLEDGMENTS

I wish again to record my indebtedness to Prof. Arthur Robinson for the loan of the specimens on which these observations were made, and to the trustees of the Moray Fund of the University of Edinburgh for financial assistance in the preparation of some of the specimens.

The major part of the investigation was carried on in the Department of Anatomy of the University of Manitoba, and my thanks are specially due to Dr C. H. Goulden, of the Agricultural College of that University, for help in the statistical work, and to Miss Nason, of the Department of Pathology, for the preparation of the photographs.

SUMMARY

Serial paraffin sections, 10 μ thick, of 101 ova of the ferret were examined, from 30 different ovaries and 19 different animals. All were at the stages between ovulation and completion of the first segmentation. The types of fixative employed were (*a*) those that did not preserve lipoid, and (*b*) osmic acid for preservation of lipoid. Special mitochondrial technique was not used. Statistical analysis was applied to the data.

The cytoplasm consists chiefly of a mass of lipoid-bearing material, this mass being surrounded by a granular zone with little lipoid. It appeared very doubtful if (as Corner suggested) the different amounts of lipoid material

in different mammalian ova are associated with the stage of development of the zygote when it is implanted in the uterus (p. 3).

The effect of osmic acid shows that the lipoid contains at least 50 per cent. unsaturated fatty acid. Arguments are adduced to suggest that saturated fatty acid radicles are also present (p. 4).

There is no evidence in favour of the suggestion that a protein framework exists in the lipoid globules (p. 5).

Four abnormal ova are discussed (p. 5), in which lipoid was almost entirely absent. One of these was definitely degenerate, and therefore this degeneration differs markedly from that of large ovarian ova, in which masses of osmic-blackened lipoid are present.

After various sources of observational error had been eliminated (p. 7), definite polarity of the cytoplasm was established in 29 out of 100 ova. Fixation in Zenker's and Perenyi's fluids demonstrated polarity equally well. Fixation in Flemming's fluid, without staining, did not demonstrate it clearly. Staining by Mayer's haemalum did not demonstrate it well, compared with staining by Heidenhain's iron-haematoxylin.

The apparent lack of polarity in some Zenker- or Pereny-fixed specimens stained by Heidenhain's iron-haematoxylin can be accounted for by the plane of section of the ovum (p. 9).

There was no appreciable loss or gain of polarity during migration of the pronuclei to the centre of the ovum.

The numbers of polar bodies found varied inversely as their distance from the centre of the granular pole, and the relationship was expressed by a rectilinear regression equation. This suggests that the granular pole was in reality the "animal" pole and the lipoidal pole was in reality the "vegetative" pole. The arrangement of polar bodies is explained, not by the reversal of polarity due to moving of the deutoplasm, but probably by migration of the polar bodies.

The relationship of the cytoplasmic polar axis to the central pronuclei and to the first segmentation spindle was investigated (p. 12-13).

Fine basiphil granules were met in the cytoplasm of some of the ova. The occurrence of these was independent of the fixatives (Zenker, Perenyi or Mann with formol). The granules were demonstrated by Heidenhain's iron-haematoxylin and eosin but not by Mayer's haemalum and eosin.

It has been shown (p. 14) that there is no reason to suggest that these granules are derived from the pronuclei. They are cytoplasmic, but their origin and function is undetermined.

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DESCRIPTION OF PLATES I AND II

PLATE I

(All the illustrations are photographs of ferret ova.)

Fig. 1. An ovum showing polarity. Fixed in Perenyi's fluid; stained by Heidenhain's iron-haematoxylin. Magnification 300.

Fig. 2. An ovum showing polarity. Fixed in Zenker's fluid; stained by Heidenhain's iron-haematoxylin and eosin. Magnification 425.

Fig. 3. An ovum showing basiphil granules. Fixed in Zenker's fluid; stained by Heidenhain's iron-haematoxylin and eosin. Magnification 550.

Fig. 4. Part of an ovum showing vacuoles left after removal of lipoid globules. Fixed in Zenker's fluid; stained by Heidenhain's iron-haematoxylin and eosin. Magnification 1200.

PLATE II

(The author is indebted to Professor Arthur Robinson for these two photographs.)

Fig. 5. Ovum showing numerous lipoid globules. Fixed in Flemming's fluid; tinted with eosin; magnification 500. It will be observed that the brittleness due to prolonged fixation in Flemming's fluid has led to rupture of the zona pellucida and part of the ovum.

Fig. 6. The same section; magnification 1000.

ABBREVIATIONS

b.g.	Basiphil granules.	*u.t.*	Uterine tube.
g.p.	Granular pole.	*v.*	Vacuole.
l.p.	Lipoidal pole.	*z.g.*	Zona granulosa.
p.b.	Polar body.	*z.p.*	Zona pellucida.
prn.	Pronucleus.		

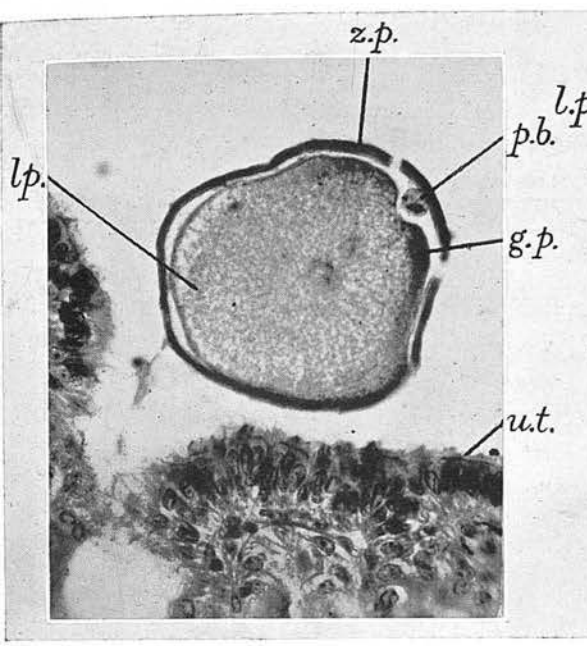

Fig. 1.

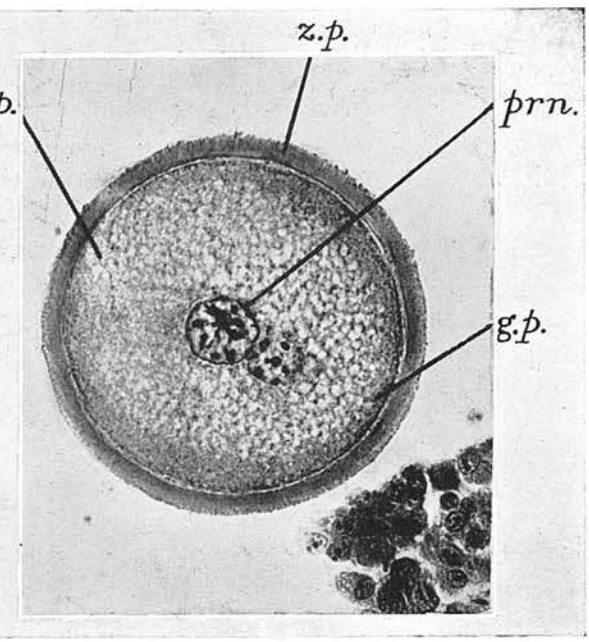

Fig. 2.

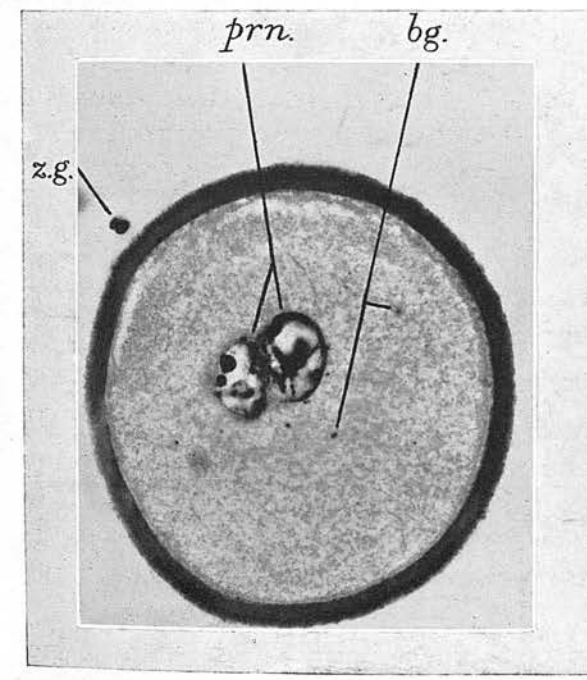

Fig. 3.

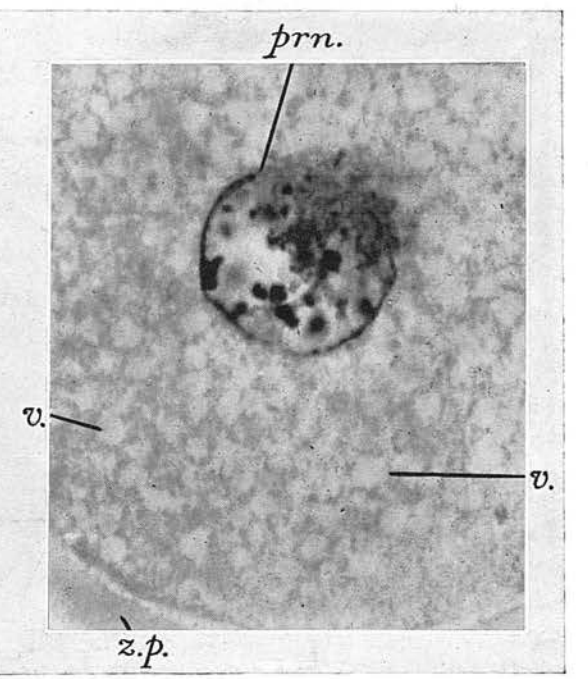

Fig. 4.

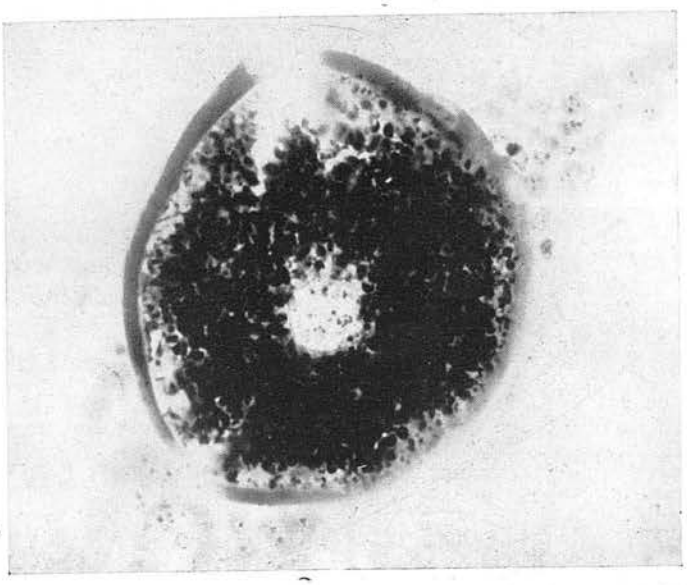

Fig. 5.

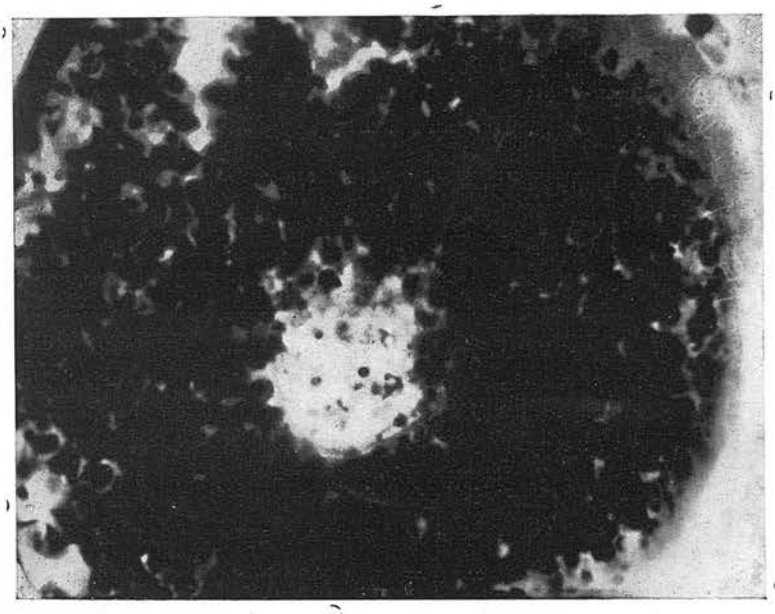

Fig. 6.

THE EARLY DEVELOPMENT OF THE FERRET;
THE ZONA GRANULOSA, ZONA PELLUCIDA
AND ASSOCIATED STRUCTURES

THE EARLY DEVELOPMENT OF THE FERRET;
THE ZONA GRANULOSA, ZONA PELLUCIDA
AND ASSOCIATED STRUCTURES

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One plate -- at end of article

INTRODUCTION

During the last few years interest in the zona granulosa and zona pellucida of Mammalian ova has waned. This is not unnatural, for it appears as if nothing very new could be learned, at least from paraffin sections, regarding the structure and functions of these membranes. The zona granulosa is generally regarded as a means whereby the ovum, at least in the ovary, is nourished (Marshall, 1922). The zona pellucida is a protective coat and in particular its late disappearance seems (Robinson, 1904) to be associated with the late implantation of the ovum.

To show that there is still something to be gained from the study of ordinary sections is partly the object of the present record. Moreover, most of the detail regarding these membranes has been obtained from a study of non-Carnivores. The present study is of a Carnivore - the ferret.

MATERIALS AND METHODS

The material used in these investigations was the same as that employed in the observations on the ferret pronuclei (Mainland, 1930) and on the cytoplasm of ferret ova (Mainland, 1931), viz., 101 ferret ova at stages from the second polar spindle to the first segmentation spindle inclusive. The ova were in the form of mounted paraffin sections cut at 10 microns. The fixatives that had been used were Flemming, Mann with and without formol, Perenyi, Zenker, and the staining methods for different ova were Heidenhain's iron-haematoxylin and Mayer's haemalum, with either eosin or orange G as counterstain.

The investigations have involved the use of a method that the author feels more and more to be essential wherever there are variations in phenomena, and, perhaps more than usual, where the number of specimens available is limited. This method is the application of biometrical tests, particularly the chi square test. In the study of the pronuclei(Mainland,1930)this method was discussed. In an appendix to the present article some further notes on and examples of its use are given. It should be pointed out here that an association between phenomena is said to be "significant" when odds are 20 or more to 1 against the association being due to chance.

ZONA GRANULOSA

The records of Robinson(1918) have shown the resemblance between the zona granulosa of the ferret and that of other mammals. It may now be subjected to a more minute examination. Out of a total of 95 ova in the early tubal stage (i.e. at the stages of pronuclei, second polar spindle or first segmentation spindle), 86 showed more or less of the zona granulosa of the follicle from which they had come. The remaining 9 showed no cells of this kind near them.

Amount of Zona Granulosa.

As is well known, the cells of the zona granulosa disappear as the ovum travels down the uterine tube. It appears desirable to demonstrate the rapidity with which this disappearance occurs, and this of special importance if, as is strongly indicated(see below), the zona granulosa is associated with the nutrition of the tubal ovum. Table I shows the relative amounts of zona granulosa found near the different ova, arranged according to the stage of development of the ovum. These data were grouped to form a 4-fold table, and the results of the application of the chi square test were: $\chi^2 = 13.6$, $n = 1$. The results were therefore very highly significant, showing that the disappearance of the zona granulosa can be definitely detected as the ovum advances from the stage of second polar spindle to the stage of first segmentation spindle. Thus there is given a criterion of development apart from the condition of the ovular nuclear material.

A further test was applied, to show the relation between the number of hours after insemination of the animal and the amount of zona granulosa remaining near the ovum. It has been shown(Mainland, 1930) that the post-inseminal period is to some extent a measure of the ovular development, for the more developed ova were in the main found in those animals that had lived longest after insemination. There was, however, no significant association between the length of the post-inseminal period

TABLE I Association between Amount of Zona Granulosa and Condition of Nuclear Material in Ovum
(The numbers of ova are recorded.)

NUCLEAR MATERIAL	AMOUNT OF ZONA GRANULOSA					
	Nil	Very Small	Small	Medium	Abundant	Total
Second Polar Spindle					15	15
Pronuclei Peripheral or Subcentral	4		3	7	1	15
Promuclei Central	5	5	13	11	5	39
First Segmentation Spindle			1			1
Total	9	5	17	18	21	70

and the amount of zona granulosa present. The post-inseminal period, as is well realized, is in the ferret, not a very satisfactory measure of development, owing probably to the great variation in the lapse of time between insemination and ovulation. In this series, therefore, there could not be demonstrated any change in the amount of zona granulosa in the first 100 hours after insemination.

Staining and Shape of Cells.

Little difference was noted in the eosinophilic staining of the cells as development proceeded. More instructive results were obtained from examination of the shape.

When the ovum is liberated it has around it the typical corona radiata, with an inner layer of pyramidal cells and two or three outer layers of polyhedral cells. Illustrations are to be found in Robinson's monograph(1918) and also in the accompanying figure(Plate-fig.7). As the ovum proceeds down the tube the zona granulosa cells degenerate. This degeneration, like the degeneration of many of the cells of the ovary, deserves attention, for it takes place in a healthy environment. It occurs in a medium composed partly of follicular fluid, partly of fluid from the tube wall and partly, no doubt, of fluid from the developing ovum itself. The degenerating cells are of various shapes(Plate-fig.2). Some are polyhedral, others more irregular, others spherical and still others ellipsoidal. Table II shows the relationship between the stage of ovular development and the shape of the zona granulosa cells. The chi square test was applied to the data grouped in a 3 x 2- table, and proved that there was a significant relationship between the shape of cell and the ovular development. Inspection of table II reveals that the significance of the association is largely if not wholly due to the greater frequency of the pyramidal cells at the polar spindle stage. It was

TABLE II Association between Shape of Zona Granulosa Cells and Condition of Nuclear Material in Ovum. (The numbers of ova are given. The table is condensed.)

NUCLEAR MATERIAL	DOMINANT SHAPE OF ZONA GRANULOSA CELLS			
	Pyramidal	Polyhedral and Irregular	Spherical and Ellipsoidal	Total
Second Polar Spindle	9			9
Pronuclei Peripheral or Subcentral	2	1	8	11
Pronuclei Central, or First Segmentation Spindle	3	12	16	31
Total	14	13	24	51

interesting to note that when the post-inseminal period was used as a measure of development, there was a highly significant association between the shape of the zona granulosa cells and the development as thus indicated. This was owing to the greater abundance of pyramidal granulosa cells around the ova at the second polar spindle stage, these ova being found in the animals with the shortest post-inseminal period. Thus the post-inseminal period appears on some occasions a satisfactory indication of development.

The next problem is the elucidation of the order of shape changes. There is no indication from the figures that spherical cells occur more abundantly at the later than at the middle stages of development, and therefore the stages might be: pyramidal, polyhedral, spherical; or: pyramidal, spherical, polyhedral. Examinations of the specimens suggested that the former series was correct. A definite answer to the question was obtained by studying the relationship between the amount of granulosa and cell-shape. It was obvious that, as would be expected, when the shape of cell was pyramidal, the amount was greater than when the cells were of other shapes; but even when all the specimens with pyramidal cells were excluded it could be shown by the chi square test that the less abundant the granulosa the more often were its cells spherical or ellipsoidal. It appeared as if more and more of the zona granulosa cytoplasm disappeared and the remainder became a spherical shell around the nucleus.

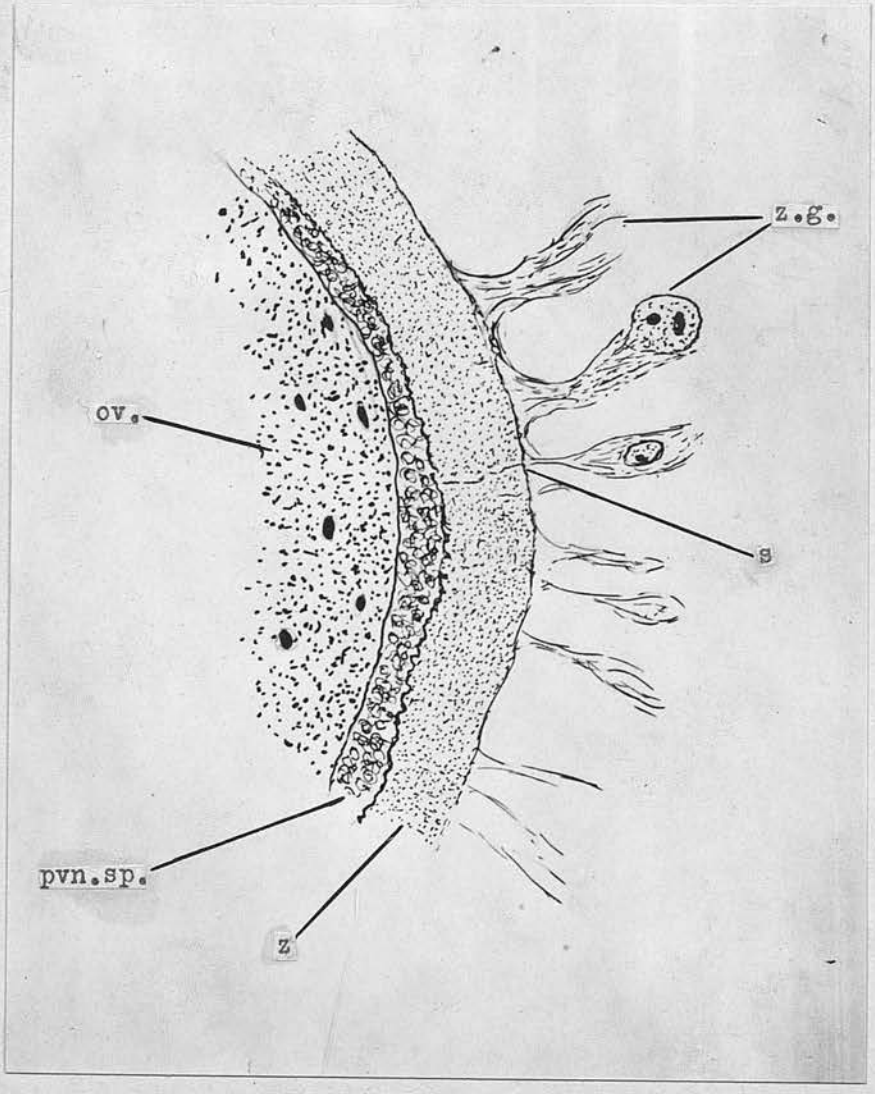
There was no association demonstrable between differences of fixative and shape of cells.

Nuclear Structure

The zona granulosa of each ovum was classified according to whether its nuclei showed only globules (or granules) of chromatin or whether some or all of its nuclei were homogeneously basiphilic (plate-figs. 2,3,4). It seemed as if the basiphilic nuclei were a sign of degeneration, but to test this supposition use was made of the fact just recorded that the shape of the granulosa cells changed from pyramidal to polyhedral and then to spherical. It was then shown that there was almost, but not quite, a significant association between nuclear structure and cell-shape. The nuclei, therefore, are of little value as an index of degeneration. There was no significant association between the nuclear structure and the stage of development of the ovum as indicated by its polar spindle or pronuclei.

The caution which is necessary in applying a chi square test is indicated by the results obtained when the association between the post-inseminal period and the nuclear condition of the granulosa was investigated. This test showed a highly significant association between the two, that is, more granular nuclei were found associated with the ova early after insemination than later on. This association appeared difficult to explain, since, as has been stated, there was no apparent association between ovular development and the condition of the granulosa nuclei. The explanation was revealed, however, when the effect of staining was observed. For each section a record was made of the depth of the basic staining of the ovum and the surrounding tissues, e.g., tube and ovary, the classification being 'dark', 'medium', 'pale'. It was then shown that there was a highly significant association between the condition of the granulosa nuclei and the depth of staining. The homogeneous nuclear appearance was much more common among deeply stained sections than among the less

Text-fig. 1 Ovum no. 24 . At point s, a strand of a zona granulosa cell penetrates the entire thickness of the zona pellucida, z. z.g., zona granulosa; pvn. sp., perivitelline space containing granular material; ov. , ovum. (Mann with formol; Heidenhain's iron-haematoxylin and orange G; camera lucida drawing photographed; magnification 1560.)



deeply stained. Moreover, it was found that, for some reason or another, deeply stained sections were much commoner among the ova taken at the late post-inseminal period. This would account for the result mentioned above. The more deeply stained sections had more homogeneous nuclei. These sections were more abundant ~~then~~ at the later post-inseminal periods, and therefore ~~is~~ a spurious association was produced between granulosa nuclear structure and the post-inseminal period.

Since the relation between depth of staining and ~~homogeneity~~ homogeneity of the nuclei is so strong, one cannot help feeling that this may mask a relationship between the nuclear structure and the degeneration of the cells. This suggestion ~~was~~ was, however, not confirmed by any tests. A test for example, was made by using the amount of granulosa present as an index of its progress towards degeneration. There was no significant association between this amount and the nuclear structure. The conclusion is that, so far as can be shown, the nuclear structure is not an index of the degeneration.

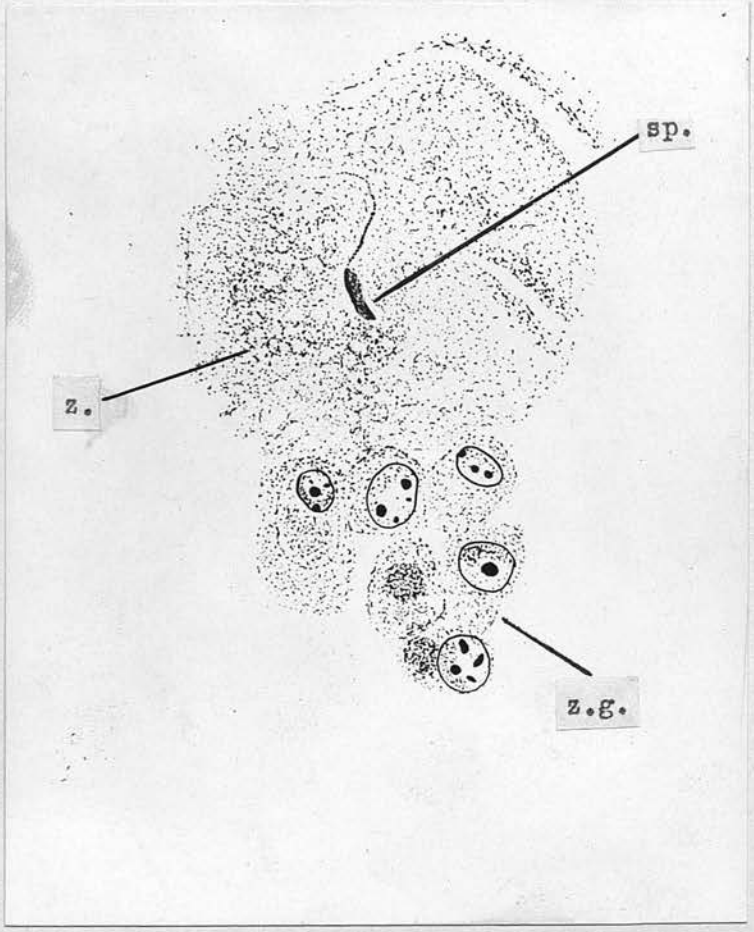
The chi square test showed no significant association between the fixative (Mann, Zenker or Perenyi) and the nuclear structure of the granulosa cells.

Relationship of Zona Granulosa and Ovum

In 10 ova of the collection some of the cells of the granulosa demonstrably penetrated the zona pellucida (text-fig. 1). In these specimens the granulosa was in the form of a corona radiata. In 9 other ova in which the zona granulosa also formed a corona, no penetration of the zona pellucida was detected. Owing to the smallness of the numbers no conclusion could be reached regarding the influence of fixative and staining on the demonstration of this penetration. In some specimens the penetration when visible was not through the whole thickness of the zona pellucida.

The interest of the proximity of the zona granulosa to the ovum lies partly in a consideration of the factors that influence the disappearance of the granulosa - dissolution by degeneration, removal by the cilia of the tubal wall and removal by the muscular contractions of the tubal wall, if, as Sobotta (1922) considers to be the case, the muscular activity of the tube is the cause of the passage of the ovum. There was a definite association between the amount of granulosa near the ovum and its proximity to the ovum, i.e. whether it was in close contact , at a little distance or at a greater distance. This was to be expected if the removal of the granulosa is largely mechanical. If degeneration alone were responsible for its disappearance, it would be expected that the cells would be near to the ovum throughout the whole course of the degeneration. They do not degenerate to the stage of forming an amorphous mass before being removed.

Text-fig. 2 Ovum no. 2. Tangential section through zona pellucida, z, showing the pitted or "cellular" appearance of its outer part. z.g., zona granulosa; sp., spermatozoon in zona pellucida. (Zenker; Heidenhain's iron-haematoxylin and eosin; camera lucida drawing photographed; magnification approx. 1000).



STRUCTURE OF ZONA PELLUCIDA

Since these investigations on the ferret were confined to ova after ovulation, discussion of zona pellucida structure may be brief. No attempt can be made to demonstrate the origin of this membrane but its structure and staining reactions deserve attention, because of the different theories of its formation and also because of the "zona pellucida bodies" discussed below. In the attempt to elucidate this question it was necessary to consult the various records of zona pellucida development. Among other records are those of Cattaneo (1922), Dubreuil and Regaud (1908), Fischer (1905), O. van der Stricht (1923) and Mjassojedoff (1923).

Texture of Zona Pellucida

Tangential and oblique sections through the zona pellucida demonstrated the "cellular" or pitted structure of its outer part (text-fig. 2). This was demonstrated by haematoxylin, but was observed also in specimens merely tinted with eosin. It was independent of the difference in fixatives. On transverse section the zona pellucida was smooth, at its inner surface, rather more rough at its outer surface, in agreement with the "cellularity" observed on tangential section.

Staining

Heidenhain's iron-haematoxylin with a light eosin counterstain gave a gray, bluish-gray, blue-black or deep purplish colour to the zona pellucida. If the basic stain was less deep as in the ova stained by Mayer's haemalum, the eosin gave the membrane a dull pink stain or an orange colour. When the specimens (fixed in Perenyi's fluid) were stained with Heidenhain's iron-haematoxylin and counterstained with orange G, the outermost part (say 1/5 or 1/6) of the zona pellucida was pale yellow, the intermediate part (the largest portion) was of a darker yellow colour, while the innermost part was a dark gray line. This

suggests a difference in texture of the different parts, especially a lack of density in the parts adjacent to the zona granulosa.

Specimens fixed in Mann's or Zenker's fluid and stained with Mayer's haemalum and eosin showed in some cases a purple outermost zone while the rest of the zona pellucida, larger in extent was orange. Similarly, among 4 Flemming-fixed specimens 2, stained moderately deeply with Heidenhain, showed the outer part of the zona pellucida blue-black, the inner part yellowish-gray.

There was no difference in staining between the Perenyi and Zenker specimens when stained with Heidenhain's iron-haematoxylin; nor between Zenker and Mann specimens stained with Mayer's haemalum and eosin; nor between Perenyi specimens stained with Heidenhain and orange G and those fixed in Mann's fluid and formol and stained in the same way.

Striae

Under the subject of zona granulosa reference is made to the presence of strands of the corona radiata penetrating the zona pellucida. Except for these, no striae were seen passing through the entire thickness of the zona pellucida. On the other hand the majority of the specimens, whatever the fixative or stain, showed numerous fine radial striae in the peripheral part of the zona pellucida (plate-fig. 5). Even Flemming specimens tinted with eosin showed these. They were present irrespective of the presence or absence of corona radiata. They presumably corresponded to the attachments of the pointed ends of the corona radiata cells.

Size

Exact determination of the thickness of the zona pellucida was not made. It is indicated in the figures of the ova accompanying the text, and may be stated as roughly 4 to 6μ.

ZONA PELLUCIDA BODIES

Occurrence and Structure

In the zona pellucida of 16 out of 99 tubal ova there were discovered spheroidal or ellipsoidal bodies (plate-figs. 1 and 2). These bodies stained usually more darkly then the zona pellucida, but rather similarly to this structure, i.e. gray or bluish-gray or dark blue in the specimens stained by Heidenhain's iron haematoxylin and a little eosin; an a dull red in the ova stained by Mayer's haemalum and strongly counterstained with eosin. Their structure was homogeneous except that a few had each a central homogeneous basiphil globule. It appeared unnecessary to determine very exactly the size of these bodies. Their diameters were frequently of about the thickness of the zona pellucida. In some specimens they were less than even the nuclei of the neighbouring granulosa cells.

The 16 ova that showed these bodies were from 4 different animals (1 tube from each animal). Other ova from the same animal or tube did not necessarily show such bodies.

Association with Fixative and Stain

These bodies were found only in specimens fixed in Zenker's fluid. Thus out of 26 specimens fixed in Zenker and stained by Heidenhain's iron-haematoxylin and eosin, as many as 12 definitely showed such bodies and 2 showed them doubtfully. Out of 15 specimens fixed in Zenker and stained by Mayer's haemalum and eosin, 4 showed such bodies. Similar bodies were not found in the 23 specimens fixed with Perenyi's fluid and stained with Heidenhain, nor in the 19 Mann-fixed specimens, nor in the 15 Flemming-fixed specimens. The association with Zenker's fixative is therefore of extremely high significance.

With regard to staining it may be pointed out that the central basiphil globule was present only in the zona pellucida bodies in specimens

stained with Heidenhain's iron-haematoxylin,and not in all of these. In this connection it should be recalled that the zona pellucida itself can, as mentioned above,take on a deep basic stain, and there is no need to suggest that the basiphil centre of the zona pellucida bodies is of the nature of a nucleus.

There was no significant differencem between the power of heidenhain's iron-haematoxylin and Mayer's haemalum tp demonstrate the zona pellucida bodies in Zenker-fixed ova.

The demonstration of these bodies by the use of Zenker's fluid and the paraffin section tecnique shows that they do not consist wholly of lipoid material. The failure of Flemming's fluid to demonstrate them shows that if any lipoid is present in them,it must have less than 50per cent non-saturated fatty acid (Parat,1927). They are presumably of protein origin. The difference between the effects of Zenker's fluid, which demonstrates them,and Perenyi's fluid,which does not, is more difficult to explain. Zenker's fluid resembles Perenyi's in possessing a chromic acid compound, but Zenker contains also mercuric chloride, which may be the cause of the demonstration of the bodies. The fact that one protoplasmic fixative and not others demonstrates them suggests that they are products of the cytoplasm rather than cytoplasm themselves. They may be either degeneration products or secretory products.

Association with Bodies in Zona Pellucida of Ovarian Ova.

In the preparationmof most of the specimens the tube and ovary had been cut and mountwd together and consequently the slides containing the tubal ova contained in many cases also sections of medium-sized or even large ovarian ova. Out of the 16 instances in which there were bodies in the zona pellucida of tubal ova,there 11 in which medium or large ovarian ova also showed similar bodies. There was shown to be a very definite association between the two occurrences.

Association with Bodies in Cytoplasm

As mentioned above, 15 ova had been fixed in Zenker's fluid, stained by Mayer's haemalum and strongly counterstained with eosin. Out of these, 13 showed bodies in the cytoplasm resembling in size and staining qualities the zona pellucida bodies. The relationship between the occurrence of these cytoplasmic bodies and the zona pellucida bodies is shown as follows (the numbers representing ova):

	Bodies in Zona Pellucida		Total
	Present	Absent	
Bodies present in Cytoplasm	3	10	13
Bodies absent from Cytoplasm	1	1	2
Total	4	11	15

None of the other ova, fixed either in Zenker's or other fluid, showed bodies that were at all comparable to these cytoplasmic bodies. There was therefore a significant association between the occurrence of the zona pellucida bodies and of the cytoplasmic bodies, inasmuch as they only occurred after Zenker fixation. There was however a definite difference between them in relation to staining. Mayer's haemalum and deep eosin staining demonstrated the cytoplasmic as well as the zona pellucida bodies, whereas Heidenhain's iron-haematoxylin demonstrated only the zona pellucida bodies. Among the Zenker-fixed ova stained by Mayer and eosin there was a significantly greater number of ova with cytoplasmic bodies than with zona pellucida bodies.

to the zona pellucida bodies
The relationship of the cytoplasmic bodies will be discussed later. At present it is desirable to give more detail regarding the structure of the cytoplasmic bodies(plate-fig. 6). Thus,in ovum No.55there lay in the cytoplasm near its edge an irregular ellipsoidal body stained orange with eosin. The surface facing the zona pellucida was more irregular in contour than the surface facing the centre of the ovum. In another part of the same ovum were 3 similar bodies. One of these,nearer the zona pellucida, had a smooth surface facing the zona pellucida;the two deeper in the ovum were more irregular all round and less homogeneous in texture.The irregularities of these bodies corresponded fairly closely to the alveolar spaces of the cytoplasm itself,spaces which in the living ovum were filled with lipoid material. If the bodies can so accommodate themselves to the masses of deutoplasm,it appears that the cytoplasmic bodies must be fairly fluid before fixation.

Differences in size between the bodies in the peripheral part of an ovum and those in the central part were not marked,and there was no evidence of absorption in the sense of a diminution in the size of the bodies as a result of a dissolution progressing from the edges towards the centre.

Association with Zona Granulosa

The Zenker-fixed ova were arranged according to the presence or absence of the zona pellucida bodies and according to the amount of zona granulosa present(table III). There was shown by the chi square test to be a significant association between the two classifications. Ova with a medium or large amount of granulosa near them more often presented zona pellucida bodies than did ova where the zona granulosa was absent or small in amount. Two possible interpretations might be placed upon this.It might be claimed as a proof that the zona pellucida bodies were products of the zona granulosa;or the amount of granulosa might be taken simply as an indication of the development of the ovum(see above),and then the relationship of granulosa and zona pellucida bodies would indicate that these bodies disappeared as the ovular development progressed.

It should be noted at this point that there was no significant association between the occurrence of the bodies in the cytoplasm and the amount of zona granulosa near the ova. If anything there was a tendency for these bodies to be more often found where the zona granulosa was small (table IV). There was therefore a distinct difference between the zona pellucida bodies and the cytoplasmic bodies in this respect. It was thought possible that this was a spurious difference due to the difference between Mayer's staining and Heidenhain's in demonstrating the bodies in the cytoplasm,but chi square tests showed no indication that this was so. The conclusion is therefore that,while the zona pellucida bodies get less frequent as the ovum progresses and the zona granulosa disappears,the bodies in the cytoplasm remain,or perhaps even increase in frequency.

TABLE III Association between Amount of Granulosa and Presence of Zona Pellucida Bodies
(Zenker specimens only. Numbers of Ova.)

Amount of Granulosa	Zona Pellucida Bodies				
	None	Doubtful	Present	Many	Total
Nil	3	-	-	-	3
Very small	1	-	3	-	4
Small	8	-	-	-	8
Medium	6	-	6	3	15
Large	3	1	4	-	8
Total	21	1	13	3	38

TABLE IV. Association between Amount of Granulosa and Presence of Bodies in Cytoplasm.

(Numbers of Ova)

Amount of Granulosa	Cytoplasmic Bodies None or Doubtful	Present or Numerous	Total
Small	3	7	10
Medium or Abundant	9	6	15
Total	12	13	25

Association with Bodies outside the Ovum

In 15 specimens spheroidal bodies resembling those of the zona pellucida in shape, size and staining reactions were discovered outside the ovum, some in contact with the zona granulosa near the tubal ovum, some in the tube at a distance from the ovum. These bodies appeared fairly homogeneous in strucure. Similar bodies were found in the liquor folliculi of some moderate sized follicles in the ovary corresponding to ovum No.65 which itself showed zona pellucida bodies. In one specimen corresponding to ovum No.67 somewhat similar bodies were found among the granulosa cells of a recently ruptured follicle, i.e. one about to become a corpus luteum.

Relationship of Zona Pellucida Bodies to Perivitelline Space

In one ovum a zona pellucida body projected into the perivitelline space causing a depression of the adjacent surface of the ovum. In ovum No.11(plate-fig.8) several bodies were seen partly inside the zona pellucida, partly outside, while some lay in the perivitelline space. The part against the ovum did not depress the latter, but was spread out on its surface. The staining of the bodies in the space was sometimes a little lighter, sometimes a little darker than that of the zona pellucida itself. There was no evidence that the zona pellucida was deficient near the bodies that lay in the space. The zona did not seem to have lost any of its substance although the bodies so closely resembled it in staining reactions.

The above data have been recorded without the introduction of any suggested explanation. The question of origin and destiny may now be discussed.

Origin and Destiny of Zona Pellucida Bodies

Before proceeding with an attempt to elucidate the genesis and fate of these bodies, it is desirable to note the references to closely similar structures in other animals. The author is acquainted with only two such descriptions. Sobotta(1908) recorded the occasional appearance of small

nucleated cells,not polar bodies,in the perivitelline space of mouse ova at the early tubal stages of development,but these bodies could not be classified definitely as follicular epithelial cells or as abnormalities in the structure of the zona pellucida. A few years later Sobotta and Burckhard(1911) described in regard to the tubal ova of the rat some roundish bodies readily stained with acid aniline dyes. These bodies were sometimes in the zona pellucida,sometimes between the zona granulosa cells, and commonly appeared first in the later stages of fertiligation. The authors could not decide whether the bodies were products of tubal secretions or of hyaline degeneration of the zona granulosa cells. At first sight it appears rather remarkable that a structure that is so common in the tubal ova of the ferret should be so seldom mentioned in connection with the ova of other animals,but technique may well be the cause of this. O.van der Stricht and his collaborators,for example,were so much concerned with cytoplasmic structure that Zenker's fluid was seldom used in their preparations,and it has been shown above that this fixative is the only one that demonstrated the bodies in the present investigations.

A brief recapitulation of the facts so far demonstrated in the ferret may now be made.

The results of fixation strongly suggested that the zona pellucida bodies were not cytoplasmic in structure,but were rather degeneration or secretory products. Similar bodies were found in relation to ovarian ova, and similar bodies were demonstrated by Mayer's haemalum and eosin in the cytoplasm of tubal ova. The zona pellucida bodies were less frequently met where the amount of zona granulosa near the ovum was small. There was if anything a tendency in the opposite direction with regard to the cytoplasmic bodies. In a number of instances structures somewhat similar to the zona pellucida bodies were found outside the tubal ova among the granulosa or elsewhere in the tube.

The bodies in the peritivelline space suggest stages of a progression into or out of the zona pellucida and out of or into the ovum. No adequate transition stages were observed between the cytoplasmic bodies and the zona pellucida bodies, but there appeared no reasonable ground for doubt of the identity of these two. It is, of course, obvious that, since they were found near the ovarian ova they could not be products of the uterine tube. The question next arises whether the bodies are products of the ovum or of the zona granulosa, i.e., the question of direction in which the bodies are passing. This is partly answered by the fact that as the zona granulosa cells get smaller in number, i.e., as the ovum advances in development, the zona pellucida bodies are less frequently met, while the cytoplasmic bodies are as abundant, or perhaps even increase in number.

Oneof the ovarian ova corresponding to the tubal ovum No.17 was very instructive in regard to the origin of the zona pellucida bodies. The specimen was a medium-sized healthy ovarian ovum, fixed in Zenker and stained with Heidenhain. In its zona pellucida small basiphil granules were detected, and by careful focussing it appeared possible to detect a connection between these and the strands of the zona granulosa around the ovum. No other example of this was observed, but the numbers of available ovarian ova of fair size were small.

When the zona granulosa is in the form of degenerating rounded cells it is sometimes very close to the zona pellucida, and may be somewhat imbedded in the outer surface of the latter. It may therefore be asked whether the zona pellucida bodies and those found in the ovular cytoplasm are not degenerate granulosa cells. This suggestion is further supported by the presence in some of the zona pellucida bodies of a central area stained blue-black like a nucleus by Heidenhain's stain. On the other hand, this area was absent from most of the bodies, even when stained by this method. Moreover, while the

some of the bodies were large enough to be granulosa cells, others could at most be fragments of granulosa cells. The presence of bodies in relation to ovarian ova is also strongly against this possibility, and it should be recalled that the fixation phenomena indicated that they were not actually cytoplasm.

The strong resemblance between the bodies and the zona pellucida is striking and important, and the conclusion of these investigations is that the zona pellucida bodies are products of the zona granulosa, in a very similar fashion to part, at least, of the zona pellucida. The bodies are absorbed by the ovum.

There is thus indicated a function for the zona pellucida beyond that of protection. During the process of development of the ovum in the uterine tube the zona pellucida contains material that is probably utilized by the ovum and passes this material on to the ovum.

PERIVITELLINE SPACE

The space between the zona pellucida and the ovum proper varied in extent from ovum to ovum. It was impossible to say whether this variation was due entirely to differences in the amount of shrinkage during preparation of the ova. This space always contained at least a small quantity of a finely granular material similar to the neighboring cytoplasm (plate-fig. 4). In some specimens, as in plate-fig. 5, the space was large and the granular material very definite. There seemed to be no reason to regard this granular material as a product of deutoplasmolysis. It could arise equally well from the precipitation of some albuminous periovular fluid.

PERIVITELLINE MEMBRANE

In the various photographs shown here the edge of the ovum proper will be noticed as marked by a line that is denser than the rest. No further evidence of the nature of this "perivitelline membrane" was obtained from a study of the sections. The most profitable way of studying this structure, like the structure bearing the same name in Invertebrate ova, is to investigate the fresh unfixed ovum in which, 50 years ago van Beneden (1880), working on the rabbit, demonstrated that the membrane could be isolated.

ACKNOWLEDGEMENTS

To Professor Arthur Robinson of Edinburgh University I am very much indebted for the loan of the specimens on which these investigations were carried out. To the trustees of the Moray Fund I wish to express my thanks for financial assistance in the preparation of some of the specimens. The observations were carried out in the Anatomy Department of Manitoba University and the work was completed in the University of Dalhousie. To Miss Nason of the Pathology Department of Manitoba University I am obliged for the preparation of the photographs.

SUMMARY

Investigations have been made of the zona granulosa, zona pellucida and associated structures of 101 ferret ova in stained paraffin sections, biometrical tests being applied to show the significance of the results. The stages examined were from the second polar spindle stage to that of the first segmentation spindle.

In spite of the great variation between different ova, the disappearance of zona granulosa could be detected as the ova advanced from the first to the last of these stages. Probably owing to the great variation in the lapse of time between insemination and ovulation it was not possible in this series to demonstrate any change in the amount of zona granulosa near an ovum in the first hundred hours after insemination.

The eosinophilic staining of the cells did not change detectably as the ova advanced in development.

There was a significantly greater number of ova with pyramidal granulosa cells at the second polar spindle stage than later in development. The order of shape changes is indicated as: pyramidal, polyhedral, spherical.

There was no indication that the nuclear structure of the granulosa cells altered as the ova developed, nor that the differences in nuclear structure indicated degeneration. The more intense the basiphilic staining of the sections, the more abundant were homogeneously stained granulosa nuclei.

The greater the amount of zona granulosa near an ovum, the nearer to the ovum did the cells tend to lie, as would be expected if the cause of their disappearance were mechanical removal rather than mere degeneration and dissolution.

The structure of the zona pellucida was studied (p. 8). The majority of the ova showed radial striae in the zona pellucida, but these

were only in its outermost part, except in those ova where cells of the corona radiata demonstrably penetrated far into or through the zona pellucida.

The thickness of the zona pellucida was roughly 4-6μ.

Sixteen of the ova showed spherical or ellipsoidal bodies in the zona pellucida. These were demonstrable only in Zenker-fixed ova, not ~~only~~ in Perenyi-, Flemming- or Mann-fixed ova. They were shown either by staining with Heidenhain's iron-haematoxylin or by staining with Mayer's haemalum. There was a significant association between the occurrence of these bodies and the occurrence of similar bodies in medium sized or large ovarian ova. Similar bodies were found in the cytoplasm of 13 out of 15 Zenker-fixed tubal ova stained with Meyer's haemalum and eosin, but these cytoplasmic bodies were not demonstrated by Heidenhain's iron-haematoxylin.

The zona pellucida bodies got less frequent as the ova progressed in development and the zona granulosa disappeared, whereas the similar bodies in the cytoplasm remained, or perhaps even increased in frequency.

In a number of instances structures somewhat similar were found outside the tubal ova in the tube and in the liquor folliculi.

It was concluded that the zona pellucida bodies were products of the zona granulosa arising in a very similar fashion to part, at least, of the zona pellucida, and that the bodies ~~are~~ were absorbed by the ovum. The zona pellucida is therefore more than a mere protection to the ovum.

The perivitelline space varied in extent in different ova. It always contained some finely granular material, not necessarily a product of "deutoplasmolysis".

The study of the sections did not give evidence of the nature of the perivitelline membrane.

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APPENDIX ON THE USE OF BIOMETRICAL TESTS

The object of such tests is to determine whether the data recorded are in agreement with some hypothesis or not. A very common instance is the test whether an observed arrangement can be due to chance, or, more specifically, what are the odds that it is not due to chance. The chi square test, used so much in this article, has a very wide application. It may, for example, be applied to embryological problems as here, or in comparing two sets of data regarding the relationship of an artery to a nerve, or in comparing the incidence of an infection among inoculated and non-inoculated individuals.

Table III of this article (p.) indicates the relationship between the amount of granulosa near the ova and the presence, absence or abundance of zona pellucida bodies. It appears as if the more abundant the zona granulosa, the more abundant the zona pellucida bodies. This is made still more striking when the data are grouped into a fourfold table, thus;

Zona Granulosa	Zona Pellucida Bodies: None or Doubtful	Zona Pellucida Bodies: Present or Many	Total
nil - small	(a) 12	(b) 3	15
medium - abundant	(c) 10	(d) 13	23
Total	22	16	38

Apart from the totals the number of squares in this arrangement is 4. For easy reference the squares have been lettered (a), (b), (c), (d). The object of the chi square test is to show whether these squares really have too few or too many individuals when they are compared with the proportions that hold among the 38 ova considered together, or whether the disproportion is only apparent, and capable of being smoothed away by investigation of more specimens. In the second column of figures the ratio 3:16 is far different from the ratio 15:38. If the proportion were that of 15:38 the 3 should be replaced by $\frac{15}{38} \times 16 = 6.3$

In a similar way all the other squares could be filled with the calculated numbers. Further manipulation would give the result, χ^2, which is a measure of the difference between all the actual numbers in the squares and the corresponding calculated numbers. For a fourfold table, however, χ^2 can be easily calculated directly by the following process (the numbers being those given here in the fourfold table):

$$\chi^2 = \frac{(12\times13-10\times3)^2\times38}{22\times16\times23\times15} = 4.97$$

It is now necessary to find whether this value might be due to chance, and what are the odds that it is due to chance. In a table of chi square, such as that of Fisher (1930), P represents these odds. In entering the table one should note the value of n, based on the number of squares (here 4) in the table of data . n is always equal to (number of rows minus one) multiplied by (number of columns minus one). Here $n = (2-1)\times(2-1) = 1$. P is therefore between 0.05 and 0.02, i.e. the odds are less than 0.05: 0.95, or 1:19 in favour of the arrangement being due to chance, i.e. more than 19 or 20 to 1 against its being due to chance. When odds are 20 or more to 1 in favour of an association (other than a chance association) between phenomena, the association is spoken of as"significant".

The calculation of χ^2 for Table IV in the text is as follows:

$$\frac{(3\times6-9\times7)^2\times25}{12\times13\times15\times10} = 2.16.$$

P is therefore between 0.2 and 0.1, and the association between the amount of granulosa and the cytoplasmic bodies is not significant.

NOTES TO PLATE

Abbreviations

c.b.,cytoplasmic body.

ov.,ovum.

prn.,pronucleus.

pvn.sp.,perivitelline space.

t.,tube(uterine).

z.g.,zona granulosa.

z.p.,zona pellucida.

z.p.b.,zona pellucida body.

Fig.1. Ovum no.17,showing zona pellucida bodies and zona granulosa. Zona granulosa nuclei are mostly homogeneously basiphil. (Zenker;Heidenhain's iron haematoxylin and eosin; magnified by 375.)

Fig.2.Ovum no. 17, a section nearer the end of the ovum than that of Fig.1. Numerous zona pellucida bodies of various sizes; zona granulosa with polyhedral cells and many homogeneous nuclei. (Zenker;Heidenhain's iron haematoxylin and eosin;magnified by 375.)

Fig.3.Ovum no.3.Granular matter in perivitelline space;zona granulosa cells massed together;many homogeneous nuclei. (Zenker;Heidenhain's iron-haematoxylin and eosin;magnified by 450.)

Fig.4.Ovum no.6. Zona granulosa with polyhedral cells;nuclei granular. (Zenker;Heidenhain's iron-haematoxylin and eosin; magnified by 425.)

Fig.5.Ovum no.93.Irregular areas in outer ~~part~~ edge of zona pellucida, less dense than the rest of it,suggesting radial striae; granular matter in perivitelline space. (Perenyi;Heidenhain's iron-haematoxylin and eosin;magnified by 1200.)

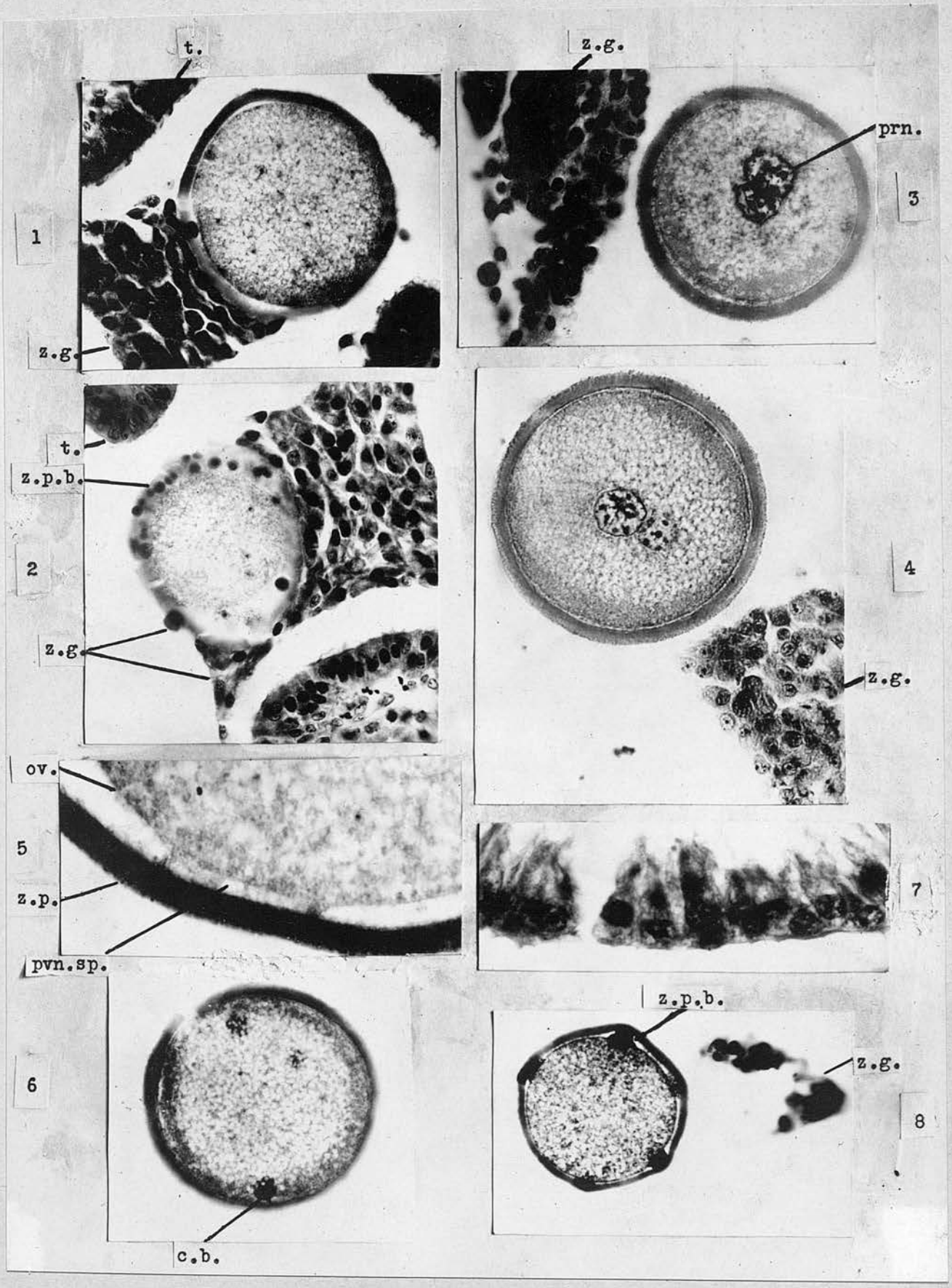
Fig.6.Ovum no.55.Bodies in cytoplasm,one appearing dense in the part facing zona pellucida,more alveolar in the part of it facing centre of ovum. (Zenker;Mayer's haemalum and eosin; magnified by 650.)

Fig.7.Ovum no.X .Zona granulosa of pyramidal cells,apices pointing towards zona pellucida. (Perenyi;Heidenhain's iron-haematoxylin and eosin;magnified by 750.)

Fig.8.Ovum no.11.Zona pellucida bodies,two in zona pellucida,two in

NOTES TO PLATE (cont.)

Fig.8.(cont.) perivitelline space. The paler granular matter in perivitelline space is probably a polar body. Remnants of zona granulosa visible.
(Zenker; Heidenhain's iron-haematoxylin and eosin; magnified by 275.)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THE VOLUMES OF FERRET OVA,

WITH SPECIAL REFERENCE TO THE METHODS

OF DETERMINATION

THE VOLUMES OF FERRET OVA, WITH SPECIAL REFERENCE TO THE METHODS OF DETERMINATION

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INTRODUCTION

In recent years a considerable literature has grown up on the sizes of cells, principally in connection with the question of nucleo-plasmic ratio. Apart from this source of interest, it would seem that the most profitable cell measurements would be carried out on the fully developed unsegmented ovum, in order to demonstrate the relationship between the ovum size and, for example, the size of the adult animal, the species of animal or the size of the mature Graafian follicle. The descriptions of Mammalian ova frequently contain either records of sizes (e.g., in the work on the cat by Hill and Tribe(1924)who give three dimensions for each ovum described); or illustrations at a known magnification from which certain dimensions can be calculated (e.g.,in the work of O. van der Stricht(1923)). What is lacking, therefore, is not measurement, but, most frequently, the establishment of facts or generalizations based on the measurements. Even where an attempt is made to establish such generalizations a second weakness becomes obvious - the failure to determine the limits of accuracy of the measurements. An example of this is contained in the work of Lams and Doorme (1908) who stated that the ova in the uterine tube are smaller than those in the ovary. Although the mean diameters of the ova, which are the only figures supplied to the reader, appear convincing, they could not be really so unless there were also supplied a measure of the variations between the individual measurements. Tests of variation are the prerequisite of all quantitative work, and to demonstrate the results of such tests is the first purpose of the present record. Since the investigations were carried out with a type of apparatus (the Edinger projection apparatus) that is very widely used in the production of tracings of tissues and embryos, the tests appear more worthy of record than if they were tests of a special technique confined to the problem in hand.

After these preliminary tests have been discussed the record is concerned with a systematic study of the volumes of tubal ova of a Carnivore- the ferret, and the demonstration of the relationship between these volumes and other quantities and phenomena.

HISTORICAL NOTES

No attempt is made to give here a historical review of the subject of cell measurement. Some valuable historical information will be found in the articles by Erdmann (1911) and Shull (1922). In this section, however, indications may be given of the type of work that has been carried out. The greatest number of these references is naturally to work on Invertebrates. Their interest in regard to the present work is chiefly in respect of method. The following is a brief list of such works:

Conklin (1912): material largely Crepidula and Fulgur; micrometer readings; calculations of volumes from formulae.

Erdmann (1909): ova of sea-urchins; axes of cells measured and volumes calculated on assumption that they were spheres or pyramids.

-------(1911): good recent history .

Godlewski (1908): material various, e.g., unfertilized ova of Echinus; one or two axes measured in drawing of magnified image; volumes calculated from formulae for sphere or ellipsoid.

--------- (1910): epithelium of Amphibia; error of measurement of tracings stated, without details, to be less than 1 per cent.

--------- (1918): tracing of sections, e.g. of unripe Echinus ova on to cardboard; areas determined by weight of cardboard, and diameters from areas, which were assumed circular; volumes calculated from formulae.

Hill and Tribe (1924): cat ova; three dimensions, one a product of known thickness of sections.

Jacobj (1925): various tissues, e.g., liver of Proteus, mouse, rat and Echidna, pancreas of rat and mouse; volumes calculated from formula for ellipsoid; eye-

piece micrometer measurements.

Koehler (1912): Strongylocentrotus lividus; eye-piece micrometer measurements mostly relied upon; emphasis on importance of determining the variability of results.

Lams and Doorme (1908): ovarian and tubal ova of mouse and guinea-pig; diameters of sections; no calculation of volumes; (see also above).

Painter (1928): eleven fresh unsegmented ova of rabbit in normal saline; diameters by eye-piece micrometer; no volume calculation.

Shull (1922): Hydatina senta; planimeter measurement of drawings of sections of cells, i.e., not actual volumes; good summary of work of other authors on nucleoplasmic ratio.

O. van der Stricht (1905): human ovarian ova; diameters of sections.

Although this list is not complete, it is representative in various ways, including its indication of the rarity with which depth of sections has been used in the estimation of volumes.

MATERIAL AND METHOD

The ova investigated were those upon which several reports have been issued (Mainland, 1930, 1931a, b, c) and consisted of part of a series of 101 ferret ova fixed at different stages after ovulation - the stages of second polar spindle, peripheral pronuclei, subcentral pronuclei, central pronuclei and first segmentation spindle. The ova were fixed, some in Flemming's fluid, some in Perenyi's, some in Zenker's and some in Mann's, with and without formol. All the ova were in paraffin sections cut at 10 microns. The staining methods were Heidenhain's iron-haematoxylin and eosin or orangeG, and Mayer's haemalum and eosin.

Althpugh more measurements were made than appear in the present record the ova that were found suitable for final inclusion were 70 in number. The others were rejected because of lost sections or for other reasons.

The apparatus, as mentioned above, was an Edinger projector. The illuminant was a carbon-arc, arranged so that the tracings of the ova could be done on a horizontal sheet of cardboard placed beneath the eyepiece of the instrument. The chief disadvantage of such a method was the impracticability of using oil-immersion lenses with the apparatus in question and with an object so large as a ferret ovum. The effect of the use of dry lenses will be referred to later, but in the meantime it may be observed that the work was carried out in such a way as to test the reliability of size records obtained from the apparatus as it is commonly employed. The apparatus was kept with all its adjustable distances (including the tube-length of 160 mm.) constant throughout the many weeks of tracing. The objective was a Zeiss dry lens (40 x) with its adjustable correction collar set permanently at the mark 17; the eyepiece was a Zeiss compensating lens (K 15 x). The magnification thus obtained was determined by a Spencer stage micrometer to be 1494.4 ± 3.31. A tracing was made of each section of the ova on to flexible cardboard of fairly uniform thickness. In the making of each tracing, except those of the end sections (upper and lower), the middle part of the depth of the section was brought into focus, so that each tracing represented a body of roughly cylindrical shape on a base equal in shape and size to the middle of the actual section. For reasons that will be explained below, the end sections were in most cases focussed so as to give the maximum area. The tracings were cut out by a small sharp Paasch swivel knife, the part cut out representing the ovum itself exclusive of the perivitelline space and zona pellucida. The pronuclei or polar spindles were marked on the tracings, but were not subtracted from the weight of these, so that the resulting ovum-volume represented cytoplasm and nuclear material.

After being cut out, the tracings were suspended and dried for from one to several weeks in rectangular specimen jars containing anhydrous calcium chloride,* the jars being made airtight by vaseline smeared between the lid and the jar. The weights of the tracings were then taken on a chemical balance

* A few discs were dried in an oven at 75°C.

sensitive to 1/5 th mgm. When the preliminary test of 25 sets of tracings was made(see below), the variation in the results due to the tracing and cutting of the cardboard and due to the cardboard itself was found to be great enough to make it unnecessary to weigh more accurately than to the nearest 0.01 gm., a procedure that was thereafter adhered to.

For the purposes of the subsequent calculation the uppermost tracings of the and lowermost sections of the ova were each weighed separately from the rest (the middle sections),which were all weighed together. The middle tracings represented a series of cylinders with irregularly curved,frequently somewhat elliptical bases,each cylinder being 10 microns high - the thickness of the sections. In finding the volumes of the parts of the ova represented by these sections,use was made of this depth of 10 microns. This was felt to be very desirable for two reasons. In the first place,the ova were presumably cut at any angle with reference to their long axes,and therefore measurement merely on the plane of the sections could give no adequate indication of the volume. Secondly,it was only by taking into account the thickness of the sections that one could use the cardboard-weight method and so allow for the variation in shape of the sections. The method,as has been pointed out above,has not been widely used,and it has in its disfavour the fact that one is trusting greatly to the accuracy of the micro-tome. The sections were, however, extremely uniform in thickness from ovum to ovum, and it was felt that the disadvantage of using thickness in the calculations was very small. It might be asked why one should not actually measure the depth of each section by the graduated fine adjustment of the microscope, as was advocated and carried out in obtaining the volumes of the pronuclei (Månsinland, 1931a). Experience in thickness measurement of the upper and lower end sections of the ova (see below), however, caused the author to think that no greater accuracy could thereby be insured.

After determining the weights of the middle sections of the ova

the next step in the calculation of the volumes was the determination of the actual area of the tracings and then of the area as it would be before magnification. The specific density of the cardboard was 0.0201 gm. per sq. cm. (mean of 25 observations). The actual area represented by the tracings was found by dividing the weight by the density. This area was divided by the square of the magnification (1494.4^2) to determine the actual area, and the result, expressed in square microns was multiplied by 10 (the height in microns of each section). Thus was obtained the volume of the middle sections of the ovum in cubic microns.

The chief difficulty arose in connection with the two end sections, and the approximation obtained for their volumes cannot be looked upon as so close an approximation as that for the middle sections. Each end (upper and lower) was considered as the segment of a sphere with one base, and its volume calculated by the formula $V = \frac{1}{6}\pi h(3r^2 + h^2)$, where V is the volume, h is the height or thickness of the section and r is the radius of the section. To find the radius , r, the area of the section was obtained from the weight and density of the cardboard, the area being assumed to be that of a circle . The formula thus became $V = \frac{1}{6}\pi h \left(\frac{3A}{\pi} + h^2\right)$, andthis became $\frac{Ah}{2} + \frac{\pi}{6}h^3$, A being the area of the section and the other letters bearing the same meaning as in the first formula. To find the height,h, measurements of thickness by the fine adjustment of the microscope were made. Since it was assumed throughout that the sections were at 10 microns, the direct reading could not be accepted. If, for example, the thickness of neighbouring parts of the section (e.g., tube wall) was read on the fine adjustment as 12 microns, and the thickness of the ovum as 6 microns, the ovum thickness was recorded as 5 microns i.e. $\frac{1}{2}$ of 10 microns. After the preliminary tests were carried out (see below), the use of the factor h^3 in the formula was dispensed with. In cases where the maximum area of the end sections had not been focussed, allowance was made in the calculation.

In actual practice the steps of the procedure were simpler than appear here. A factor, k, was employed, and the rules followed were:

For the upper end section take $\frac{1}{2}$ its thickness in mm. and multiply by the weight of the tracing.

For the lower end section do likewise.

For the middle sections multiply the total weight of the tracings by the thickness of one section in mm., i.e. $\frac{1}{100}$ mm.

Add these three quantities. The result is density of cardboard x apparent area in cm. x actual height of ovum.

To find the volume in cubic mm. divide by density of cardboard and by the square of the magnification and multiply by 100, i.e., instead of these manipulations multiply by k=0.002229.

TESTS OF VARIABILITY DUE TO METHOD

(a) Tracing, Measurement, Variation in Cardboard, etc.

The drawback to the use of cardboard in work of this kind, as has been pointed out by Scammon and Scott (1927), is its tendency to vary from piece to piece in density and also to absorb moisture rapidly. The authors named recommended the use of kodaloid instead of cardboard. In the carrying out of the present research the expense of the kodaloid was a deterrent and cardboard was adopted. Precautions were taken, however, to weigh as quickly as possible before much moisture could be absorbed. Moreover the danger from this was much less when the weighing was done, as in the main part of the investigation, only to the nearest 0.01 gm., than in the series of preliminary test weighings, where the accuracy was carried to the fourth decimal place. It was further realized that a very great source of error lay in the variability of the different tracings of any one section and therefore the adoption of a minutely accurate but expensive medium instead of cardboard was considered unnecessary.

In any case it was needful to determine the extent to which differences in results were produced by differences in tracing, cutting,

measurement of thickness, weighing and cardboard density. To do this, 25 sets of tracings of one typical ovum were made and the volumes were calculated by the method shown above (involving the use of h^3). It will be noted that these tests were to some extent supplemental to those of Scammon and Scott (1927) who showed the variability due to cardboard alone. The results of the tests were as follows:

Mean volume of ovum obtained from 25 sets of observations: 0.000,296 cub. mm.
Probable error of mean: ± 0.000,001,1
Standard deviation of series: ± 0.000,008
Coefficient of variation: 2.7 per cent.

The error due to the factors mentioned is therefore reasonably small.

It was next shown that no significant difference was introduced into the result by neglecting the factor h^3 in the formula given above, and this factor was ~~[illegible]~~x omitted in the calculations of the volumes of the other ova.

(b) Personal errors

Since it was not found convenient for one observer to cut out all the tracings of the ova, tests had to be made to compare the results obtained by the author, who cut out all the tracings in the series of 25 sets just mentioned, with the results obtained by an assistant who cut out nearly all of the tracings of the rest of the ova . The method of obtaining relative areas and volumes by weighing cardboard is widely used, and it must sometimes happen, as in the present instance, that two observers find it convenient to share the work of cutting out the tracings, so that the question of personal errors becomes important. For this reason some detail is here given regarding the tests made in this study.

The object of the tests was to found out whether the results of the 25 sets of observations of the preceding section would have differed if the assistant instead of the author had cut out the tracings. In the first place two sets of 25 tracings were made by the author by drawing a pencil on cardboard round the edge of a circular tin about 4 inches in diameter. The tracings of one set were cut out by the author and those of the other set by the assistant, and each disc after being dried was weighed separately by the author.

Discs cut by the author

Mean weight of discs in grams: 2.516

Standard deviation of series: ± 0.0747

Probable error of mean: ± 0.010

Probable error of standard deviation: ± 0.007

Discs cut by the assistant

Mean weight of discs in grams: 2.513

Standard deviation of series: ± 0.0788

Probable error mean: ± 0.011

Probable error of standard deviation: ± 0.007

Difference of means: 0.003

Difference of standard deviation: 0.0041

Both these differences are less than the probable errors of the corresponding quantities, and therefore for tracings of this size there was no significant difference between the results of the two workers.

These discs were similar in size to the average discs representing ovular sections, and it was thought that when the discs cut were larger or smaller than this the personal error might have and influence. Two sets of 25 circles were therefore made by the author with a compass on cardboard, so that each pair of circles should have the same diameter, but should differ from every other pair in this respect. The circles of one set were cut out by the author, those of the other by the assistant, and after being dried the discs were weighed separately. The weights varied from 0.52 gm. to 5.30 gm., so that the range was great enough to compare with that of the tracings of ova. The weights of the members of each pair of corresponding discs were compared, and the results were as follows, the discs (cut by the assistant) being arranged in ascending order of magnitude.

Numbers of Discs cut by Assistant

	I (lightest)	II	III	IV	V (heaviest)	Total
Exceeding author's discs in weight	2	1	3	1	4	11
Below author's discs in weight	3	4	2	4	1	14
Total	5	5	5	5	5	25

The ratio 11 to 14 was shown by the chi square test not to differ significantly from the ratio 12.5 to 12.5 or 1 to 1 (chi square = 0.36;

n = 1; P = approx. 0.6); i.e., the assistant's cutting is equally liable to exceed or fall short of that of the author. Moreover, there is obviously no tendency for the difference between the two to go in any one direction according to the size of the discs concerned.

The conclusion is, therefore, that tracings cut by the assistant are directly comparable with those cut by the author.

(c) Influence of Coverglass Thickness

Whenever a micro-projector is used, as in the present investigations, without oil-immersion lenses, an error is introduced into the measurements because of the variation in the thickness of coverglasses on the different slides. The vertical Edinger apparatus, while convenient in many ways, was not found suitable for the use of immersion oil at the magnification employed, even when a mechanical stage was affixed so that the slides could be moved with precision or kept perfectly stationary at will. There must be many other investigations presenting similar difficulties, and it seems desirable, therefore, to give careful attention to the elementary optical principles that result in the introduction of the error and also to the determination of its magnitude.

In the micro-projection apparatus rays of light leave the object on the slide, pass through the coverglass and are refracted when they meet the air at the surface of the latter because the refractive index of glass differs from that of air. After passing through the objective and eyepiece of the microscope tube the rays form a real image on the screen. The effect of the coverglass is virtually to bring the object nearer to the objective than it would be if the coverglass were replaced by air, just as a coin at the bottom of a tumbler appears nearer to the observer when it is seen through water than when seen through air. If a thicker coverglass is placed over the object, the latter will be, virtually, nearer still to the objective, and consequently the final image will be altered in size.

To determine the magnitude of the error the following observations* were made.

* See note on next page.

Footnote to page 10B.

It would be possible from theoretical considerations to calculate the influence of coverglass thickness, but with such a system of lenses the calculation would be very complicated.

A fine mark was made with India ink on a microscope slide and covered for protection by a thin layer of flexible collodion. The thickness of a number of coverglasses was measured by the micrometer sold for that purpose and five of the coverglasses were selected so as to give a range of thickness from 0.09 mm. to 0.235 mm. The India ink mark was covered by Canada balsam and on it was placed the thinnest coverglass. The slide was laid in the usual position on the stage of the micro-projector, and 25 tracings were made to show the breadth of the projected image of the mark at the usual magnification of approximately 1500, the lenses and distances being as in the investigations upon the ova. Between each tracing the image was thrown out of focus and then refocussed. From the 25 readings the average breadth was obtained. The other four coverglasses were in turn used in the same way. The data are recorded hereunder.

Coverglass Thickness in mm.	Reading on Correction Collar of Objective	Mean Breadth of Mark in cm.	Probable Error of Mean
0.090	10	7.784	0.0135
0.090	17	7.854	0.0129
0.115	11.5	7.672	0.0147
0.115	17	7.916	0.0164
0.15	15	7.776	0.0140
0.15	17	7.954	0.0143
0.20	17	7.924	0.0200
0.20	20	7.938	0.0193
0.235	17	7.996	0.0181
0.235	20	8.032	0.0104

Attention was paid chiefly to the readings taken with the correction collar at the mark 17, for in tracing ovular sections this was the position at which the collar was always kept. It will be observed that in general the thicker coverglass gave the broader image.*

* It may be noted that, with a simple convex lens giving a real image, the reverse appears to be the case.

It was shown by the probable errors that the differences were significant. The exception to the rule just stated was that the coverglass of thickness 0.20 mm. gave a smaller reading than the coverglass of thickness 0.15, but in reality there was no significant difference here, and the fact that there was not a significant increase in size with the thicker coverglass may be have been due to the fact that the correction collar for both was at 17, i.e., too high for the coverglass 0.15 and too low for the coverglass 0.20, for it may further be noted that, the higher the reading on the collar, the greater the breadth of the image.

From the data the following values were calculated (correction collar at 17).

Differences in breadth (cm.) due to coverglasses in ascending order of their thickness: + 0.06, + 0.04, -0.03, + 0.07

Each of these changes in breadth was divided by the increase in coverglass thickness responsible for it, e.g., 0.06 was divided by 0.115-0.090, i.e., 0.025. The resulting four items were added together and the mean value 1.225 was obtained, i.e. the mean increase in breadth in cm. caused by 1 mm. of increase in thickness of coverglass. The experiments were, however, concerned with much smaller increases in coverglass thickness, and the change is preferably expressed as 0.01225 cm. per 0.01 mm. increase in coverglass thickness. The mean breadth of the line for the five coverglasses was 7.889 cm. (correction collar at 17). The mean increase in breadth or linear measurement, just found, for each 0.01 mm. of added coverglass thickness was expressed as a percentage of this mean breadth, the result being 0.155 percent.

There was no way of properly determining the thickness of the coverglasses used on the specimens from which the tracings had been made, but all the specimens had been covered by thin coverglasses and therefore the subsequent calculations were based on the assumption that the cover-

glasses corresponded to those of no. 1 thickness, which in the catalogues of Zeiss, Leitz, the Central Scientific Co. and the Spencer Lens Co. were recorded as varying from 0.13 mm. to 0.17 mm. Since it was recognized that the variation might be greater than this (see, for example, "Watson's Microscope Record", Sept. 1930, p.29), the range was considered as 0.12 to 0.18. The difference between the extremes is therefore 0.06 mm. , and , if the two specimens were compared, one mounted under the thinnest and the other under the thickest of these coverglasses, the difference in linear measurement due to coverglass thickness alone would, according to the results just reached, be nearly one percent of the mean — a result which should be borne in mind in all such work. In other words, a magnification of 1500 with the thinner glass might be 1515 with the thicker.

The next step in the application of these results to the specimens investigated was the conversion of this effect on linear measurement to an effect on volume. If it had been possible to incorporate variation in coverglass thickness with the tests of the influence of tracing, weighing, etc. (subsection (a) above), the result would have shown directly the possible and probable effects of all the experimental errors in the investigation, but to apply and remove several different coverglasses to the sections used in the test would have seriously jeopardized valuable material. Therefore the results obtained from measuring the India ink mark had to be utilized.

In introducing the variation due to coverglass thickness, it should be remembered that in tracings of the sections of ova, only the length and breadth are affected by coverglass thickness, for the estimation of thickness of the sections is made by reference to the microtome and, in the case of the end sections, by the fine adjustment of the microscope with oil immersion.

The ovum can be considered as a sphere of volume $V=\frac{4}{3}\pi r^3$, where r is the radius. When the length and breadth of a section are altered, r will become r_1 and the new volume, V_1 will be $\frac{4}{3}\pi r_1^2 r$, one of the original r's being retained because the thickness measurements of the sections are unaltered by the change in coverglass thickness. V_1-V will therefore be $\frac{4}{3}\pi r(r_1^2-r^2)$.

The coverglass range was taken as 0.12 mm. to 0.18 mm., with a middle value at 0.15 mm. Each 0.01 mm. increase in coverglass thickness means an increase of 0.155 percent in linear measurement. It may be assumed that the original volume was determined from a section with coverglass 0.15 mm. thick. Then the greatest possible increase would be $(0.18-0.15)\times 0.155 = 0.47$ percent or nearly ~~0.05~~ 0.5 percent. r_1 would then be $(r+\frac{0.5r}{100})$, and V_1-V would be $\frac{4}{3}\pi r(\frac{0.25r^2}{10{,}000}+\frac{r^2}{100})$. The first item in the bracket is so small that it may be neglected, and the formula becomes $V(\frac{1}{100})$, i.e. the volume is increased by one percent when a coverglass of 0.18 mm. is substituted for one of 0.15 mm. Roughly, the difference in volume if a coverglass of 0.18 mm. were substituted for one of 0.12 mm. would be 2 percent.

It may still be assumed that the tests for tracing-variations, cutting-variations, etc. were carried out on the sections covered with a coverglass of 0.15 mm., for, even if this were not true, the changes calculated would be relatively the same, and it is only relative changes that concern this investigation. If the coverglass were 0.18 mm. instead of 0.15 mm., the new volume would be 0.000296 c.mm. (i.e. the original volume) plus $\frac{1}{100}$ of 0.000296, i.e. 0.000299 c.mm.

The ~~result~~ value 0.000296 was obtained as the mean of a series of observations scattered symmetrically (in a normal frequency curve) on either side of the mean. From the standard deviation of this series it would be possible to find the range of variation, for the extreme

values are obtained by taking roughly three times the standard deviation and adding the result to or taking it from the mean. In the same way, if a set of measurements had been taken with the thickest coverglass (0.18 mm.) in position, the highest possible value for the series could be calculated. As has been said, it was not possible to make these observations directly, but the mean of this series is known, for it has been shown to be one per cent in excess of the mean actually obtained i.e. it is 0.000299 c.mm. The standard deviation is not known, but it is fair to assume that the variation in this series of measurements would have been the same as in the set actually taken, that is the coefficient of variation would have been 2.7 per cent. In other words ,

$\frac{\text{Standard deviation}}{\text{mean}} \times 100 = 2.7$. Therefore standard deviation $= \frac{2.7}{100} \times 0.000299$. Three times the standard deviation is therefore 0.0000242. When this is added to the mean the extreme possible observatin is found to be 0.000323. But, since the coverglass (0.18 mm.) is the thickest to be employed, this observation would form the highest limit of a series of observations built up by employing coverglasses of different thicknesses on the sections of the same ovum. If such a series of observations had been made it would have been necessary to select a series of coverglasses of regularly graded thicknesses, since there are equal chances, so far as is known, of a specimen being mounted under a thin or a thick coverglass within the limits 0.12 to 0.18 mm. The mean volume of the ovum would in that case be the same as in the actual test, i.e. 0.000296 c.mm., the extreme highest value possible would be 0.000323 c.mm., giving a difference from the mean of 0.000027. Therefore the standard deviation would have been 1/3 of this, i.e. 0.000009. The calculation involving the lower limit would give similar results, and it is sufficient for the present purpose to utilize the upper limit only.

It has therefore been shown that, if a test had been made to estimate the error due to variation in cardboard, tracing, cutting, weighing and also variation in coverglass thickness, the standard deviation of a series of mean 0.000296 c.mm. would have been 0.000009, and the coefficient of variation would have been 3.1 per cent instead of 2.7 per cent — the coefficient of variation where the coverglass thickness differences were not involved.

It is desirable at this juncture to point out a certain difference that existed between the conditions under which all the tracings of ova were made and those under which the experiments on coverglass thickness were carried out. In the latter/ the slides were supported, with a view to prevent the coverglasses from touching the stage of the microprojector. The coverglasses on the ova were, however, so large that when the slide was placed on the stage the coverglass had to come directly in contact with the latter, and therefore a thicker coverglass would remove the object farther from the lens than would a thinner coverglass, this effect being partly counteracted by the refractive properties of the coverglass, so that, if an increase in coverglass thickness, t, were introduced, the object would be virtually $\frac{2t}{3}$ units farther from the objective (1.5 being roughly the refractive index of the glass). This reversal of the process that has been so far discussed does not, however vitiate in any way the argument, the only important facts being the change in size and the relative amount of this change, for, wherever an increase in size would occur in the tests made on the coverglasses, a decrease would occur in the work on the ova, and vice versa.

In the subsequent records, wherever there is found an apparently significant difference between two sets of ovum volumes, it is necessary to question whether the difference exceeds that due to the influence of all the experimental errors combined, represented here by a coefficient of variation of 3.1 per cent, and a probable error of 0.000002 for a mean of 0.000296.

TABLE I Ovum Volumes and Associated Data.

Abbreviations

Z., Zenker
M., Mann
M.F., Ma^nn with formol
Per., Perenyi
Fl., Flemming
prn., pronucleus (-ei)

Serial no. of Ovum	Animal	Side (Right or Left)	Fixative	Vol. of Ovum (units)	Nuclear Material with combined area of pronuclei if central (μ^2)
3	FDA33-15	L.	Z.	5006	prn.cent. 2248
4	"	L.	Z.	3912	" 2453
5	FDA38-15	L.	Z.	4630	" 2939
7	PGA3	L.	Z.	5437	prn.peripheral
8	"	L.	Z.	5778	prn. subcentral
9	"	L.	Z.	5610	prn.periph.&subcent.
10	"	L.	Z.	6339	prn. peripheral
11	"	L.	Z.	5807	prn. peripheral
14	FGB4	R.	Per.	6830	prn. central 2002
15	"	R.	Per.	5909	prn. cent. and periph.
16	"	R.	Per.	5294	prn.cent. 1594
18	FGB5	R.	Z.	6081	prn.cent. 2995
22	"	R.	Z.	5466	prn.cent. 3042
24	PDC	R.	M.F.	3436	-
25	"	R.	M.F.	3622	-
26	"	R.	M.F.	3221	-
27	"	L.	Per.	5365	2nd polar spindle
28	FDG	L.	Per.	4750	prn. peripheral
29	"	L.	Per.	4333	" "
30	"	L.	Per.	4739	" "
31	"	L.	Per.	4587	?
32	FDI	L.	M.F.	2933	2nd polar spindle
36	"	L.	M.F.	3923	prn. peripheral
37	"	L.	M.F.	4141	2nd polar spindle
38	"	R.	Per.	5519	" " "
41	FDJ	R.	M.F.	2973	-
43	"	L.	Per.	5992	prn.periph.&subcent.
44	FDK	R.	Per.	3867	subcent. prn.
46	PDA5	R.	Per.	3805	prn.cent.
48	"	R.	Per.	3747	prn.periph.
49	FZ1.19	-	Z.	3564	2nd polar spindle
50	"	-	Z.	3473	" " "
51	"	-	Z.	5283	" " "
52	FM1.18	-	M.	3475	" " "
53	"	-	M.	3283	" " "
54	"	-	M.	2653	" " "
57	FH21	-	Z.	4639	prn.cent. 1973
58	"	-	Z.	4351	" " 2255
59	"	-	Z.	3836	" " 2230
60	"	-	Z.	4621	" " 2514
62	FG9	-	M.	3208	" " 1678
63	"	-	M.	2035	" " 1383
64	"	-	M.	2073	" " 1546

TABLE II (continued).

Serial no. of Ovum	Animal	Side (Right or left)	Fixative	Vol. of Ovum (units)	Nuclear Material with combined area of pronuclei if central (μ^2)
69	FGB5	L.	Fl.	4342	prn.cent.
72	FGB4	L.	Fl.	4084	prn.subcentral
73	"	L.	Fl.	4306	prn.cent.
75	"	L.	Fl.	3977	prn.cent.
76	FGA6	R.	Fl.	4964	prn.subcentral
77	? GB3	? R.	Fl.	3410	prn.cent.
79	PDA5	L.	Fl.	5811	prn.cent.
80	"	L.	Fl.	5205	prn. ? cent.
81	PDA31	L.	Fl.	5539	prn.cent.
83	PDA5	L.	Fl.	5829	prn.cent.
84	"	L.	Fl.	5136	prn.cent.
85	F26.7	R.	Per.	4202	prn.cent. 2798
86	"	R.	Per.	5002	prn.cent. 2839
87	"	R.	Per.	3787	prn.cent. 1690
88	"	L.	Z.	3582	prn.cent. 2962
89	"	R.	Per.	3584	prn.cent. 2571
90	"	R.	Per.	3557	prn.peripheral
91	"	R.	Per.	2920	prn.peripheral
92	"	R.	Per.	4162	prn.cent. 2195
93	"	R.	Per.	3907	prn.cent. 1811
94	"	L.	Z.	4133	prn.cent. 1722
95	"	L.	Z.	4485	prn. cent. 2286
96	"	L.	Z.	2930	prn.cent. 3072
97	"	L.	Z.	4090	prn.peripheral
98	"	L.	Z.	3397	prn.cent. 3394
99	"	L.	Z.	4750	prn.cent. 3259

Notes: The animals, except 26.7, are from Professor Robinson's collection in Edinburgh University. Data regarding the weights, ovary volumes, etc., of most of them appear in his monograph (1918).

The unit of ovum volume adopted for convenience is $\frac{1}{10,000,000}$ c.mm.

For the mean volume and other statistical functions reference should be made to the section on the Influence of Fixatives (p.).

The pronuclear areas are repeated from a recent article (Mainland, 1931b). These values in the case of Flemming-fixed ova could not be accurately calculated, for most such specimens were merely tinted with eosin.

RESULTS OF VOLUME CALCULATIONS

After the volumes had been calculated, the units employed were for convenience changed from cubic mm. to $\frac{1}{10,000,000}$ of a cubic mm., i.e., the first four significant figures were taken from the volumes in cubic mm. The results expressed in this form are given in table I, arranged according to the serial number of the ovum and accompanied by references to the animals from which the ova were obtained and data regarding the sizes of the pronuclei, etc.

FREQUENCY DISTRIBUTION

For a first analysis, the results may be grouped according to frequency as follows:

Volumes (units)	Number of ova
2001 - 2500	2
2501 - 3000	5
3001 - 3500	8
3501 - 4000	14
4001 - 4500	11
4501 - 5000	8
5001 - 5500	9
5501 - 6000	9
6001 - 6500	2
6501 - 7000	1
7001 - 7500	-
7501 - 8000	-
8001 - 8500	-
8501 - 9000	-
9001 - 9500	1
Total	70

There is observable a tendency for the numbers to rise fairly quickly as the volumes increase and then to fall much more gradually as the volumes still increase. With so few data further analysis of the curve is not desirable. The largest ovum in the series was so large compared with the rest that it was excluded from further treatment. Even when it had been omitted, however, there still remained ova that were three times the size of others. The question that naturally arises is whether or not these great differences are found in nature or are

due to technique. A partial answer to that question is found in the next section.

INFLUENCE OF FIXATIVES

The ovum volumes were arranged in groups according to the fixatives as follows:

Fixative	Flemming	Mann	Mann with Formol	Perenyi	Zenker
No.of ova	11	6	7	21	24
Mean vol.	4,782.1	2787.8	3463.7	4,564.6	4633.6

It appears that the mean volumes of the ova fixed in Flemming's, Perenyi's or Zenker's fluids resemble each other much more than they resemble the volumes of ova fixed in Mann's fluid, with or without formol. To prove whether there is a significant difference, however, a statistical test has to be applied. The test is an analysis of variance, whereby it is determined whether there is more variance among the individual ova within the classes (Flemming-fixed, Mann-fixed, etc.) or between the classes. If the variance within the classes were as great as that between them, the apparent difference in the means would be unreal. The method of analysis used was that of Fisher(1930,p.196). The result was: $z = 1.055$, $n_1 = 4$, $n_2 = 64$, where z is a quantity indicating the excess of the variance between the classes over the variance within the classes; n_1 and n_2 are based on the numbers of the specimens and classes. Reference to Fisher's table(1 per cent points of z) shows that the value of z is highly significant, for there are hundreds of chances to 1 against such a value of z being due to accident. There is therefore a real difference between the volumes of the ova fixed by either Flemming, Perenyi or Zenker on the one hand and the volumes of the ova fixed by Mann on the other.

Inspection showed that this association between Mann's fixative and the smallness of the ovum was not an apparent association due to the chance selection of ovaries of certain animals for fixation by the differ-

ent methods.

The variations shown below indicate clearly that experimental errors, including those due to coverglass thickness, are not great enough to account for the difference between the volumes where the fixatives are different.

The mean volume of the ova fixed in the common fluids(Flemming, Perenyi and Zenker) was for 55 ova: 4659.1 units, i.e. 0.00046591 c.mm.
Probable error of mean: ± 82.15 (i.e. ± 0.000,008,215 c.mm.)
Standard deviation of series: ± 903.27
Coefficient of variation: 19.4 per cent.

It will be observed how much this variation exceeds that due to the technique alone, where the coefficient of variation was 3.1 per cent, and the probable error (in the selected units) was ± 12.0 (see above, p. 10H).

Recently in a study of the polar bodies of the ferret(Mainland, 1931c) it was shown that, roughly, the number of sections per ovum was 10. At 10 microns for each section, the depth of an ovum is 1/10 mm. If the ovum is considered spherical, the volume,calculated from the formula $\frac{4}{3}\pi r^3$, with r = 1/20 mm., will be 0.000,5 c.mm. This is very nearly the same as the mean volume just mentioned.

In order to show whether there was still further difference in ovum due to difference in fixatives, the following data were secured:

Fixative	Flemming	Perenyi	Zenker
Mean volume of ova	4,782.1	4,564.6	4,633.6
Standard error* of mean	± 231.8	± 208.0	± 123.8

* This quantity has the same function as the probable error, and is roughly 1½ times the latter.

Differences between means

Flemming and Perenyi: 217.5

Flemming and Zenker: 148.8

Zenker and Perenyi: 168.7

Even without calculating the standard errors of the differences, one may see that there is no significance in any of these differences. The three fixatives concerned are, so far as can be shown with the present data, similar in their effects on ovum size.

RELATIONSHIP BETWEEN VOLUME AND DEVELOPMENT

The index of ovular development is the condition of its nuclear material, i.e., whether this is in the form of second polar spindle, peripheral pronuclei or central pronuclei. The relationship between this and the volume of the ova was shown as follows (only Flemming-, Perenyi- and Zenker-specimens were used, since these alone were comparable with each other):

Nuclear material	2nd polar spindle	Pronuclei peripheral	Pronuclei central
Number of ova	5	10	32
Mean volume	4,640.8	4571.9	4,523.2

There appeared to be a slight tendency for the volumes to diminish as development progressed, but the numbers of ova were too few to allow this to be proved. There was, moreover, no significant difference between the two extreme volumes (4,640.8 and 4,523.2)

RELATIONSHIP BETWEEN OVUM VOLUME AND PRONUCLEAR SIZE

In a recent investigation (Mainland, in preparation) there was shown the great variation in pronuclear size among the central pronuclei of the ferret, the size being expressed by the areas of surface of the two pronuclei added together, since area was a less variable

quantity than the volume. It was further shown to be impossible to correlate these variations with the structure of the central pronuclei, and it was then suggested that the size of the ovum might have to be taken into account, since the pronuclear size might be closely correlated with this, and, when allowance was made for that correlation, it might be possible to show some association between the size of the central pronuclei and their structure. Now that the ovum volumes have been estimated it is possible to test this suggestion. The correlation coefficient between volume of ova and pronuclear combined area (23 ova) is -0.072, but its standard error is $\pm$0.2, and therefore there is no significant correlation between the two. This confirms the author in his previous conception regarding pronuclear development in the ferret. The central pronuclei, so far as can yet be shown, alter structurally independently of size. Some large pronuclei are at the stage, structurally, of some much smaller. Moreover there is little association apparent between the structural changes themselves (Mainland, 1930).

RELATIONSHIP BETWEEN OVUM VOLUME AND ANIMAL

The ovum volumes were arranged as shown below according to the animal from which the ova came, only Flemming-, Perenyi- and Zenker-fixed ova being used. The data are placed as follows:

Reference number or letters of animal;

Variety of animal (white ferret, F; or "polecat" ferret, P);

Number of ova;

Mean volume of ova.

26.7	FDK	GB 4	DA 5
F	F	F	P
15	1	6	6
3899.2	3867.0	5066.7	4922.2
FZ 1.19	FH 21	GA 3	DA 31
F	F	P	P
3	4	5	1
4106.7	4361.8	5794.2	5539.0
GA 6	GB 5	PDC	DA 33
F	F	P	F
1	3	1	2
4964.0	5296.3	5365.0	4459.0
FDJ	DA 38	FDG	FDI
F	F	F	F
1	1	4	1
5992.0	4630.0	4602.3	5519.0

To these data the analysis of variance test was applied, in order to show whether there was more variation in ovum volume from animal to animal than among the different ova of each animal. In other words, the question was, do some animals produce large ova and some small ova? The result of the analysis of variance was: $z = 0.5207$, $n_1 = 15$, $n_2 = 39$. Fisher's (1930) table showed that the chances of this value of z being due to accident were less than 1 in 100. z indicated the excess of the variations of volume from animal to animal over the variations among the individual animals. Therefore there was a highly significant difference between the animals in respect of ovum volume.

The next problem was to examine possible causes of this lack of homogeneity. Only the ova fixed in Flemming, Perenyi and Zenker had been used in this investigation, and it has been shown above that there is no significant difference between these fixatives in respect of ovum volume. The effect of coverglass thickness could only be dismissed inferentially. There was not sufficient resemblance in volume among the ova prepared in any one year or on any one slide.

to indicate that the cover glass thickness was responsible for the variation from animal to animal. Since the technique appeared not to be responsible for this variation, an attempt was made to prove relationships between ovum volumes and other factors in which the animals might differ from each other.

RELATIONSHIP BETWEEN VARIETY OF ANIMAL AND VOLUME OF OVUM

Some of the animals from which the ova were obtained were white ferrets and others were dark or "polecat" ferrets. A comparison was made between the mean volumes of the ova obtained from these two varieties of animal, only the Flemming-, Perenyi-and Zenker-fixed ova being used.

Mean Volume of ova obtained from white ferrets: 4448.57 (42 ova)

Probable error of mean: ± 88.0 Coefficient of variation:20.0;prob.error:1.5.

Mean volume of ova obtained from"polecat" ferrets: 5339.07 (13 ova)

Probable error of mean: ± 137.0

Difference between means: 890.5

Probable error of difference: ± 162.8

Since the difference was much more than three times its probable error, one would feel justified in asserting that it was significant, but the fact that the "polecat" ova were only 13 in number makes it necessary to apply Fisher's t-test. The results of this were highly significant ($t = 3.34$; $n = 53$; P is less than 0.01). There was therefore a very strong suggestion that ova from"polecat"ferrets were larger than those from white ferrets, for the difference was much greater than one that could be caused by the experimental errors which gave a variation coefficient of 3.1 per cent and a probable error of 12 (see above , p. 10H). It was thought possible, however, that the "polecat" ferrets, being only four in number, might by chance have possessed in common some characteristic responsible for the ovum size, apart from their racial similarity. When the mean volume

of the ova per animal was calculated, it was not possible to show any significant difference between the means obtained in the two classes -- white ferrets and "polecat" ferrets. It is regrettable that there is not in existence a sufficiently large collection of "polecat" ferret ova to prove the point conclusively, but the suggestion of a difference remains very strong.

RELATIONSHIP BETWEEN WEIGHT OF ANIMAL AND VOLUME OF OVUM

The volumes of the ova were arranged in groups and the weights of the animals (in ounces) were arranged similarly. Table II shows this grouping with the corresponding numbers of ova, so as to indicate the relationship between the volumes and the weights. The coefficient of correlation between ovum volume and animal weight was -0.23, but its probable error was ± 0.087, and therefore it was not quite significant. Hence it was not possible to show any correlation between ovum volume and the weight of the animals.

RELATIONSHIP BETWEEN VOLUME OF OVARY AND OVUM VOLUME

The date recorded by Robinson (1918) included the volumes of the ovaries from which many of the specimens used in the present investigation were obtained. It was therefore possible to arrange 37 of the ova according to their volumes and according to the volumes of the ovaries from which they came, thus forming a correlation surface. Inspection of this arrangement, without calculation of the coefficient of correlation, was sufficient to show that there was no significant correlation between ovum volume and ovary volume. So far as the present data are concerned, this is as far as can be gone, but it should be pointed out that the final relationship between ovum volume and ovary volume could only be reached if consideration were made of the numbers and volumes of the large follicles and corpora lutea present in the ovaries, for one or two more or less of these in an ovary makes a considerable difference to its volume. If, however, a very large number of specimens were available it might be possible to neglect this factor, because in such a large number there would be equal chances for an ovary with many large follicles (or corpora lutea) to have large ova or to have small ova, and any more fundamental relationship between ovum volume and ovary volume would emerge into view. It is, of course, impossible to state what the requisite number of specimens would be.

TABLE II. Relationship between Volume of Ova and Weight of Animal (Numbers of Ova)

Volume of Ovum (selected units; middle values of classes)

Weight of Animal (oz.; middle values)	2750	3250	3750	4250	4750	5250	5750	6250	6750	Total
13.5			1	3	3	1	1		1	10
14.5					1					1
15.5	1						1			2
16.5			1			1				2
17.5		2	1	1		2		1		7
18.5					1					1
19.5						1	3	1		5
20.5	1		3	1		2	3			10
21.5							1			1
22.5										—
23.5										-
24.5										-
25.5	2	1	5	5	1	1				15
Total	4	3	11	10	6	8	9	2	1	54

CONCLUSION

A survey of the preceding records will probably suggest that a great amount of labour has had meagre results, and those results chiefly negative. It may be desirable,,therefore, to indicate in general what has been achieved.

In the first place, there has been evolved and tested a method for determining in mounted sections the volumes of ova. This method can be extended to ova of other animals, including Invertebrates, and it can also be extended to investigations of structures other than ova, but of similar shape. This work may thus be the starting point of various researches, such, for example, as an investigation of the relationship between the growth of the ovarian follicle and the growth of the ovum contained in it.

In the second place, there has been shown the greatness of the ordinary variation between ova. When the difference in the influence of fixatives has been eliminated and the differences due to racial type have been excluded,the coefficient of variation of volume in white ferrets is 20.0. per cent, ± 1·5. The establishment of this can form the basis for comparison with ova of other animals, with the sizes of adult animals and with the sizes of organs. One or two such comparisons may be made here.

From Robinson's (1918) data there have been calculated the mean weight in ounces of mature female ferrets and the corresponding coefficient of variation, viz, mean: 17.0; coefficient of variation: 32.7 ± 3·1. The variation in weight between adult females is therefore greater than that between the ova. Unfortunately, it was not possible to determine the ages of these animals, and it is presumably the variation in age that caused their great variation in body weight. More detail is obtained from Donaldson's (1924) table 121 (King's data), concerning white rats. This table shows that the coefficient of variation in of body weight

each of 10 consecutive age periods from 90 days to 485 days was very similar, varying from 12.6 to 15.0 for males. It is probable that ferrets do not vary much more than this when allowance is made for breed and age. Moreover Donaldson's (1924) table 142 (Jackson's data) shows that the coefficient of variation in total body weight of rats at birth was 12 per cent. It is perhaps of interest to compare these variations with that found in human stature. As an example, from Grant's (1929) data the coefficient of variation in stature among Island Lake Indians has been found by combining the information relating to males with that relating to females, since the ferret ova studied contained potential females and potential males. The results were: for individuals of ages 29-59, mean: 162.3 cm.: coefficient of variation: 4.9.

Further comparisons of like nature are desirable, but these instances will suffice to show the tendency for the ova to vary more in size than adult individuals, and more also than newly-born individuals. Taking into consideration the great amount of prenatal mortality (see, e.g. Robinson, 1921) one may suggest that the ova that most widely differ from the average are those that die before the end of the gestation period, for the variation between animals at birth appears less than that between the ova at ovulation.

SUMMARY

These investigations concern the volumes of ova of the ferret obtained after ovulation and before segmentation, the material being in the form of paraffin sections stained and mounted. A brief list of references is given to illustrate chiefly the types of method employed in cell measurement.

In the method employed here the images of each section of the ova were projected on to cardboard at a linear magnification of 1,494 ± 3.31, the tracings were cut out, dried and weighed. The specific density of the cardboard and the thickness of section (cut very consistently at 10μ) were then employed to calculate the volumes of all the sections of the ova except the rounded end-sections, which were considered as segments of a sphere with one base, the height being obtained directly by the fine adjustment of the microscope.

The error due to variability in tracing, cutting, weighing, cardboard density, and measurement with the fine adjustment were represented by a coefficient of variation of 2.7 per cent. The cutting out of tracings by another worker did not alter this variability. Alteration of coverglass thickness from 0.12 mm. to 0.18 mm. (i.e about the range of no. 1 coverglasses) altered the linear measurement (or magnification) by about one per cent, and the volume measurements by about two per cent. The total coefficient of variation due to all the experimental errors was calculated as 3.1 per cent.

The volumes of 70 ova were considered. When these specimens were arranged in ascending order of volume, there was a tendency for the curve of frequency to rise fairly quickly and then fall more gradually.

The ova fixed in Mann's fluid, with or without formol, had a significantly smaller average volume than those fixed in Flemming's,

Perenyi's or Zenker's fluids. These fixatives did not differ significantly from each other in their effects on size, and the mean volume of the ova fixed by them was approximately 0.0005 c.mm.

There was no significant change in average ovular size between the second polar spindle and the stage of central pronuclei.

There was no significant correlation between ovum volume and the size of the central pronuclei.

A highly significant difference existed between animals in respect of the volumes of the ova that they produced.

There was a very strong suggestion that "polecat" ferrets produced larger ova than white ferrets, but this was not proved conclusively because of the insufficiency of the numbers of "polecat" ferrets.

There was no definite correlation between the ovum volume and the weight of the animal producing the ovum, but there was a possible tendency towards a negative correlation.

It was not possible to show with such small numbers a correlation between ovum volume and the volume of the ovary from which the ovum was liberated.

The positive value of these investigations lies partly in the establishment of a tested method of volume determination that can be applied to other ova in mounted sections and to other similar structures.

One of the most important values established is the coefficient of variation in ovum volume at ovulation, *i.e.*, 20.0 per cent. This is greater than the size variation among adults of various species (when allowance is made for age), and greater also than the variation among rat foetuses at birth. Hence it is suggested that the ova that die during gestation are those that vary most from the average in size.

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A STUDY OF THE SIZES OF NUCLEI IN OVARIAN STROMA

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A STUDY OF THE SIZES OF NUCLEI IN OVARIAN STROMA

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FOUR FIGURES

INTRODUCTION

In histology two main routes of advance are open. The first is the change and improvement of preparation, fixation, and staining of tissues. The second, less used route is the application of strict quantitative methods to specimens prepared by ordinary technique. This second method has to its disadvantage great laboriousness, the natural antipathy of many toward mathematical methods, and the distrust with which statistical methods especially are viewed. On the other hand, this method is technically simple, consisting chiefly of careful measurement. The statistical treatment of the data may be mastered without great difficulty, and the distrust of statistics diminishes as one becomes familiar with the principles. Moreover, for any method in human histology to become of diagnostic or prognostic value to the pathologist or the surgeon, it is essential that it be easily applied. The measurement of cells or nuclei in a paraffin section of a formalin-fixed tissue, stained by haematoxylin and eosin, is much more convenient in this respect than complicated processes of fixation and staining. The final and perhaps most convincing argument in favor of an exact quantitative method is its precision. Minute differences of color, shading, and shape are hard to distinguish, and the recording of them is

influenced by personal factors in the observer. Even comparison of the sizes of objects without actual measurement becomes unreliable when many tissues have to be examined by different observers.

Such arguments point to the desirability of more thorough quantitative investigations, with regard at least to actual measurements of length, breadth, and area of cells and nuclei, and probably with regard also to colorimetric tests, which have not been used in histology as much as appears possible. Considerations of this kind prompted the author to carry out the examination of human ovarian stroma here recorded. While one of the objects was the observation of possible interstitial cells, the primary purpose was the discovery of the type of results to be obtained and the establishment of a method simple enough to be used in further investigation with regard to changes incident to the menstrual cycle, age, and disease.

MATERIAL AND METHODS

Among the specimens chosen for investigation was a section of human ovary from the collection in the Department of Anatomy of Manitoba University. This section was a paraffin section (stained with haematoxylin and eosin) of the formalin-fixed ovary of an unmarried woman of twenty-three who died in convulsions during tonsillectomy. Death was apparently caused by hypnotics and a local anaesthetic. This ovary was chosen, first, because of the cause of death and the absence of serious disease, and secondly, because, in spite of the presence of some large cysts, it appeared to be a well-fixed, well-stained, typical ovary. The autopsy notes recorded a thin-walled cyst about the size of a tangerine orange in this ovary, which, therefore, must not be considered normal, even although the part investigated appeared so. This does not, however, vitiate the results, for such an investigation may start as well from a pathological specimen as from an apparently healthy one.

The section was set up under immersion oil in a horizontal microscope and the image was projected by means of a carbon-arc light onto a cardboard screen, 3 or 4 feet from the eye-piece of the microscope. The lenses used were a Zeiss apochromatic objective (numerical aperture 1.30) and a Zeiss compensating eye-piece (10×). The position of all the apparatus was left constant throughout the series of measurements, which occupied several days. Measurements were recorded of 1000 nuclei in the cortical stroma, the section being moved by a mechanical stage, so that no field was examined twice. The measurements were commenced on nuclei near the germinal epithelium, and from there the investigations were carried deeper and deeper into the cortex. Five different times in the examination of the 1000 nuclei such a commencement at the germinal epithelium was made. All the stroma nuclei met were measured, except such as focused badly or were so bent as to introduce serious errors into the measurements. The nuclei were focused so as to give the best definition and at the same time a maximum reading of the longitudinal or transverse[1] axis, as the case might be. The measurements were taken by means of fine-pointed screw dividers which were placed on a steel scale graduated in half-millimeters, the scale being set up under a binocular microscope and strongly illuminated. The magnification used was 1824, approximately, but at this magnification the ruling on even a fine stage micrometer appears coarse, and therefore, to facilitate the resetting of the apparatus in future, the image of a finely drawn mark on a microscope slide was copied onto a card. In setting up the apparatus again, adjustments can be made until the new image of this mark coincides with the image previously recorded on the card. In all subsequent records the sizes in millimeters of the magnified images are given.

The observations made were: 1) Longitudinal axis measured to the nearest one-tenth of a millimeter; 2) transverse

[1] Where a localized swelling of the nucleus was found, it was considered best to give a reading for the transverse axis so as not to include the swelling.

axis, i.e., at right angles to the long axis, measured with the same degree of accuracy; 3) depth of staining, expressed as 'pale,' 'medium,' or 'dark'; 4) structural appearance, expressed as 'uniform,' 'mottled,' or 'shaded'; 5) outline, expressed as 'regular' or 'irregular.'

From the longitudinal and transverse axes of each nucleus there was calculated the index:[2] $\frac{\text{transverse axis}}{\text{longitudinal axis}}$.

An investigation conducted on these lines may appear to have several serious sources of error. In the first place, it may justly be criticized, in that, owing to the varying obliquity of the nuclei, the so-called longitudinal axes are not true lengths of the nuclei; that the 'length' would seem to vary on that account, and that the average or mean length would be too small. It should be pointed out that the assumption on which the work was based was that the nuclei were arranged in random manner at all possible angles to the plane of the section—an assumption that was borne out as far as could be ascertained by inspection. If the nuclei were all of the same length, there should be approximately the same number of nuclei in all the different classes of longitudinal axis. As will be shown below, this was not the case.

The second criticism is that the transverse measurement is not one of width of a nucleus always, but of width in some and of thickness in others, according to the position in which the nucleus lay. It is to emphasize these distinctions that the terms 'longitudinal axis' and 'transverse axis' have been applied. In the case of the transverse axis also, the assumption is that when so many nuclei are examined the nuclei are placed at random in all different positions, so that as many widths as thicknesses will have been recorded as transverse axes.

In connection with any such criticism, the principles underlying this type of investigation should be definitely formulated. In the first place, the investigation is not concerned with actual length or breadth or thickness, but with quantities related to these, and the quantities in question must be such

[2] It appeared unnecessary to multiply this index by 100, as is the custom in anthropological work.

as to afford a means of distinguishing, in the present instance, one type of nucleus from another, and, later on, distinguishing one ovary from another. Secondly, the mode of treatment of the data must be essentially statistical, so that variations due to 'chance' may be allowed for, and significant differences may emerge. The statistical methods used in this paper have not been explained in detail, but the results have been set forth in technical and also in less technical language. References have been made to a description of the methods used, commonly those of Fisher ('30).

TYPES OF STROMA CELL AND NUCLEUS

It was noted as the examination proceeded that most of the nuclei met belonged to the class of 'fibroblastic' nuclei, fusiform or cylindrical in shape. These are the nuclei commonly described in the cortical stroma (Schafer, '20; Maximow and Bloom, '30, etc.). There were also seen, however, flat, faintly stained nuclei, frequently of mottled appearance and always elliptical in shape, i.e., broader for their length than the fusiform or cylindrical nuclei. To these the term 'lamellar connective-tissue' nuclei may be provisionally applied. In addition to these, a few cells were seen of spherical shape, resembling some of the white cells of the blood. No cells were seen that suggested the interstitial cells of such animals as the rabbit or the ferret. This finding is therefore in agreement with the statement of Corner ('28) that there are no true interstitial cells in the human ovarian cortical stroma.

It should be noted that the differentiation of fibroblastic and lamellar connective-tissue nuclei has been used for convenience of description without any assumptions as to the relationship or mutual independence of the two classes.

TABLE 1

Frequency distribution of nuclei according to sizes of longitudinal and transverse axes. (The 'class values' are assumed values used for ease in calculation.)

LONGITUDINAL AXIS		TRANSVERSE AXIS																
		Range (millimeters)																
Range (mm.)	Class value	0.1–1.0	1.1–2.0	2.1–3.0	3.1–4.0	4.1–5.0	5.1–6.0	6.1–7.0	7.1–8.0	8.1–9.0	9.1–10.0	10.1–11.0	11.1–12.0	12.1–13.0	13.1–14.0	14.1–15.0	15.1–16.0	Total
		Class value																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
0.1– 2.0	1																	0
2.1– 4.0	2				2													2
4.1– 6.0	3			1	3	5	1											10
6.1– 8.0	4		2	6	15	23	24	12	2	1								85
8.1–10.0	5	1	8	29	25	37	19	25	12	1								157
10.1–12.0	6		16	50	42	34	25	13	9	3	1							193
12.1–14.0	7		17	28	44	22	17	12	6	5	1	1						153
14.1–16.0	8		6	21	22	12	18	5	6	2	2		1	1				96
16.1–18.0	9		1	17	19	15	5	5	3	2	1	1		2				71
18.1–20.0	10		4	9	18	8	6	4	5	4	7	2	5	3				75
20.1–22.0	11			7	6	7	6	4	3	5	4	4	3	3	4			56
22.1–24.0	12		1	9	12	3	1	2		4	2	4	1				1	40
24.1–26.0	13		2	5	4	5	2	3	4	3	1	2	1	2	1	1	1	37
26.1–28.0	14			2	3	1	1	1	3		1			2				14
28.1–30.0	15			1	2	4	1	1	1									10
30.1–40.0	16–20																	0
40.1–42.0	21					1												1
Total		1	57	185	217	177	126	87	54	30	20	14	11	13	5	1	2	1000

QUANTITATIVE RESULTS

In table 1 are set forth the longitudinal and transverse axes of the 1000 nuclei. Calculated from this table were the following data:

Longitudinal axis of nuclei

Mean: 14.028 mm.
Probable error of mean: ± 0.116
Standard deviation of series: ± 5.42
Probable error of standard deviation: ± 0.082
Coefficient of variation: 38.6 per cent
Probable error of coefficient of variation: ± 0.66

Transverse axis of nuclei

Mean: 4.812 mm.
Probable error of mean: ± 0.052
Standard deviation of series: ± 2.45
Probable error of standard deviation: ± 0.037
Coefficient of variation: 51.0 per cent
Probable error of coefficient of variation: ± 0.95

Coefficient of correlation between longitudinal and transverse axes: + 0.27
Probable error of coefficient of correlation: ± 0.~~0006~~ ± 0.02

It will be observed that in both sets of figures the probable error of the mean is small, since the number of observations (1000) is fairly large. The coefficient of variation, i.e., the variation expressed as the percentage of the mean, is very great. The correlation between the size of the two axes is small but significant. There is therefore a slight tendency for a greater longitudinal axis to be associated with a greater transverse axis.

Figure 1 is a graph of the lengths of the longitudinal axes arranged according to frequency. The graph has not been smoothed to form a curve, and it is not desirable for the present purpose to calculate the equation for the curve, or even to determine its classification. It is clear, however, that the curve of frequencies would be skewed in such a way as to show that the mean (14.028 mm.) is pulled to the right of the mode, i.e., the region of greatest density (the class 10.1–12). This is due to the occurrence of individuals much farther to the right of the mode than to its left. It will be

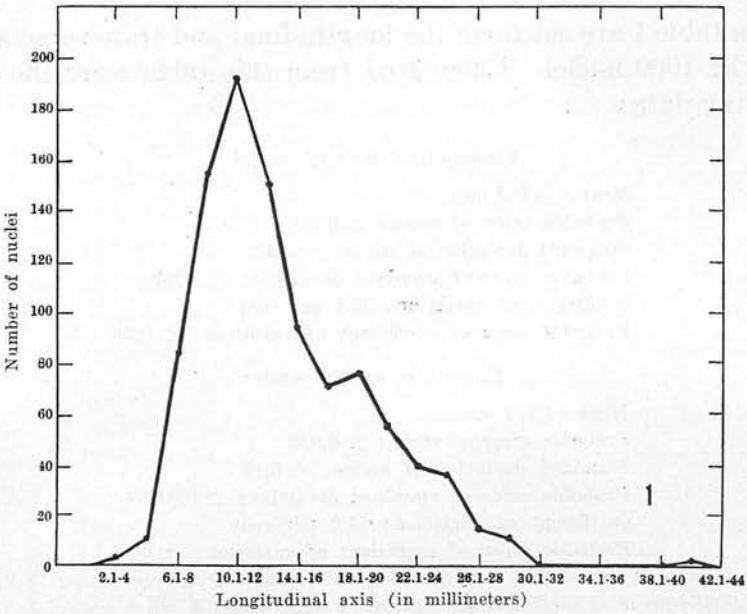

Fig. 1 Frequency of longitudinal axes.

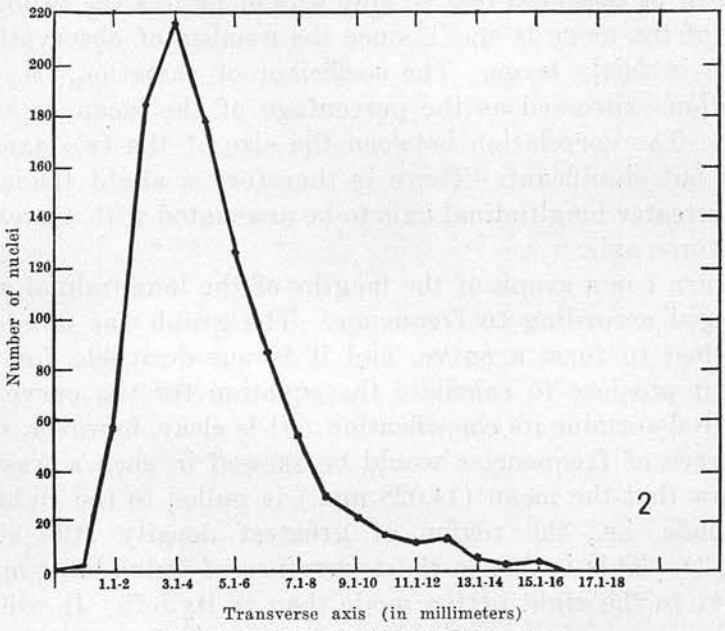

Fig. 2 Frequency of transverse axes.

noted that there is a minor mode in the class 18.1–20. The significance of this is questionable and the investigation of it has no obvious bearing on the present work.

Figure 2 is a graph of the lengths of the transverse axes arranged according to frequency. There is a skewness of a type similar to that in the graph of longitudinal axes.

In the description of nuclei given above there were mentioned nuclei of lamellar connective-tissue cells. Apart from structural differences, the difference between these nuclei and the fibroblastic nuclei is in shape. It appeared obvious that the lamellar connective-tissue nuclei were broader for their length than fibroblastic nuclei. It was to obtain a numerical criterion of shape that the index, viz., $\frac{\text{transverse axis}}{\text{longitudinal axis}}$, was used. It was to be expected that the average index of the lamellar connective-tissue nuclei would be greater than that of the fibroblastic nuclei. Table 2 shows the 1000 nuclei arranged according to the lengths of transverse axes and indices, and figure 3 is a graph of the average index for each class of transverse axis. This graph shows an ascent to the class 6.1–7, and thereafter the line becomes almost horizontal. These two parts of the graph can be first treated separately, part I being the classes of transverse axis from 0 to 6 mm., inclusive, and part II the classes from 6.1 to 16 mm., inclusive.

For each part the method of treatment was as follows: There was calculated from the data of table 2 the linear regression equation for index on transverse axis, i.e., the equation for the straight line representing the mean index for each class of transverse axis (Fisher, '30, p. 112 seq.). The straight line obtained in each part is represented by the broken lines of figure 3. For each part a test was made (Fisher, loc. cit., p. 117) to determine whether the line differed significantly from the horizontal, i.e., whether the data could be as adequately represented by horizontal lines as by the sloping lines of the graph. For each part a test was made of the goodness of fit of the calculated straight line to the actual figures obtained from the data. This was done by the analysis of variance method of Fisher (loc. cit., p. 216

TABLE 2

Frequency distribution of nuclei according to transverse axis and index: $\frac{\text{Transverse axis}}{\text{Longitudinal axis}}$. *(The 'class values' are assumed values used for ease in calculation.)*

TRANSVERSE AXIS		INDEX										
		Range (millimeters)										
		.01–.1	.11–.2	.21–.3	.31–.4	.41–.5	.51–.6	.61–.7	.71–.8	.81–.9	.91–1.0	
		Class value										
Range (mm.)	Class value	.1	.2	.3	.4	.5	.6	.7	.8	.9	1.0	Total
0.1– 1.0	1		1									1
1.1– 2.0	2	9	41	7								57
2.1– 3.0	3	2	83	81	16	3						185
3.1– 4.0	4		43	87	56	22	4	2	1	2		217
4.1– 5.0	5		11	35	43	45	19	18	3	2	1	177
5.1– 6.0	6		2	16	21	36	9	20	11	11		126
6.1– 7.0	7			7	14	12	10	16	13	8	7	87
7.1– 8.0	8			5	8	7	10	8	4	7	5	54
8.1– 9.0	9				7	10	3	5	1	4		30
9.1–10.0	10				3	10	3	2	2			20
10.1–11.0	11					9	3	1		1		14
11.1–12.0	12					1	7	2	1			11
12.1–13.0	13					3	4	3	2	1		13
13.1–14.0	14						1	4				5
14.1–15.0	15						1					1
15.1–16.0	16							2				2
		11	181	238	168	158	74	83	38	36	13	1000

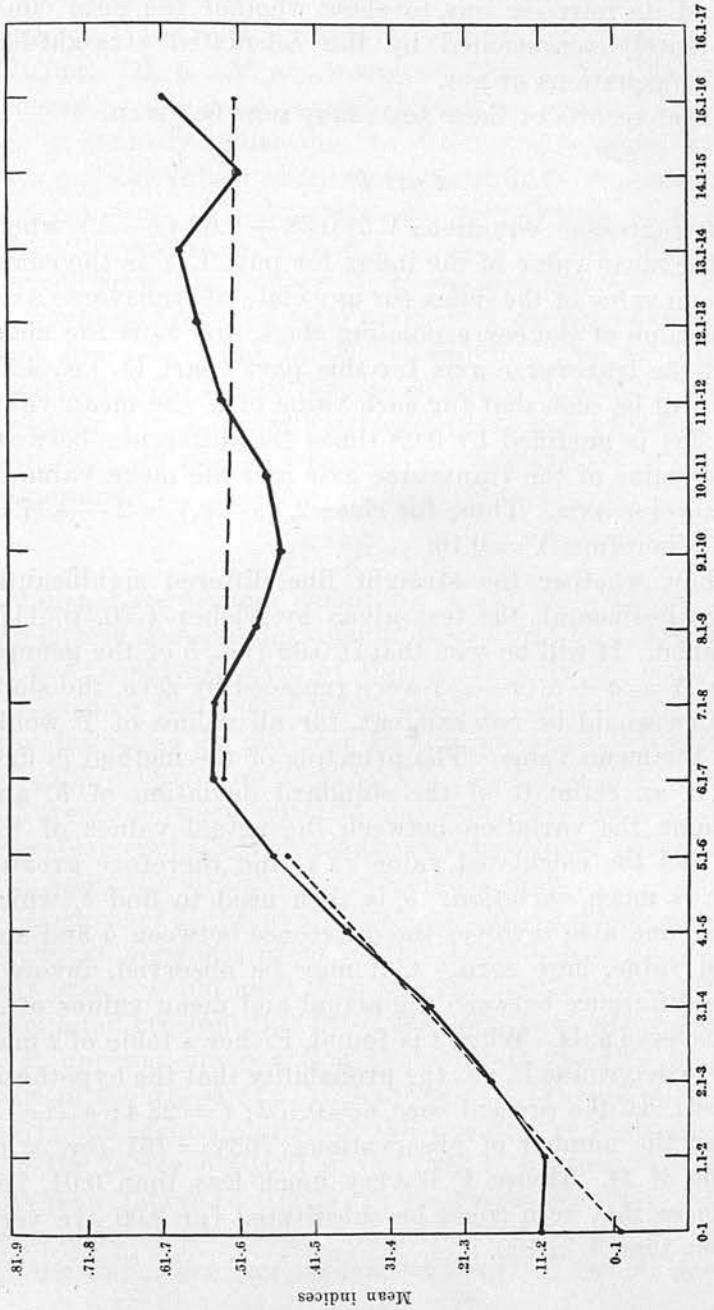

Fig. 3 Graph showing mean index for each class of transverse axis.
The unbroken line represents the actual data. The broken lines represent the regression equations.

seq.), and its purpose was to show whether the data could be adequately represented by the calculated straight-line regression equations or not.

The chief results of these tests may now be given.

Part I

Linear regression equation: $Y = 0.38 + 0.09\ (x—\bar{x}_1)$, where 0.38 is the mean value of the index for part I, Y is the calculated mean value of the index for any class of transverse axis, x is the value of the corresponding class, and $\bar{x}_1$ is the mean value of the transverse axis for this part (part I), i.e., 4.17. Thus it will be seen that for each value of Y the mean value of y (0.38) is modified by 0.09 times the difference between the class value of the transverse axis and the mean value of the transverse axis. Thus, for class 2, $(x—\bar{x}_1)$ is $2 — 4.17 = —2.17$. Therefore $Y = 0.18$.

To show whether the straight line differed significantly from the horizontal, the test given by Fisher ('30, p. 117) was applied. It will be seen that if 0.09 (i.e., b of the general formula, $Y = a + b\ (x—\bar{x})$) were replaced by zero, the slope of the line would be non-existent, for all values of Y would be 0.38, the mean value. The principle of the method is first to find s, an estimate of the standard deviation of b, and based upon the variation between the actual values of the indices and the calculated value (Y), and therefore greater if there is much variation. s is then used to find t, which involves $\frac{1}{s}$ and also involves the difference between b and any assumed value, here zero. t, it may be observed, involves also the difference between the actual and mean values of x, the transverse axis. When t is found, Fisher's table of t may be used to determine P, i.e., the probability that the hypothesis is correct. In the present case $s = 0.132$; $t = 22.4$; n (i.e., 2 less than the number of observations, 763) $= 761$ (or $\propto$ in the table of t). Hence P is very much less than 0.01, i.e., the chances that zero could be substituted for 0.09 are very much less than 1 in 100.

To show whether the calculated straight line could account sufficiently for all the data, the analysis of variance method (Fisher, '30, p. 216 seq.) was employed. The steps of the process need not be given. The final result, z, was 0.291. z is a quantity indicating the difference between the actual data and the values calculated according to the linear regression equation. To determine the significance of z, Fisher's tables were used, with $n_1 = 4$, $n_2 = 757$, these numbers being derived from the number of classes into which the data were divided and from the total number of nuclei recorded in this part (part I). The tables showed that the chances of the deviation from linear regression being significant was much less than 1 in 100, the 1 per cent point being 0.5999. Therefore the data can be adequately accounted for by the linear regression.

Part II

Linear regression equation: $Y = 0.62 - 0.0024\ (x - \bar{x}_2)$, the letters being as for part I, except that $\bar{x}_2$, the mean value of the transverse axis of this part (part I), is 8.79. The assumption that b (—0.0024) differed significantly from zero was tested as in part I. In this case $s = 0.179$, $t = 0.43$, $n = 235$. Therefore $P < 0.7 > 0.6$, i.e., there is no significant difference between —0.0024 and zero. The data in this part therefore could be as adequately represented by a horizontal line as by the straight line calculated above.

As in part I, the analysis of variance method was used to show the goodness of fit of the calculated straight line. In this case z would have been less than zero, and therefore the calculated straight line fitted the data quite adequately.

It appears obvious that the slope of the line in the first part of the graph (fig. 3) is different from that of the second part; but adequate proof of this can only be made by comparing the factor b (0.09) in the first regression equation with the same factor (—0.0024) in the second equation, for, as has been shown above, these factors are responsible for the slope of the line. The comparison was carried out by calculating

t for the second regression line[3] as when comparing b and zero above, and it was shown that 0.09 could not be substituted for —0.0024 in the second regression equation.

The results of this statistical analysis may be expressed as follows. The mean index increases in proportion to the transverse axis as this axis increases from 0.1 to 6 mm. As the transverse axis increases from 6.1 mm. to 16 mm., the mean index remains unchanged. The interpretation of these results is not difficult when the definition of the index is recalled, viz., $\frac{\text{transverse axis}}{\text{longitudinal axis}}$. As the transverse axis increases up to 6 mm., the longitudinal axis must not increase proportionately, because, if it did so, the index would remain constant—a state of affairs that holds as the transverse axis increases from 6.1 mm. upward. A graph, not here reproduced, was drawn showing the relationship between the average longitudinal axis and the various classes of transverse axis. The statement just made was substantiated by the appearance of this graph. Figure 4 has been drawn to represent schematically the differences between the nuclei of the different classes. (It should be noted that the average (mean) indices have been used in the production of these diagrams.)

It will be recalled that the coefficient of correlation between the longitudinal and transverse axis of the whole series of 1000 nuclei was +0.27. When the nuclei of transverse axis 6.1 mm. and over are removed, the coefficient of correlation is —0.083, with a probable error of ±0.0004. Thus the nuclei of transverse axis greater than 6 mm. were wholly responsible for the positive correlation.

COMPARISON OF QUANTITATIVE WITH QUALITATIVE RESULTS

It is fairly obvious that the nuclei called above the lamellar connective-tissue nuclei must be mostly found among the nuclei of transverse axis over 6 mm., i.e., the nuclei that are

[3] In the calculation of s, allowance had to be made for the fact that both 0.07 and —0.0024 had standard errors. The combined effect of these was obtained by letting s^2 be the sum of the squares of the values of s for the two values of b. $s^2 = 0.179^2 + 0.133^2 = 0.05$.

less elongated than the rest in proportion to their transverse axes. The lamellar connective-tissue nuclei were noted as being in the main pale and mottled in appearance. It is now desirable to show the relationship between these qualitative features and the quantitative results already secured. A table was prepared representing the classification according to transverse axis and depth of staining. The proportion of pale nuclei was much greater in the class of over 6-mm. transverse axis and the χ^2 test showed that there was a very highly significant association between pallor and length of transverse axis ($\chi^2 =$ approximately 90, $n = 1$, $P =$ much less than 0.01). Another table was prepared showing the relationship between transverse axis and structural appearance of

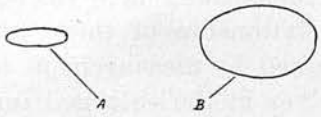

Fig. 4 Schemata of nuclei. Nucleus A from ascending part (I) of graph in figure 3. Transverse axis class, 2.1–3 mm.; index, 0.3. Nucleus B from horizontal part (II) of graph in figure 3. Transverse axis class, 9.1–10 mm.; index, 0.62.

the nuclei. The proportion of mottled nuclei was very much greater in the class of over 6-mm. transverse axis ($\chi^2 =$ approximately 600).

It should be observed how coarse these qualitative tests are, as compared with the quantitative tests—a very strong argument in favor of pursuing the quantitative methods here introduced.

DISTRIBUTION OF NUCLEI

When the 1000 nuclei were arranged in ten consecutive groups of 100, the numbers of nuclei of over 6-mm. transverse axis varied from 4 per 100 to 53 per 100. It appears obvious that the cortex is not homogeneous in respect of the proportion of these nuclei, but to demonstrate this the test illustrated by Fisher ('30, p. 70) was applied. $\chi^2 =$ approximately 130, $n = 9$. P is therefore much less than 0.01, and

the distribution of the broader nuclei is definitely non-uniform. Even when the nuclei are taken in groups of 200, the distribution is still not uniform. The nuclei concerned, therefore, occur irregularly in clumps with variable intervals. It would therefore be impossible to form an estimate of the number present in the 1000 nuclei examined from an examination of 200, and indeed it is highly doubtful if the 1000 nuclei indicate the distribution of the broad nuclei in the whole cortex.

CONCLUSIONS AND DISCUSSION OF FURTHER INVESTIGATION

The methods here used have enabled one to divide the nuclei of the cortical ovarian stroma into two classes by measurement of the transverse axes of the nuclei. It should be noted that no conclusions have yet been drawn regarding the origin and relationship of these two types. The data have been determined by measurement of the nuclei as they presented themselves in the enlarged image projected on to the screen. To determine how far the results are dependent on the obliquity of the nuclei will be the object of further investigation.

The obvious application of the results here set forth is to the study of age changes, of fibrosis, of cystic disease, and of connective-tissue tumors. The method of such application would be the measurement and counting (under the magnification here employed, or under a known multiple of this magnification) of the nuclei of over 6-mm.[4] transverse axis out of a total of, say, 1000 consecutive nuclei. This would entail much less labor than did the present investigations, where over 75 per cent of the nuclei were below 6.1 mm.

Since the size of the nuclei may vary significantly according to the fixation and other treatment of the ovary, it would be necessary in each ovary to establish the means for the longitudinal and transverse axes. Finally, it would be necessary to determine the variability in the distribution of the nuclei under consideration, for it has been shown that the ovarian

[4] If the magnification employed differed from that used here, it would be necessary to modify this number, 6 mm., accordingly.

cortex here examined was not by any means homogeneous in respect of the distribution of these nuclei.

SUMMARY

The use of quantitative methods in histology is desirable because they are more precise than qualitative methods, and because they can be easily applied to specimens prepared by the ordinary techniques of fixation and staining, and therefore can be of use to the pathologist and surgeon for diagnosis and prognosis. The purpose of the present study is to indicate the type of results obtained by such a method applied to the ovary. Measurements have been made of the longitudinal and transverse axes of 1000 nuclei in the projected magnified image of the cortical stroma of a paraffin section from a formalin-fixed human ovary. The method is criticized and the results, set forth in graphs and tables, are subjected to statistical analysis. At the magnification used ($\times$ 1824) the mean longitudinal axis is 14.028 mm.; standard deviation of series, $\pm$5.42. The mean transverse axis is 4.812 mm.; standard deviation of series, $\pm$2.45; coefficient of correlation of these two axes, +0.27. An index, viz., $\frac{\text{transverse axis}}{\text{longitudinal axis}}$, has been calculated for each nucleus. By means of regression equations and the analysis of variance method, it is shown that the mean index increases in proportion to the transverse axis as this axis increases from 0.1 to 6 mm.; but as the axis increases from 6.1 mm. to 16 mm., the mean index remains unchanged. These two classes of nuclei correspond respectively to: 1) the cylindrical or fusiform nuclei of 'fibroblastic' type, and, 2) the flat, pale, mottled 'lamellar connective-tissue' nuclei. No suggestion can yet be made regarding the relationship of these two classes.

The nuclei of over 6-mm. transverse axis occur irregularly in clumps at variable intervals.

Suggestions are made for the use of these methods and results in a study of age changes and pathological conditions.

ACKNOWLEDGMENTS

To my wife, who has assisted throughout, especially in the laborious tasks of measurement and of sorting and classifying the cards on which the data were recorded, I wish to express my special gratitude. My sincere thanks are also due to Dr. C. H. Goulden for great assistance in the statistical work, to Prof. J. C. B. Grant for valuable criticism, and to Prof. William Boyd for kind permission to use data secured in the Pathological Department of Winnipeg General Hospital.

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POSTERIOR DUPLICITY IN A DOG,

WITH REFERENCE TO MAMMALIAN TERATOLOGY IN GENERAL

Appen-
dix

POSTERIOR DUPLICITY IN A DOG, WITH REFERENCE TO MAMMALIAN TERATOLOGY IN GENERAL

BY

DONALD MAINLAND, M.B., CH.B. (EDIN.)

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POSTERIOR DUPLICITY IN A DOG, WITH REFERENCE TO MAMMALIAN TERATOLOGY IN GENERAL

By DONALD MAINLAND, M.B., Ch.B. (Edin.)
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In a valuable contribution to teratology, Stockard (1921) stated his belief that the only benefit to be derived from descriptions of isolated monsters was the aid which could possibly be obtained from them in studying the normal sequence of development. The admission of the embryological value of monsters in itself appears to justify the continued publication of reports on these specimens, and in fact necessitates considerable minuteness in the description of them. Moreover, although the large amount of recent experimental work on Fishes, Amphibia and Birds has led to a certain amount of agreement on the general nature of the mode of origin of monsters, there is still difference of opinion upon detail, nor is it by any means clear how far the knowledge obtained can be applied to Mammals. It is obvious that, unless some reasonably coherent theory of the mode of origin of the abnormal specimens be adopted, there will be little justification for an attempt to elucidate normal development by means of monstrosities; and it is equally obvious that, unless there is a considerable amount of information upon the normal development of the species, there can, as regards that species, be no adequate proof or disproof of any teratological theory. It is of interest, therefore, to ascertain in any given instance how far our present knowledge of development and our present views of teratogenesis elucidate each other. This is in part the object of the investigations recorded here, their primary aim being the description of an instance of posterior duplicity in a Mammal. This is a type of duplicity which appears less prominently in teratological literature than do some other forms, either because instances of it are less frequently met, or because, being less striking than anterior duplicities, instances of it are more frequently allowed to pass unrecorded. As will be mentioned later, however, this form of abnormality possesses features of special interest with regard to the mode of origin of duplicities in general.

The animal which forms the subject of the present communication was a newly-born French bulldog. Of the two other pups in the same litter both were unusually large, and one was born dead; no other gross abnormality was recorded in either[1].

[1] I am indebted to Dr Crew, Director of the Animal Breeding Research Department of Edinburgh University, for this specimen, and to A. D. Buchanan Smith, Esq., of that Department, for passing on to me the above information concerning its birth.

To Messrs Richard Burrell and W. F. Jenkins I am obliged for the drawing reproduced in fig. 5.

After injection of the arteries through the left ventricle of the heart with vermilion-urea-gelatine, the whole animal was preserved in formalin.

EXTERNAL APPEARANCE

The animal was photographed while fresh, and a drawing, prepared from the photograph, is shown in fig. 1. The part of the body on the cephalic side of the umbilicus showed no abnormalities. Four hind-limbs were present,

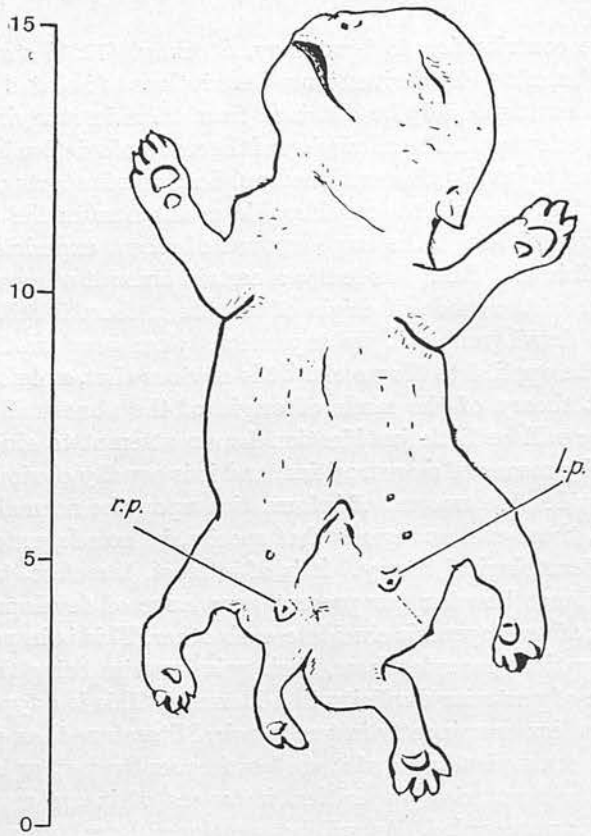

Fig. 1. (Drawn from a photograph; scale marked at intervals of 5 cm.)
r.p., right penis; *l.p.*, left penis.

which from their positions were designated right lateral, left lateral, right central and left central. In all four the segments, thigh, leg and foot, could be distinguished. The lateral hind-limbs were proportionate in size to the body and to the fore-limbs. The left central was slightly smaller than the left lateral, and the right central was thinner and shorter than any of the others, its length being approximately two-thirds of that of the left central. The two central limbs lay very close together at their roots. The right central hind-foot had

two digital pads, instead of the four possessed by the other hind-feet. One of these two pads was large, and this toe bore a double nail.

The umbilicus was single but occupied a broad area, from which a shallow pouch of peritoneum protruded. On the abdominal wall postero-lateral to the umbilicus on each side was a complete penis. Between the roots of the left pair of hind-limbs an anal orifice was situated, and dorsal to this was a tail of about one centimetre in length. There was no right anal orifice and no right tail.

OSSEOUS AND ARTICULATORY SYSTEMS

The numbers of vertebrae in the cervical, thoracic, lumbar and sacral regions were normal, as was also the number of ribs. There were only five

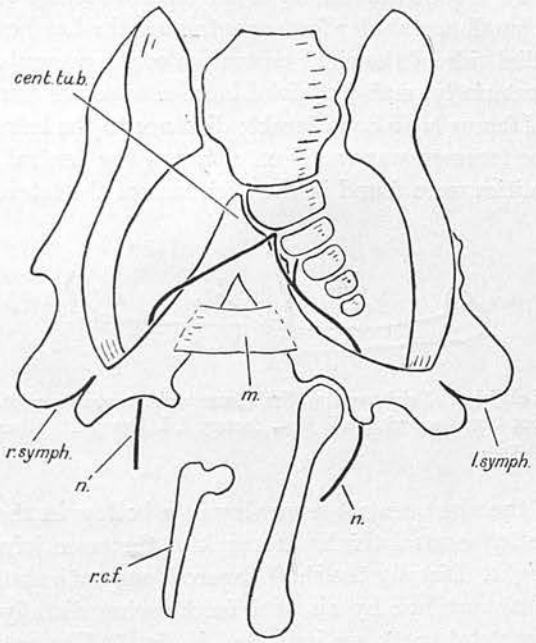

Fig. 2. Diagram of double pelvis from ventral aspect. *cent. tub.*, central tuberosity; *l. symph.*, left symphysis pubis; *r. symph.*, right symphysis pubis; *r.c.f.*, right central femur; *m.*, interpelvic membrane; *n.*, nerve to left central thigh; *n'.*, nerve to region at roots of right hind-limbs.

coccygeal vertebrae[1]. The tail was permanently pointed somewhat to the left, and the vertebrae composing it were shaped in conformity with this.

The bones and joints of the fore-limbs and skull were normal.

The sacrum articulated as in the normal with the iliac part of an os coxae

[1] No specific information on the number of coccygeal vertebrae in the French bulldog is available. For the dog in general, Ellenberger and Baum (*Anatomie des Hundes*, Berlin, 1891) give the variations as from 20 to 22.

on each side. The ventral parts of these laterally placed ossa coxae, instead of meeting in the mid-line, remained about 1½ cm. apart (fig. 2). Each articulated by its medial side with another bone, and thus two symphyses were formed, the long axes of which sloped dorsally and laterally. Each of the two extra bones (central ossa coxae) was rather more than half the length of one of the lateral ossa coxae, and each passed dorsally and medially, so that the two met and articulated with each other, slightly to the right of the mid-line. In the region of union the two bones formed a prominent tuberosity, which lay immediately to the right of and dorsal to the base of the tail. The first two coccygeal vertebrae articulated by their right sides with the left ventral portion of the tuberosity. The triangular interval between the central ossa coxae was partly filled by a strong quadrilateral membrane (the interpelvic membrane, fig. 2). About the middle of the medial surface of the left central os coxae was a small acetabular fossa, containing the head of the left central femur. The medial side of the right central os coxae presented a fossa which was deficient posteriorly, and contained loose connective tissue. The head of the right central femur lay a considerable distance to the left of this.

No obturator foramen was found on either of the central ossa coxae.

No abnormalities were found in the skeletons of the lateral hind-limbs.

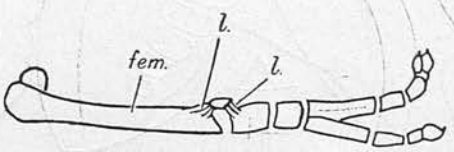

Fig. 3. Diagram of skeleton of right central limb from right ventral aspect. (The plantar surfaces of the foot bones are visible.) *fem.*, femur; *l*, *l*, ligaments attached to a structure resembling a patella.

The head of the right central femur lay in a hollow in the muscles of the right side of the left central thigh. The neck of the bone joined the shaft by passing to the right. Distally the shaft tapered, and articulated with a much shorter cylindrical cartilage by an oval facet facing distally and ventrally. The remaining skeletal parts are indicated in fig. 3. The extreme deformity of this limb rendered it difficult to determine whether the member corresponded in symmetry to a left or to a right normal limb. The body of the femur lay to the right of and distal to the head (fig. 2), but the plantar aspects of the metatarsals faced somewhat to the right as well as ventrally (fig. 3). The tarsal bones do not assist in the elucidation of this matter, and it is improbable that the small structure resembling a patella (fig. 3) was really such. On the whole the appearance of the limb suggested that it very probably corresponded to a right normal limb, that is, that it was of right "asymmetry."

The head of the left central femur articulated with the corresponding os coxae at the acetabulum. The distal end of the bone was broader than the shaft, but did not show definite condyles. With this end articulated a bone

comparable to a normal tibia. To the right of and ventral to this lay a flexible strip of bony or pre-osseous tissue which joined its neighbour proximally and distally, and apparently represented a fibula. If this interpretation is correct, then, as shown in fig. 4, the structure in question was placed abnormally in relation to the bones of the foot. The tarsals, metatarsals and phalanges were well defined and strictly comparable to those of a normal right foot.

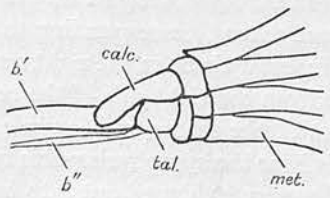

Fig. 4. Diagram of tarsus and adjoining parts in left central hind-limb from ventral aspect. (The plantar surfaces of the foot bones are visible.) *b′*., principal bone of the leg (tibia); *b″*., adjacent, fibula-like strip; *calc.*, calcaneus; *tal.*, talus; *met.*, metatarsal.

It may be concluded that this limb was a somewhat irregular limb of right "asymmetry." It was much more normal as regards skeletal parts than its neighbour on the right.

MUSCULAR SYSTEM

No abnormalities were noted in the muscles of the fore-limbs, head, neck or thorax.

A musculus praeputialis ran, laterally and caudally, to each penis. The oblique and transverse muscles of the abdomen were normal laterally. Those of the right side were widely separated from those of the left at the umbilicus, but posteriorly formed a continuous sheet, composed principally of obliquus internus and transversus abdominis. This sheet was attached to the bones of the right and left symphyses pubis, and to some extent to the membrane joining the two pelves. Each of the two recti abdominis was attached to the corresponding lateral os coxae, and for only a very short distance to the adjacent central pelvic bone. There was thus a considerable interval between these muscles in the pubic part of the abdominal wall. This interval was bridged by the aponeuroses of the lateral muscles of the abdominal wall.

The muscles of the lateral hind-limbs presented no abnormalities, and were easily defined. Those of the central limbs were more indefinite, and consisted of fibro-muscular masses more or less fused with each other. In the space between the two pelves, caudal to the interpelvic membrane, lay such a mass, with feeble attachments to bone.

In the left central limb, a thin layer of muscle passed from the mass just mentioned down the ventral aspect of the femur to the knee. This represented apparently the extensor muscle of the thigh. A better developed muscle passed from an attachment on the right of the left pubic symphysis to the dorsal aspect of the femur, and resembled an adductor. From the dorsal part of the central tuberosity (*cent. tub.*, fig. 2), and from the part of the os coxae which led from it to the left symphysis pubis, there arose a fairly well-defined muscle which was inserted partly into the dorsal aspect of the left central

femur and partly into bone of the leg distal to this. Except for this, muscular tissue in the leg was slight. In the sole of this limb was a fleshy mass subdivided to some extent, corresponding to the toes.

Muscular tissue in the right central thigh was poorly defined. Muscle which arose from the right side of the central tuberosity was inserted into the dorsal aspect of the femur, and muscle which arose from the left side of the right pelvis near the symphysis pubis lay on the right side of the femur. Practically all the muscle thinned out into fascia at the knee.

In these two central limbs, therefore, the degree of the abnormality of the muscular system corresponded closely to that of the osseous system. In so far as ordinary examination of the injected arteries allowed a conclusion to be formed, it appeared that poor development of the muscular tissue was not correlated with deficiency in the large vessels of supply to the limbs. It was observed that the definition of the muscles was greater where attachment to bone was possible.

ALIMENTARY SYSTEM

No reduplication of the alimentary canal or of its glands was observed in passing from the mouth posteriorly until the most caudal portion of the small intestine was reached. From the pylorus onwards the small intestine measured 38 cm. Four centimetres from its entrance into the colon, the small

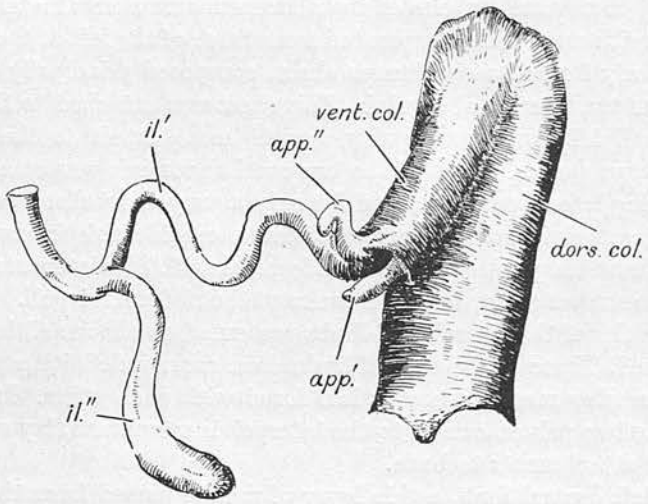

Fig. 5. Reduplicated part of intestine. *app'*., *app''*., caecal appendices; *dors. col.*, dorsal limb of colon; *il'*., ileum; *il''*., blindly ending branch of the ileum; *vent. col.*, ventral limb of colon.

intestine bifurcated (fig. 5). The narrower and thinner walled limb of the bifurcated part passed to the colon. The other limb, of equal length, lay beside the first, in a mesentery derived from the chief mesentery, and then ended blindly. The large intestine lay in the caudal part of the abdomen, to the left

of the median plane, and was shaped like an inverted U. The ventral limb of the U was about 1·5 cm. long. It received the small intestine, and presented two caecal appendices near the ileo-colic orifice. This limb was held by peritoneum to the surface of the dorsal limb, which was about 3·5 cm. long and was much distended by meconium. The terminal third of this dorsal limb bifurcated into a smaller, shallower right portion, which passed towards the right pelvic outlet, and a larger, deeper left branch, which passed towards the left pelvic outlet. The cavity of each branch was continuous with that of the corresponding urethra. The anal orifice present on the left side ventral to the root of the tail did not communicate with the intestine. On the right side there was no anal opening whatsoever.

Reduplication of the alimentary canal was therefore present in the distal part of the ileum, the caecum, and the terminal part of the rectum. The question might be raised whether the blind tube connected with the small intestine was a Meckel's diverticulum. Apart from the associated reduplicated caecum, two facts render this interpretation unlikely. One of these is the length of the tube, and the other is the presence of the remains of the vitelline arteries, which were found, as is usual in the newly-born Dog, at the umbilicus, quite unconnected with this tube.

UROGENITAL SYSTEM

There was both a right and a left bladder, each with a urachus. Each urethra communicated with the corresponding part of the divided rectum, the communication on the right side being much smaller than that on the left. The penile part of each urethra was patent. As the anus was not patent, the condition on both sides would be described as *atresia ani urethralis*. In the left lumbar region was a kidney, from which a ureter led to the left bladder. There was no kidney on the right, but a fine double cord-like structure attached to the right bladder near the base possibly represented the ureter of this side. The structure mentioned lay entirely in the pelvis, and the part of it remote from the bladder merely ended in fascia.

There was one testis in the lower lumbar region of each side. The epididymis of each was very imperfect. The two penes have already been noted. Each received a urethra through the corresponding pelvic outlet.

In the region between the two bladders, closely attached by peritoneum to the ventral surface of the rectum, was a lobulated gland-like structure, similar in size to each of the bladders. Caudally it was narrow and attached to the fascia of the pelvic floor. This structure and also one of the testes were examined histologically. Comparison was made with the testis of a normal pup removed from the uterus of a dog near the end of gestation. The lobulated structure in the pelvis of the six-legged dog was mostly composed of fibrous tissue, containing some small lymphocyte-like cells and some broad cells quadrilateral in outline. Identification either with post-natal or with foetal tissue was found to be impossible.

ARTERIAL SYSTEM

Before the body was preserved, the arteries were injected from the heart by a vermilion-urea-gelatine mass, and some of the finer points in the distribution of the limb arteries could be observed.

The abdominal aorta bifurcated opposite the posterior part of the lumbar region of the vertebral column. The two branches again divided, and contributed to the lateral hind-limbs arteries which corresponded in course and distribution to those of a normal animal. In addition, branches arose as indicated in the diagram (fig. 6). Among the arteries which resulted from the

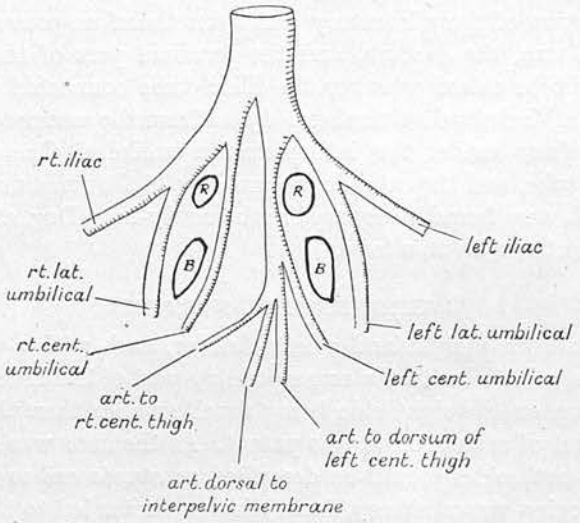

Fig 6. Diagram of posterior part of arterial system. *BB*, right and left bladders; *RR*, right and left subdivisions of rectum.

subdivision of the left terminal branch of the aorta were: (1) a left lateral umbilical; (2) a left central umbilical, which was smaller than the lateral, but adhered similarly to the left bladder; (3) arteries which emerged from the pelvis dorsal to the interpelvic membrane and supplied the fibro-muscular tissue between the two central limbs, and also the muscle of the left central limb which resembled an adductor; (4) a large artery which emerged from the left pelvic outlet, supplied the dorsal muscles of the left central thigh, and passed along the leg to the plantar aspect of the foot; (5) an artery which emerged through the right pelvic outlet and supplied the right central limb.

Among the branches of the right terminal division of the aorta were a right lateral umbilical and a smaller right central umbilical, both adherent to the right bladder.

It will be noted that both of the central hind-limbs were supplied by arteries which belonged to the left part of the double pelvis.

NERVOUS SYSTEM

The arch of the third sacral vertebra was thin and membranous. Distal to it was a small meningocele, which lay dorsal and to the left of the first two coccygeal vertebrae.

The nerve supply of both lateral hind limbs was normal. Dissection with the aid of a lens failed to reveal conclusively nerves to the central limbs except as mentioned below. A nerve emerged on the right side from between the first and second coccygeal vertebrae, and joined another which emerged, also on the right side, from between the second and third coccygeal vertebrae. The common trunk crossed the pelvic surface of the left central os coxae (fig. 2), and passed through the left pelvic outlet, to become the principal nerve of the left central limb. With the more cephalic root of this nerve lay another nerve, which emerged from the same intervertebral space, but left the pelvis through the right outlet, and supplied the skin of the region at the roots of the two right hind-limbs. No undoubted branches from it could be found supplying the muscles of the right central limb.

In contrast to the arterial supply of the central limbs, it is notable that the nerve supply of the left central and perhaps (indeed probably) that of the right central limbs originated in series with the spinal nerves of the right side of the body.

DISCUSSION

General scheme

The study of the mode of origin of any abnormality arising in embryonic life is necessarily based upon knowledge of the normal embryology of the species concerned; but in many groups of animals observation can be supplemented by experiment. At present a technique for the experimental production in Mammals of abnormalities such as that described here does not exist; and therefore comparison between the naturally produced and the artificially produced abnormality must entail reference to experiments on other groups of animals. It would not be profitable to discuss at length the various types of teratological experiments that have been performed, and the various theories that have been introduced to account for double monsters. Detailed information on these aspects of the subject is available in the work of Schwalbe (1907), and this can be supplemented from various subsequent papers, some of which contain useful bibliographical references (Anders, 1921 b; Stockard, 1921). Some twenty years ago, in spite of the numerous experiments that had even then been performed, Schwalbe (*loc. cit.*) felt that the only permissible generalisation was the limited one, that duplicities were due to the division of the material forming the egg, or, in the words of Rauber, that the germinal protoplasm was in such cases multi- instead of uni-centred. This statement referred to the "formal genesis" of duplicities (i.e. what might be called their mode of

origin). As regards their "causal genesis" (or actual cause), Schwalbe justly held that the existing knowledge was strictly nil.

At the present time it is sufficient to consider more recent views, which are based chiefly on experimental evidence, but which have themselves in part evolved out of the older knowledge and beliefs. An attempt can then be made to apply these views to the specimen described, to correlate them with the embryology of the Dog, and finally to seek further elucidation by brief reference to records of closely similar abnormalities.

Sources of embryological evidence

For a description of the early development of the Dog dependence has chiefly to be placed upon the work of Bonnet (1897, 1901). The numerous and carefully recorded observations of Bischoff (1845) afford considerable information, but they naturally do not afford evidence of minute differences in cellular activity or of the earliest stages of differentiation. Although the information is thus limited, sufficient is known to show that, as in other Mammals and in other non-mammalian Vertebrates, differentiation of the embryo proceeds first in a cephalo-caudal direction, and in particular that the hind-limb buds appear later than those of the fore-limb (Bischoff, 1845). Bonnet's records concern the stages from the formation of the embryonic area onwards. They are among the most minutely detailed of any in Mammalian embryology.

Experimental evidence

Within recent years extensive experimental investigations on the production of monstrosities have been carried out, especially in America, and some of the conclusions reached are of obvious importance to the present discussion, in which an attempt is made to apply the results of the experiments to another group of animals, Mammals, and to argue from these cases, where the causal agents were known, to a case where they are unknown. Thus, it appears now to be quite generally accepted: (1) that the type of the agent which causes the abnormality is a secondary matter, whether, for example, it is magnesium chloride, lowered temperature, or ultra-violet radiation (see Stockard, 1921; Marie Hinrichs, 1925; Bellamy, 1922); (2) that any one agent can produce a great many types of abnormality (see Stockard, 1921; Marie Hinrichs, 1925, 1927); and (3) that the resulting abnormalities reveal great similarity to each other among the various groups and species of animals.

A considerable amount of light has been thrown upon problems of teratogenesis by a number of authors who have applied to it Child's conception that during development the regions of high metabolism are regions of dominance which control the other regions. (For a concise critical statement of Child's position, especially regarding the cephalo-caudal metabolic gradient in Vertebrates, see a recent article by that author, Child, 1925. References to some of his previous publications are there given. A fuller bibliography is supplied by Conklin, 1924, p. 602.) As representative of investigations along

these lines mention may be made of the experiments of Bellamy (1922) on Frog embryos, and of Libbie Hyman (1921) on those of Teleost Fish. Although complete unity is not displayed in the conclusions reached by the various investigators, two main points of agreement may be stated as follows: (1) abnormalities arise because of the imperfect co-ordination between the parts with higher metabolism and the rest, as a result either of inhibition—an arrest of development (Stockard, 1921; Libbie Hyman, 1921; Bellamy, 1922)—or of acceleration (Riddle, 1923; Bellamy, 1922); (2) the parts most susceptible to interference by a teratogenetic agent are those of which the metabolic activity is highest, whether the interference causes the affected region to fail to regain its control of other regions (Stockard, 1921), or whether the activity of the susceptible region is directly accelerated (Bellamy, 1922), or whether the susceptible region shows more power of recovery than other regions (Libbie Hyman, 1921)[1].

Whatever be the ultimate destiny of the theory which has prompted these investigations, the value of their results is considerable, and, if these main points of agreement are accepted as at least the basis for a theory of teratogenesis, the mode of correlation of normal embryology with teratology is broadly indicated. It will not be permissible to assume that all the organs arising from a certain part of the embryo will be reduplicated or malformed. On the other hand it may be assumed that if regions or organs were in or about to enter a state of high metabolism when the teratogenetic factor became operative, then they will manifest abnormalities. Difficulty in the interpretation is, however, very liable to arise. At least four factors may be responsible for confusion and obscurity: (1) the possibility of the influence of several inhibiting or accelerating factors at different times; (2) the difference in susceptibility of the different germinal layers, shown experimentally by Libbie Hyman (1921); (3) secondary fusion of the parts of the embryo after primary separation (see reference to Fischel, below, and, especially as regards posterior duplicity, the reference to Hertwig in Schwalbe, 1907, pp. 60–1, also Marie Hinrichs, 1925); (4) the secondary changes which may occur during subsequent growth as a result of lack of room, alteration in blood supply or other factors (see Wilder, 1904). In the first instance, however, it is desirable to ignore these possibilities as far as possible, in order to test the validity of the conceptions mentioned, namely, that the causal agent operates in virtue of the susceptibility of the areas of high metabolism, and renders imperfect the co-ordination between these areas and the rest.

[1] Misconception may be avoided if it is stated that these references, which appear to suggest diversity of opinion, are intended to be representative, and not to be exhaustive either of the investigations or of the opinions held by the investigators named.

Period of origin of duplicities in general

Before these results of experiment are utilised in an attempt to determine in the present instance the time of operation of the teratogenetic factor, it is desirable to raise the question, within what period teratogenetic factors in general are supposed to act. Schwalbe (1907) stated as a widely accepted view the belief that gastrulation marked the end of the period during which most of the symmetrical duplicities were produced. This view has been reaffirmed more recently on experimental evidence (Stockard, 1921), and on morphological grounds (Lebedinsky, 1923), but individual specimens have been described which have been thought to demand a much later period of formation (Werber, 1917). Certain experiments (Kopsch, 1899; Baldwin, 1915; Marie Hinrichs, 1925) are of special interest because they demonstrate the possibility of production of posterior reduplications without anterior reduplication by interference with the ovum definitely before the embryonic area has formed. If this belief is accepted, as appears to be necessary, namely, that in most cases the teratogenetic agent operates not later than the gastrulation period or what corresponds to it in Mammals, and, further, if the assumption mentioned above of co-ordination and susceptibility be made, then our conception of the exact mode of action of the causal agent is more strictly defined. It is necessary to suppose that the effects of the agent will be manifested, not in virtue of incipient metabolic activity of various anlagen—for example, hind-limb buds—but on account of an earlier activity of the portions of the ovum destined to give rise to these anlagen.

Period of origin in this specimen

In the dog at present under discussion, it is noteworthy, firstly, that the abnormalities discovered were limited to the caudal part of the body, and, secondly, that, while the hind-limbs and pelvis were multiple, the tail was rudimentary. The vascularity of the parts about the pelvic outlet was considerable, and there appeared no reason to suppose that the tail, if able to demand greater nutrition, would have failed to receive it. More probably, the activity of the elements forming it was in the first instance depressed, and never completely recovered. The restriction of the abnormalities to the caudal portion of the body renders this and other cases of posterior duplicity of special interest in connection with the beliefs that high metabolism accounts for susceptibility to the causal agent, and that this agent does not act after the gastrulation period (or what corresponds to it in Mammals). After the gastrulation period, when the embryonic area becomes trilaminar, the region of high activity is in the future cephalic part. Therefore, where the ultimate reduplication is, as in the specimen described, purely posterior, it must be supposed either that secondary changes have taken place, or that the effect of the causal agent was not originally uniform on all parts of the ovum or gastrula. In accordance with the views of co-ordination and susceptibility stated above,

it may in this case be suggested that the agent, whatever it was, affected the ovum during a period of activity of the parts destined, at a later stage, to give rise to hind-limbs, alimentary tract, bladder and urachus; the effects being the prevention of the normal co-ordination in development with consequent reduplication of parts on opposite sides of the future median plane.

From the experimental evidence referred to, it appears quite reasonable to ascribe the deficiency of the tail to the same factor, which in this instance exerted a purely depressing effect. The defective and deformed structure of the central hind-limbs may be accounted for by restricted room (Wilder, 1904), or by deficiencies in the anlagen, induced at the time of reduplication.

In an attempt to correlate in further detail the course of development in the normal with the abnormal occurrences in this animal, appeal may be made to the facts of comparative Mammalian embryology, but here care must be exercised, owing to the known divergence in detail between the development in various species. For this purpose reference will be made to the embryology of Man and of the Pig, both of which have been studied attentively. Bischoff's (1845) records show that in the Dog the tongue manifests early activity after the appearance of the hind-limb buds, but apparently before the genital tubercle arises. That this is not an exceptional sequence is shown by records concerning Man (Keibel, 1910, pp. 68–9—hind-limb; McMurrich, 1912, pp. 343–4—tongue; Felix, 1912, p. 947—genital tubercle) and the Pig (Arey, 1925, p. 355—hind-limbs; *ibid.*, fig. 84, p. 95—tongue; *ibid.*, p. 378; and Bühler, 1906, fig. 495, p. 840—genital tubercle). In the specimen examined there was reduplication of the hind-limbs and of the external genitals, but there was no obvious abnormality of the tongue. Visible development of an organ is only a late result of metabolic activity, and therefore the weakness of the embryological evidence is readily admitted; but the line of argument indicated is definite. If the facts are as stated, a period of high metabolism in an anlage that apparently was not affected intervened between the high metabolic periods of two anlagen that were reduplicated. The reduplication of organs appears to be determined by their situation, not by their known metabolic history. If the causal agent was single and yet acted on parts in virtue of their high metabolism, then it is suggested either that a specially localised fusion of certain reduplicated organs took place, or, as is less hypothetical, that the cause operated before differentiation had proceeded far, and that at some period the cells destined to give rise to the caudal part of the embryo were highly active, while the rest were not. This view, supplemented by the fact of the high primary cephalic activity mentioned above, would indicate that the causal agent was effective at a period before the trilaminar stage of the embryonic area. The result of this argument would be to show that the assumptions of co-ordination and susceptibility made above, when applied to a case of posterior duplicity, practically pointed to the general conclusion already stated, that such abnormalities do not arise after the gastrulation stage or what corresponds to it in Mammals (p. 484).

Even Bonnet's (1897) minute embryological observations do not justify any more strict definition of the time of origin.

As the lobulated fibro-cellular mass in the pelvis of this dog was not identified, it is hardly justifiable to make any assumption that would exclude the possibility of its being either nephrogenic or genital tissue. This difficulty affords an instance of an essential weakness in teratological records based upon ordinary dissection and naked-eye observation, and this weakness is not always overcome by histological methods, such as were used in the present case. Evidence of the absence of reduplication of any organ or tissue may be untrustworthy because an originally doubled portion may have regressed so far as not to be readily recognisable (Stockard, 1921), or even so far as to be undetectable. This source of error can be removed probably only by a graded series of strictly controlled experiments (not necessarily on Mammals), followed by a histological examination of the products.

If, however, it was assumed that the testes in this specimen were unreduplicated, and if the assumption was supported by reference to Horsley's specimen (see below, p. 491), the line of argument from embryology would be the same as in the case of the tongue, the sequence of appearance being, according to evidence from Bischoff (1845), hind-limb buds, genital glands, genital tubercle. (Compare Man—references to hind-limbs and tubercle as above; genital gland: Felix, 1912, p. 885.)

The earliest time of action of the cause

In the search for evidence of the earliest activity of the causal agent in this specimen, attention may be turned to the urachus. Reduplication of this structure as far as the umbilicus has been noted. In the Dog, Bonnet (1901) detected cellular changes in the entoderm of the future allantoic region before any mesodermal somites had appeared, and he stated that in embryos with from eight to ten somites this allantoic entoderm was for the most part very clear. Differentiation in this part, therefore, arises before the appearance of either the buds of the fore-limbs, which were not reduplicated here, or of the buds of the hind-limbs, which were reduplicated. Even if it be assumed that the agent responsible for the reduplication was effective only during high metabolic activity of the parts concerned, it is, on this evidence alone, impossible to state whether the agent operated at the period when the urachus commenced to differentiate, or during its period of high metabolic activity immediately subsequent to that (Bischoff, 1845), or at a much earlier period in the history of the developing zygote.

Although the first incidence of the teratological change on this specimen cannot be more nearly determined than has been stated, it has been found (e.g. by Marie Hinrichs, 1925, experimenting on Fishes) that reduplication, including posterior reduplication, can occur when ova are subjected to the teratogenetic agent within the first few minutes of fertilisation. Attempts to account for this may take one of several forms. Very early physiological or

constitutional differences in the zygote were suggested by Marie Hinrichs herself. It would be unprofitable to enter into a discussion of the differentiation of the unsegmented Mammalian ovum in connection with the present specimen; but this explanation cannot on that account be rejected. To adopt the theory advocated at some length by Fischel (see Schwalbe, 1907, and Werber, 1917), namely, complete separation of the ovum into two followed by partial reunion, would be to introduce an additional assumption to those already made; and there appears to be as much evidence in support of a suggestion based on the assumptions of co-ordination and dominance. It might be supposed that the effect of the interference with the ovum was to produce a greater or lesser degree of inco-ordination, and that at the first differentiation the part which usually dominated and controlled the growth of the rest had to some extent lost this power, the degree and even the type of the deformity depending on the extent of the loss, both in the case of the part which should dominate first, and of the parts which should dominate later.

The alimentary system

Reduplication of the alimentary system of this specimen has been noted as confined to the caudal portion of the small intestine, the caecal region, and the terminal part of the large intestine. The part affected, therefore, appears to be wholly or almost wholly caudal to the position of the vitello-intestinal duct. While it is known that, during the developmental activity of the tail region of the embryo, all the germinal layers are more or less involved, detailed knowledge of the metabolic relationships is still small. Moreover, in experiments to test the metabolic activity of developing ova by toxic solutions, entodermal structures frequently cannot be studied (Libbie Hyman, 1921). It is therefore impossible even to attempt to decide whether the incompleteness of the reduplication of the large gut was due to a secondary fusion, or to a primary difference in the metabolic activity of the cells which were destined to form it. Variations in the extent of doubling of the intestine in cases of posterior duplicity are considerable, as may be seen by comparing the present case with those of Anders (1928, p. 421), Sara Conrow (1917), and Horsley (1920).

Fission and hyper-regeneration

Schwalbe (1907, p. 306) remarked, in connection with a case of posterior duplicity in a dog described by Otto, that the absence of reduplication of the vertebrae rendered it doubtful whether the specimen ought to be considered a true posterior duplicity or a product of fission of the pelvis with subsequent hyper-regeneration, such as was produced by the experiments of Tornier on Amphibia. In these experiments (Tornier, 1906) the hind-limb buds were cut through, and the results, carefully described and illustrated, afford a basis for comparison with naturally occurring duplicities of the pelvic region. In the possession of an unreduplicated vertebral column the present case resembles that of Otto, and, moreover, Schwalbe's remark raises the important question

of the relationship between experimentally produced accessory limbs and cases of duplicity occurring in nature. Since Tornier's work a large amount of research has been carried out on the regeneration and other changes resulting from the splitting, excision and transplantation of limb-buds (Harrison, 1921; Spurling, 1923; Swett, 1924; etc.). Much attention has been paid to the question of symmetry. If redundant limbs in any natural specimen are to be regarded as the products solely of such a process of fission and hyper-regeneration, then the rules of symmetry demonstrated by experiments of that kind would necessarily be assumed to act in such a specimen also. Swett (1924) gives a summary of the conclusions justifiably drawn from his own investigations and those of others, to the effects: (1) that both primary limbs developing from isolated portions of a limb-bud always exhibit the same "asymmetry" (both being left limbs or both right); (2) that a secondary limb formed by budding is a mirror image of the primary (in accordance with Bateson's rule of minor symmetry); (3) that, however, the boundary line between these two methods of double limb formation is very indistinct. That unity upon this question is not reached is indicated by the article of Lataste (1926). The great extent of the question and the value of analogies obtained in remote fields may be illustrated by the investigations of Goldschmidt (1921) on naturally occurring reduplications of the copulation apparatus of the gipsy moth (*Lymantria dispar*). The conclusions from a study of symmetry are that fission and budding occur in these cases.

Swett's immediate purpose in the paper just referred to (Swett, 1924) was the discussion of exceptions to Bateson's rules of minor symmetry, and in the course of it he expressed the belief that many cases of extra limbs recorded among the higher Vertebrates, including Man, are exceptions to these rules. Instead of two adjacent members in sets of double or multiple limbs forming a mirror image of each other in accordance with the rule, limbs of the same "asymmetry" may lie side by side—two right limbs or two left limbs may be adjacent. There is no detailed treatment in his paper of the examples in higher Vertebrates.

If it becomes possible to subdivide duplicities of limbs into those which present the mirror image relationship and those which do not, the results may have some etiological importance, but will not necessarily prove Schwalbe's (*loc. cit.*) suggestion correct. One of the chief obstacles to such a classification is emphasised by Lataste (1926), and is illustrated by the present specimen—the difficulty of determining the symmetry of the limbs. The left central hind-limb in the dog described was a (slightly irregular) right limb, and therefore a mirror image of its left lateral neighbour; but the true "asymmetry" of the right central hind-limb is still somewhat in doubt. If it was of right "asymmetry," which appeared to be very probable, then the sequence of hind-limbs is, from left to right: LRRR. The difficulty in deciding this question is partly due to the extreme deformity of the limb; but it is possible that it may be to some extent due to an incomplete process of rotation of developing limbs,

by which Swett (1924) accounts in part for the exceptions to the rule of mirror images.

Even if it should be shown that cases such as the present are due to such a process of "fission" of a limb-bud with hyper-regeneration, it might well be that the fission was simply a physiological isolation of parts, a conception quite closely akin to that adopted above as the basis of this discussion, namely a lack of co-ordination. If such an explanation were adopted in regard to the dog described here, it would imply that from one limb-bud three limbs developed; from the other, only one limb. It might be expected that the nerves supplying or associated with the limbs would afford a clue to the side of the embryo from which the limbs arose, and therefore that the right lateral and both central limbs of this specimen were products of the right hind-limb bud. It might, however, reasonably be supposed that the products of fission or reduplication acted as a transplant in such experiments as those of Detwiler (1924), and became supplied by nerves from parts of the cord not normally supplying limbs at all. The only nerves supplying or associated with the central limbs in this specimen actually made their appearance in series with the nerves to the right lateral hind-limb, but more caudally.

The chief arguments against the suggestion of fission of the limb-bud in this specimen are, firstly, the extent of the abnormalities (involving alimentary system, urogenital system and the coccygeal part of the vertebral column), and, secondly, the probability indicated above that the time of operation of the cause must be referred to an earlier period in the ontogeny of the specimen than the period of limb-bud formation.

Minor accompanying abnormalities

It may be asked whether the other abnormalities recorded in this dog (meningocele, *atresia ani urethralis*, unilateral absence of a kidney) can be accounted for in such a way as to harmonise with the principal lesions. The cause of the defective tail may well have been the cause also of the imperfect formation of the vertebral canal; but to ascribe directly to the same cause the imperfect development of the uro-rectal septum would hardly be more than a conjecture. It is an interesting coincidence that in both of the recently recorded specimens of posterior duplicity mentioned below, there were kidney defects. In Horsley's specimen (1920) there was only one kidney; in Sara Conrow's (1917) one of the two kidneys was rudimentary.

With regard to the *atresia ani*, one may recall the recent work of Anders (1921 a), who came to the conclusion that where the hind-gut opened into the cloaca or ended blindly high up in the pelvis, the ectodermal depression or anus was absent. This was attributed to the fact that the distance between the entoderm and the ectoderm prevented a chemotactic action of the former. In the dog described above it is interesting to note that the larger, left branch of the rectum, which penetrated more deeply into the pelvis, corresponded to the single anal depression. There was, however, no very great difference

in depth between the two blind ends of the rectum, and if the theory of chemotaxis be assumed, then the absence of a second anus is perhaps more adequately explained by the lesser degree of activity of the right half of the rectum.

Differences between components

The great variation in the degree of similarity between the two components of double monsters is at once striking. The dog here discussed is, comparatively, symmetrical, but is not quite so. It is not possible to do anything more than surmise the cause of these differences; but their existence becomes important in connection with the more completely reduplicated human monsters such as the Siamese Twins. A short time ago, Regnault (1925) remarked upon the great differences, physical and psychological, between the two components of such double human monsters, and pointed out how, by excluding the effects of heredity, of the external environment, and of the vascular factor in the internal environment (all of which were common to both components), it was possible to attribute certain phenomena definitely to the nervous factor in the internal environment. Among these were hunger, sleep and the menstrual flow. From brief consideration of minor differences, such as those present in the dog described, where there is only one nervous system, it is obvious that at a very early period of development differences arose in the structure and obviously in the future function of the component parts. Differences of this fundamental kind, but varying in degree and involving other organs and other functions, obviously exist in the more nearly complete duplicities. Therefore, whatever will ultimately be found responsible for these differences, it is impossible to use them as a proof of certain functions of the nervous system.

Other cases of posterior duplicity

For comparison with the specimen described above, brief reference may be made to several other instances of posterior duplicity in Mammals. Concerning the instances which have occurred in the human species little more is known than the peculiarities of external form. Records of these may be found in the writings of Wilder (1904, 1908), Schwalbe (1907) and Broman (1911). As regards the non-human Mammals[1], reference has already been made to the example quoted from Otto by Schwalbe (1907) (a female dog, single from head to umbilicus, with single vertebral column, single left anus, double pelvis, four hind-limbs, double vulva, double caecum, small gut apparently for the most part single). Two instances of animals, each with six hind-legs, are a male rat described by Sara Conrow (1917) and a female dog described by Horsley (1920). The latter case, occurring in a dog, is of special value for comparison with the bulldog pup described above. In both of these

[1] Schlegel (1921) has published a series of short abstracts of many of the cases of monsters (chiefly non-human) described in the first two decades of this century. His article forms, therefore, a valuable supplement to the earlier volumes of Schwalbe's *Die Morphologie der Missbildungen des Menschen und der Tiere.*

last-mentioned dogs the reduplication affected the pelvic girdle, the external genitalia and urinary bladder; the genital glands were apparently unreduplicated. In Horsley's specimen, however, the vertebral column was apparently doubled in the sacral region; whereas in the bulldog described here this was not so. On the other hand, in the latter specimen reduplication extended into the caudal part of the small intestine; in Horsley's dog it was restricted to the large bowel. If it be supposed that rates of metabolic activity in the different parts of the ovum are the primary factors in determining the distribution of the abnormalities, the question arises whether the normal sequence of embryonic development would account for these differences. In the rat described by Sara Conrow (1917) the vertebral column was single throughout, and there were two pelves and two penes as in the bulldog described here; but in the rat there were four testes, while the urinary bladder was incompletely doubled, and, although there were two ani (one a false one), the alimentary canal was otherwise little affected. When this specimen is compared with the dog described by Horsley and with the bulldog pup described here, it may appear questionable whether the differences in times of development between the two species, or between individuals of the same species, are sufficient in themselves to account for the differences in distribution of the duplicities. It may be suggested that some process of secondary fusion of parts, or of absorption of reduplicates, or some specific difference in the causal agents, must be postulated. While it cannot be denied that some or all of these factors may be operative, it may still be possible to account for the differences in the results solely by differences in the state of metabolism of cells or cell-masses in the developing zygote, the causal agent being assumed to act only on the more susceptible, that is, the more active cells or masses. It is known, in general, how cells and cell groups of a zygote can pass rapidly from a state of activity to one of quiescence, and how one mass may be active and a neighbouring mass apparently inactive; but to show in detail the normal sequence of these changes in Mammalian ova by modern cytological, especially cytoplasmic, technique, is a task for the future.

Future investigation

In the preceding discussion there have been shown some of the problems that arise and the difficulties that are encountered when an attempt is made to correlate an instance of reduplication in a Mammal with the results of the more recent teratological experiments on other groups of animals. It is obvious how few justifiable inferences can be made from such a comparison. Lack of embryological information may appear to render the Dog a rather unfortunate species for this type of investigation; but it is very questionable whether the existing embryological knowledge concerning any Mammal is sufficiently detailed as regards the cytological changes to afford satisfactory evidence on the point at issue, namely, the normal sequence of metabolic activity from the stage of fertilisation onwards. On the other hand, experiments on other groups

do not yet afford sufficient information to enable descriptive teratology to contribute to the question of cell-lineage in Mammals, as, sooner or later, it probably will do.

It may, perhaps, not be out of place to consider briefly certain points with regard to the future of Mammalian teratology. Such advanced degrees of abnormality as the one here described are, of course, comparatively rare, perhaps so rare in human development that their causation may be held of little moment. There appears, however, no justification for the supposition that they are, causally, at all widely removed from many of the minor abnormalities which are more common and of which some have practical importance in human medicine. From the purely biological standpoint, and therefore ultimately also from the medical standpoint, the importance of all these abnormalities in all species of animals becomes greater as the purely mechanical theories of their causation become less plausible.

In most groups of animals the determination of possible causes and of the mode of production of abnormalities can be facilitated by experiment; and there is even the danger that repeated observations of natural specimens and of the normal development may be neglected. In Mammals, while attempts at experimental production should be carried out, the careful dissection and histological examination of natural specimens is of the first importance. It is, however, obviously undesirable that space in anatomical journals should be frequently occupied by such detailed descriptions, which have their chief value as part of a large series. It is perhaps desirable that the instances studied should be restricted at first to one species, of which the early development was known in great detail by the investigators. The rarity of certain forms of terata might, however, render it necessary that more than one species of animal be included. Throughout the investigations correlation with recent experimental work should be attempted. Minute examination, both histological and biochemical, of the mother of the abnormal specimen should be made. To insure the possibility of this, research would probably have to be restricted to animals bred in large numbers for research. These lines of approach all seem necessary if light is to be thrown, not only upon the mode of origin of these abnormalities in Mammals, but upon the very obscure question of their actual cause.

SUMMARY

The specimen was a French bulldog, newly born.

Externally it was reduplicated only caudal to the umbilicus.

There were two pelves, two normal lateral hind-limbs, and two centrally placed hind-limbs. The left central hind-limb was more nearly normal than the right central, in osseous and muscular structure, and corresponded in symmetry to a normal right limb. The right central limb was small and de-

formed. It was of doubtful symmetry, but probably also corresponded to a normal right limb.

The rest of the skeleton was not reduplicated. The coccyx was very short and deformed. The abdominal muscles were only slightly abnormal. The arteries supplying both central hind-limbs came from the vessels in the left pelvis. There were four umbilical arteries.

Only two nerves could definitely be shown to be associated with the central hind-limbs: one to the left central, and one to the skin at the root of the right central limb. These nerves appeared in series with the lumbar and sacral nerves supplying the right lateral hind-limb; but were more caudal than these, for they corresponded to coccygeal nerves.

In the alimentary system the reduplications affected only the terminal one-tenth of the small intestine, the caecum (two caecal appendices), and the rectum. There was only one anus. It corresponded to the left branch of the rectum, and was imperforate.

There was only one kidney. It was present on the left side. There were two urinary bladders, each with a urachus. Each urethra communicated with the corresponding part of the divided rectum.

There were two penes; but only two definite testes were found. Between the urinary bladders and the colon lay a fibro-cellular structure, covered with peritoneum. It was not identified.

Facts are mentioned which support the application to a mammalian monstrosity of the results of experiments employing known causal agents upon non-mammalian animals (p. 483). The assumptions based on these experiments (mostly recent) are: (1) that abnormalities arise because of imperfect co-ordination in the developing zygote between parts of high metabolic activity and the other parts; and (2) that these highly active parts are most susceptible to the causal agents.

The sources of information upon the early embryology of the Dog are not numerous; but the general scheme of development is known, and, concerning certain aspects, a considerable amount of minute detail is available.

Posterior reduplications are of special interest in view of the assumptions of co-ordination and susceptibility, because, after the trilaminar stage of the embryonic area has arisen, metabolic activity is highest first in the future cephalic region. To this fact is added the observation that, in the specimen described, parts (tongue and perhaps testis) apparently unaffected by the teratogenetic agent, had, so far as available embryological evidence shows, a period of high initial activity in the interval between the periods of activity of organs or anlagen here reduplicated (hind-limbs, genital tubercle). The conclusion indicated is that, on the assumptions made, the cause, if single, operated at some time when all the future caudal parts of the embryo were active, and the rest were not, i.e. before the trilaminar stage. This is in agreement with the widely held belief that gastrulation (or what corresponds to it in Mammals) is the latest period of origin of symmetrical duplicities.

More definite determination of the time of action of the cause was impossible; but experiments on other groups of animals show that this period may be immediately after fertilisation.

Reduplication in the alimentary system apparently was confined wholly or almost wholly to the part caudal to the vitello-intestinal duct.

Lack of evidence of reduplication may vitiate any teratological record such as the present one, because reduplicated parts may have regressed. Histological methods are not always sufficient to obviate this difficulty, which can probably only be overcome by experiment.

Even although the vertebral column was not reduplicated here, it is improbable that, as Schwalbe (1907) suggested, fission and hyper-regeneration of a limb-bud would account for this type of abnormality. Relations of symmetry between limbs are important in this connection. A common source of difficulty in the determination of the symmetry of a limb is illustrated by the deformed right central hind-limb of this specimen.

Differences between the components of double monsters are fundamental, whatever their origin. Those present here, where the nervous system was single, are comparable with those between the two components of more nearly complete double monsters, with separate nervous systems. Therefore the latter do not, as Regnault (1925) has suggested, enable one to ascribe certain functions to the nervous system.

The differences in distribution of reduplications between the various cases of posterior duplicity are considerable. They may have to be accounted for by some factor other than the primary cause acting as assumed here; but it may well be that minute metabolic differences in the developing zygote are sufficient. Knowledge of this detail is yet inadequate.

Reasons for the continued study of such Mammalian monsters are indicated, and methods of systematic investigation are suggested.

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