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CONTENTS

No. 1. JANUARY

No. 2. JULY

No. 3. AUGUST

STUDIES ON SEX IN THE HERMAPHRODITE MOLLUSC CREPIDULA PLANA

I. HISTORY OF THE SEXUAL CYCLE

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EIGHTY-FIVE FIGURES

CONTENTS

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INTRODUCTION

It has been shown by various authors whose work will be briefly reviewed, that protandric hermaphroditism is common in that group of Prosobranch Molluscs known as the Calyptraeidae, the animals functioning as males during the early part of their lives and as females during the later part. The male and female phases are connected by a transitional period. It was suggested to the writer by Prof. E. G. Conklin in 1913 that if a detailed histological study were made of the development and history of the gonad in these protandric hermaphrodites, new facts might come to light bearing on the early differentiation of the male and female germ cells, and perhaps on other questions relating to hermaphroditism. Those curious structures found in the testis of so many Prosobranchs, known as the atypical spermatozoa, are also of interest in this connection. The problem has opened up even more possibilities than could have been anticipated, and I take this occasion to express my thanks to Professor Conklin for his suggestions and constant oversight of the work.

There are three species of the genus Crepidula, listed among the Calyptraeidae, obtainable along the Atlantic Coast; viz., C. plana, C. convexa and C. fornicata. The two last show some overlapping of the male upon the female phase, while in C. plana no resting oocytes are normally found in the gonad until the production of spermatozoa has entirely ceased. Upon C. plana the work described in this paper has been chiefly concentrated. The choice of this form for investigation was finally determined by the discovery that it possessed a most striking peculiarity, the dependence of the male phase upon conditions of the environment. The instability of the male phase will be discussed in a report shortly to follow the present paper; experiments will be recorded there to show how the male characters may appear and disappear as the environment is changed.

MATERIAL AND METHOD

The material was obtained from the Marine Biological Laboratory at Woods Hole, Massachusetts, and work was carried on there during the summer. Small compartments were arranged in one of the floating live-cars of the Laboratory, where the hermit crabs with which the Crepidulas are associated could be kept at about the same depth of water as that in which they are normally found. They could thus be under constant observation, and the Crepidulas could be transferred from one shell to another with some chance of their making a secure attachment in the new situation. It was also found feasible to transfer the Crepidulas to small glass dishes which were then put in the small compartments of the live car or in the salt water aquaria of the laboratory.

During the winter, some hermit crabs with Crepidulas were brought from the coast and kept in the salt water aquaria in the vivarium of Princeton University, where they lived fairly well, some for over a year. This situation did not prove so favorable, however, as the more nearly normal one in the live-car at Woods Hole.

In killing the specimens, a number of fixing fluids were used, of which the most favorable for general work was Bouin's picro-formol-acetic. This mixture will decalcify the shells as well as harden the tissues, and thereby avoid the distortion of the organs often caused by pulling the Crepidulas from their shells. For some of the finer cytological work Flemming's stronger mixture was used, and occasionally gave beautiful preparations, but it was very unreliable. Fixation with Flemming to which urea had been added, fixation at low temperatures, or in the dark, and other recent methods of technique with osmic acid fixing fluids, did not insure good preparations.

The results of the study are drawn from over a thousand slides, each containing sections of the gonad and goniduct of one individual Crepidula. Material was put up during every month of the year, with a view to observing any seasonal differences in the sexual condition.

Of all the common stains, iron haemotoxylin, either alone or in combination with eosin or light green, was most satisfactory. Delafield's haemotoxylin was occasionally used.

NATURAL HISTORY OF CREPIDULA PLANA WITH DISCUSSION OF FORMER WORK ON HERMAPHRODITISM IN CALYPTRAEIDAE

A. Relation with hermit crabs

Although the natural history of C. plana has been described by Conklin ('97) it is necessary for the sake of clarity in subsequent parts of the discussion to state some facts regarding its mode of life, which is intimately connected with and greatly dependent upon the activities of the large hermit crab, Eupagurus bernhardus. The hermit crabs may be picked up just below low-tide mark along flat, sandy beaches where the water is comparatively quiet. The Crepidulas adhere to the inside of the empty Gastropod shell which the hermit crab occupies, usually with their anterior ends directed toward the opening of the shell. Associated with each hermit crab may be a varying number of Crepidulas. Some of the shells used by the hermits are very large and afford room for the attachment of a great number of the small Molluscs. Those shells which give evidence of having been long occupied by the crab bear larger and more numerous Crepidulas than the shells where a glossy, pearly appearance indicates that the Gastropod has only recently died and the hermit assumed possession.

B. Formation of the shell

It was shown in Conklin's "Embryology of Crepidula" ('97) that the individual differences in the shape of Crepidula's own shell vary according to the surface on which the animals live and grow. It is convex on a convex surface, concave on a concave surface, thick when the mantle cannot be readily extended, thin when it can; for the shell is deposited by the mantle, and the latter readily adjusts itself over the substratum.

The shape of the Crepidula shell naturally shows striking differences in the sparsely populated 'new' hermit crab shells

and in the older, crowded ones; for as the swimming veliger settles and commences its growth, crawling about to secure a favorable location, it has a smooth, uninterrupted, slightly concave surface in the 'new' shell on which to rest; while in the already thickly populated 'old' shell it must adjust its growth over the shell of some large individual already in possession, or between two of them, or behind them. If it moves it will find itself, at the end of each small journey, on a new sort of surface, to which it must again adjust itself if it remains there long enough for the mantle to be extended and the shell substance secreted. In the 'new' hermit shells, inhabited by only a few Crepidulas which do not interfere with one another, those Crepidulas have thin, smooth, flat shells with few lines of growth or none; while in the crowded colonies the shells are more convex, thicker, and marked with numerous lines of growth, each of which indicates a movement to a new position, a growth over an irregular surface, or some other hindrance to the continuous free extension of the mantle.

Frequency of movement is a factor which affects the shell of Crepidula. It can be observed, in large colonies, that individuals close to the mouth of the hermit's shell do not often change their position, and their shells become intimately fitted to the surroundings. There is more shifting about among the individuals behind them in the deeper recesses of the hermit's shell; it is quite probable that these latter have a less advantageous position for securing food. The edges of their shells are smooth and rounded, not fitted to the underlying surface.

Conditions are different in 'new' hermit shells. When a swimming veliger of C. plana settles there, it finds a smooth uninterrupted surface; it quickly reaches an advantageous position and henceforth seldom moves. A thin, smooth shell, with almost indistinguishable growth marks, is evenly deposited and closely applied over the substratum.

C. Environmental polymorphism

Conklin has described the condition of 'environmental dimorphism' in C. plana. In the dead shells of Ilyonassa or Lit-

torina, inhabited by the little hermit crab Eupagurus longicarpus, are found 'dwarf plana.' They are exactly like the typical form but average only one-fifteenth the volume, have fewer cells in their tissues, and lay a little more than one-third the number of eggs. Their tissue cells and eggs are, however, as large as those of the typical form. The organs only are smaller. When these dwarfs are removed to a situation where they have plenty of room to grow, their size increases far beyond its former limits. The conclusion is that they are a 'physiological variety' merely, their smaller size being due to a stoppage of cell growth and division by the cramping of the environment.

From the writer's own experience it may be added that every possible gradation may be found between the dwarfs and the typical forms. Comparison can be made between adult females only, for up to the adult female phase the sexual condition, whether immature, male or transitional, has little relation to the age and size. The females differ most markedly in size even in the largest shells, and this variation is not all to be ascribed to age differences, as the lines of growth show; there are instances of cramping environment here as well as with the smaller hermits. When the dwarfed individuals are transferred to a fingerbowl or aquarium one observes with surprise how quickly they extend the mantle and begin to deposit shell in a thin layer. The thin rim of new shell may be evident the day following the transfer.

Now such an increase in growth rate may be inaugurated not only in dwarfed females, but in almost any of the small Crepidulas having a thick and convex shell and living in the crowded colonies. The new growth is most striking when they are placed in glass dishes of sea water with a perfectly smooth surface and nothing to interfere with the extension of the mantle. It is evident that in all crowded colonies there is a suppression of growth of the animals because they do not have unlimited surface on which to extend the mantle.

It has been found that the small Crepidulas kept in the inland vivarium during the winter have a very limited growth

compared with those kept at Woods Hole during the summer months. For instance, about fifty very small specimens (2 to 3 mm. in length) were taken from a consignment sent from Woods Hole to Princeton in November, 1915, and placed in a tumbler in the salt water aquarium. During five months following they showed hardly any growth at all. At the last of March no specimen in the tumbler was more than 4 mm. in length. A similar lack of growth was observed in larger specimens. At Woods Hole in the summer swimming larvae settled on some of the hermit shells in the float car and grew to considerable size, some up to 10 to 15 mm. length, within two months. How much of the difference in amount of growth in the two situations ought to be ascribed to the season of year will not be perfectly clear until the animals have been studied during the winter in their natural environment. It was noted, however, in June, 1916, that many of the specimens wintered in the vivarium without growing were beginning to exhibit a considerable margin of new shell, and it is probable that there is a marked seasonal difference in normal growth. Indeed this must be the case; for if growth went on as rapidly during the winter as in the summer, very few small specimens could be found in the spring, since the production of eggs does not continue to any extent during cold weather; but many small individuals can be found in the spring. The shape and color of the shell indicates clearly that they are not young, but belong to a brood of the previous autumn. Their growth has been retarded during the winter.

This ready response of the organism to the environment in the matter of growth is one of the conditions which so greatly complicate the study of the sexual cycle. In the large and crowded colonies, where many individuals are wedged in between and lying upon one another, possible dwarfing is so confusing a factor that it is necessary almost to disregard size when selecting material.

Summing up the facts already discussed in regard to growth, it may be said: 1) Apparently no rapid growth of the body of Crepidula plana can take place without free extension of the mantle and the deposition of new shell. a) Frequent movement

will interfere with this extension of the mantle. b) Cramped quarters will hinder or prevent it. 2) The amount of growth varies according to the season of the year.

D. Variations in motility of males

Males and other small individuals of C. plana are usually more motile than the large females (Conklin, '98). The writer has found many exceptions to this rule, however. Occasionally males are found immovably fixed. They are either wedged between two large individuals, or between a large one and an angle of the hermit shell, or they are placed on top of larger individuals where the irregularities of the surface of attachment were mirrored by the irregularities in the shells of the males. In all these cases the shell of the male was so conformed to the surroundings that there was no doubt of its having remained motionless during the growth of the shell. That they were functional males was confirmed by sections through the gonad of several of them. One small animal, which, as will be explained in a subsequent paper, was developing the male phase under experiment, remained for several weeks in the same position on top of the shell of a female, and in the meantime its own shell grew somewhat and fitted itself to the irregularities of the surface beneath it. The majority of males are undoubtedly motile however. In Crepidula fornicata the mature males in the 'chains' described by Conklin and Orton (see below) are completely sedentary.

E. Variations in sexual development

There is a peculiarity of Crepidula plana, already mentioned by Conklin ('98), which is as follows: Many individuals of this species are found, of the same sizes as the males, which do not exhibit the male condition. Neither do they yet show any indication of assuming the female phase. They are what may be called 'sexually indifferent' or 'neuter' animals. The gonad is rudimentary and resembles that of the very small and evidently immature specimens. The penis, sperm groove and seminal

vesicle are not developed. The shell is often flat, thin, symmetrical and without growth marks. From what has been said on shell formation it will be clear that the neuters must have developed on a smooth, unrestricted surface, and this is really the case; for they are most often found on 'new' hermit shells with no larger individuals present. They are the first occupants.

The writer finds that there are not only these sexually inactive animals of the same sizes as males, but there are also *newly developing males* and *males with the testis in a state of degeneration.* Evidently the gonad does not necessarily undergo male development, nor the accessory male organs appear, when the animal has reached any certain size. Given several specimens of the same size, some may be sexually inactive; some may have an immature testis developed to the spermatogonial, or spermatocytic, or spermatid stage, as the case may be, with the sperm groove and seminal vesicle forming and the penis growing out behind the right tentacle; some may be mature males; some may be males with the testis in any conceivable state of retrogression—one, several or all the stages of spermatogenesis lacking—and with the penis partly atrophied. Examples of all the above conditions are obtainable, in any size from 2 to 20 mm. length or even outside those limits, and they may all be found at many different seasons of the year. A partial analysis of this phenomenon by means of experiment has been attempted in a paper to follow the present one. At present it is necessary only to state that the conditions occur and that the case seems to be unique.

Orton has described ('09) the sex relations in the 'chain formations' (Conklin) of Crepidula fornicata. "Individuals of this species associate permanently in linear series to form 'chains.' All lengths of chain composed of as many as 12 individuals have been found." Orton showed that the largest, most proximal individuals in such a colony were usually females, the smallest and most distal ones males, and between the two were all stages from the male to the female phase including hermaphrodite forms. He pointed out that the proximal animals were the oldest members of the colony, and had undoubt-

edly been passing through the transition from male to female while the younger members were successively adding themselves to the chain. Orton found that in going from the male end of the chain to the female end there was a "transitional series from maleness to femaleness, both in primary and secondary sexual characteristics."

Orton believes that as C. fornicata grows older it gradually loses the power of attaching itself after being accidentally detached; that the foot muscles cannot relax and grasp a new surface after a certain age. Many observations have convinced the writer that the only difficulty in the way of re-affixation is the shape of the shell which has been deposited by the old specimens; if the edges of the shell are trimmed away, the animals will re-attach themselves and move about.

F. Genital organs of the Calyptraeidae

It will be of advantage to have a brief sketch of the anatomy of the reproductive organs in the Calyptraeidae. Reference should be made to the recent work of Scheidig ('13), Kleinensteuber ('13) and Giese ('15) as well as from the early researches of Haller ('92) and Plate ('94).

The gonad is a yellow or brown organ of an irregular, lobulated shape lying along the intestine and between the liver lobes in the short visceral sac. It may be relatively compact as figured by Giese for Calyptraea or it may be very diffuse, the lobules long and widely separated from one another, as in Crepidula plana. Anteriorly the gonad narrows down to form the duct. The latter is strikingly different in the male and the female phase.

In the male condition the proximal part of the goniduct is very much widened and twisted upon itself to make a convoluted seminal vesicle, for the storage of sperm. It is continued anteriorly into a much narrower, ciliated vas deferens which opens into the mantle cavity in the region of the shell muscle. From this opening a ciliated sperm groove runs forward along the right side of the neck until it reaches the large muscular penis just behind the base of the right tentacle. The groove is con-

tinued along the posterior side of the penis to the tip. The organ contains transverse and longitudinal muscle fibers and is capable of great extension.

In the female phase the reproductive organs are much increased in size corresponding to the increased size of the whole body. The goniduct is now transformed into a large ciliated tube with its inner wall thrown into longitudinal folds; it is about the same diameter throughout. It runs directly forward, without turning upon itself, to the region where the vas deferens formerly opened into the sperm groove; but we now find there a large uterus with thick glandular walls. It is usually ciliated on the inner surface. Several seminal receptacles for the storage of sperm open into it. They consist of non-ciliated sacs connecting with the uterus by small ciliated tubes. There may be one common tube or as many individual ones as there are receptacles. The shape of the uterus varies in different genera. It may be nearly straight or may be bent upon itself; it often shows a transverse segmentation externally. Two or three kinds of gland cells can be recognized in its thick walls by their structure and peculiar staining reactions. In some forms (e.g., Crepidula peruviana [Haller]) the uterus is said to have accessory glands which lie upon its surface.

A structure connected with the reproductive system and of doubtful function is the gonopericardial duct. It is present only in the female phase. It connects the pericardial chamber with the oviduct, joining the latter approximately midway between the ovary and the uterus.

The Calyptraeidae, like many other Prosobranchs, have, so far as described, the 'atypical' as well as the typical or true sperm.

OBSERVATIONS ON CREPIDULA PLANA

Establishment of the gonad and efferent duct

In the earliest stage in which the gonad has been observed, the primordium of the organ occupies a position in the anterior region of the visceral sac, underneath and to the right of that portion of the intestine, which after leaving the stomach runs

obliquely across the visceral sac. The gonad is minute. In one of the smallest specimens 39 nuclei of active germ cells were counted in serial sections. Allowing for those which may have been overlooked, there were at least no more than 50 germ cells present. At this time the gonad is triangular in cross section (fig. 1) with one side parallel to the intestine. The angle opposite this side is drawn out gradually into a strand of cells which runs forward and to the right, until it meets the extreme right posterior angle of the mantle cavity, where it ends. This strand is the anlage of the goniduct (fig. 2, *d*, and fig. 62).

The gonad is surrounded by a thin layer of connective tissue. The germ cells are contiguous to the connective tissue sheath, and are not all of the same nature. In the youngest specimens which it has been possible to obtain two types of germ cells may already be distinguished. Since the presence of dimorphic germ cells in so early a stage of development is significant in the light of the "indifferent germinal epithelium" theories, the cells will be described in some detail at this point.

Types of germ cells

Throughout the life of Crepidula plana, up to the assumption of the adult female condition, two types of germ cells, which we may call 'type A' and 'type B', are at all times present in the gonad and may be distinguished from each other in the following ways: 1) The type A cells are larger than the type B cells and have a different nuclear pattern. 2) The type A cells occur only in the periphery of the gonad, while the type B cells occur both in the wall and in the lumen. 3) The two types of cell have different times of maximum activity. 4) Two types of division figures are present which correspond to the two different kinds of germ cells.

1. In the type A series the nucleus is very transparent and the chromatin is disposed either in deeply staining masses or in strands which suggest a persistent spireme (figs. 1, 2, 3, 4, 7, etc.). Good fixation reveals the fact that these chromatic elements are of a duplex nature; they are composed of two short rods or strands lying side by side. The type B cells (figs. 1, 2,

3, 4, 14, etc.) have a reticular nucleus, the chromatin being disposed upon an indistinct network of linin whose meshes are dense enough to give the whole nucleus an opaque appearance.

Measurements of type A and type B cells have been made at different stages of the sexual cycle, and the results have been brought together in table 1. The average diameter of the A cells in any specimen is always greater than that of the B cells. The two series are grouped about different modes.

2. The type A cells, though they may become ever so numerous, do not ever come to lie in the lumen of the gonad. Their divisions always leave the daughter cells attached to the periphery. The type B elements, on the other hand, when undergoing a period of mitotic division, proliferate cells like themselves into the lumen of the gonad where they lie free, which continue to divide.

3. The significance of the dimorphic germ cells becomes evident when one observes their times of activity. As male development takes place great numbers of type B cells are produced and fill up and enlarge the lumen of the gonad. After their period of multiplication they become transformed into the spermatocytes and the cells which are destined to give rise to the atypical sperm.

The type A cells undergo their period of greatest activity after the male phase is over (if that occurs at all), and when they have become very numerous, are gradually transformed into the oocytes. Knowing the fate of the two series of germinal elements, we may speak of the type A cells as the primordial egg cells and the type B's as the primordial sperm cells. Crepidula plana produces its sperm and eggs at widely separated times, and correspondingly its primordial male and female sex cells undergo their periods of activity at different times. The presence of large numbers of either type of cell is accompanied by a paucity of cells of the other type.

4. There is no possibility that we are dealing with different phases in the activity of a single series, for two different series of division figures have been found, the behavior of each of which identifies it with one of the types of cells above described. Just

before the beginning of female differentiation, mitotic figures like those shown in figures 8 to 12 may be found in the germinal layer of the gonad, and only in the germinal layer. The direction of division may be parallel to the wall of the gonad, or somewhat oblique to it; in any case the daughter products always lie close to the connective tissue sheath; they are never found in the lumen. The chromosomes are comparatively large and very distinct. The only period of the sexual cycle when these mitoses have been observed in large numbers is the transitional stage which precedes the assumption of the female condition.

Contrasted with the dividing cells shown in figures 8 to 12 are those of figures 15 to 20. The difference in size is striking. The character of the metaphase figure is a distinguishing mark also; for in the larger mitoses (fig. 9) the metaphase plate is flat and the chromosomes are somewhat separated from one another, while in the smaller (fig. 16) it is sheaf-like and the chromosomes are densely packed together so that their individual forms cannot be traced, except in cells which the microtome knife has cut in two. These figures are found in great abundance during the period of spermatogonial multiplication and all through the male phase as long as the testis continues to produce spermatozoa. They represent the method of division of the primordial male cells and spermatogonia.

It has been said that in Crepidula convexa (as well as C. fornicata) there is an overlapping of the male upon the female phase. Perhaps it would be better to say that in these species the egg cells develop relatively early, attaining the condition of the resting oocyte while the male period is still at its height. The writer was interested to know whether there is any cell in these species comparable to the primordial egg cell of C. plana. Figure 5 shows a section through part of the gonad of a minute C. convexa, less than 1 mm. long, and probably only a few weeks after hatching. As the figure shows, there are cells in the germinal layer which resemble the primordial egg cells of C. plana, although they are smaller, relative to the spermatogonial cells in the lumen, than the type A cells of C. plana. Actually, all the germ cells of C. convexa are larger than those of the former species.

The diameters of primordial male and primordial female cells group themselves about different modes. Further, the mean diameter of each type is different at different periods in the life of the animal. In table 1 each mean diameter is taken from 65 cells chosen at random and measured with an ocular micrometer.

TABLE 1

	Mean diameter (in micra)
Minute animals	
Primordial egg cells	11.9
Primordial sperm cells	9.6
Spermatogonia in lumen	8.5
Developing males	
Primordial egg cells	12.6
Primordial sperm cells	10.8
Spermatogonia in lumen	8.7
Mature males[1]	
Primordial sperm cells	10.2
Spermatogonia in lumen	9.8
Neuter animals[2]	
Primordial egg cells	11.9
Primordial sperm cells	10.0
Neuter animals with large numbers of primordial egg cells	
Primordial egg cells	11.3
Primordial sperm cells	9.7
Incipient females (with oocytes going through nuclear changes)[3]	
Primordial egg cells	12.0

[1] In mature animals primordial egg cells are difficult to identify and no attempt was made to measure them.

[2] In neuter and young female specimens there are ordinarily no spermatogonia in the lumen of the gonad.

[3] Primordial sperm cells become very rare at this stage, and no attempt was made to measure them.

The follicle cells

At the early stage of development first described above there are besides the primordial male and female germ cells certain other elements in the germinal layer, in the nature of follicle

cells (figs. 2, 3, etc., *f*). The nuclei are sometimes hard to distinguish from the smallest of the primordial male germ nuclei, but can usually be identified by their deeper stain. After prolonged staining in iron haemotoxylin they will remain a dense black when the germ nuclei have been so destained that their chromatic pattern is clear; and in Delafield's haemotoxylin the follicle nuclei take a deeper blue than the germ nuclei. No mitotic figures have ever been found which could be ascribed to the follicle cells, although there are certain periods in the sexual cycle (e.g. at the beginning of the female phase) when their number is rapidly increased.

In this connection we must consider the relation of the germ cells to the so-called 'basal cells' described in certain Molluscs. The authors of the earliest work on the origin of germ cells in Molluscs were of the opinion that the spermatogonia of the male arose from the large 'basal nuclei' found in the germinal layer. Some even derived the spermatogonia from these elements by amitosis (Von Brunn, '84). Other writers on the contrary described the basal nuclei as arising from spermatogonia. Lee ('97), Ancel ('03), de Bruyne ('03) and Kleinert ('09) interpret their material as showing that the germ cells and basal cells have a common origin out of an indifferent germinal epithelium. Kuschakewitsch ('13) states that he has followed the formation of the basal cells in Vermetus out of an indifferent layer; and is of the opinion that the young, relatively small basal cells have the power of mitotic division; but when they have reached their full development, though they may make an effort at such a division, it is never completed. Earlier authors have also expressed the opinion that the basal cells have lost the power of mitotic division. These basal cells are generally considered to be nutritive elements. Reinke ('14) traces the apyrene spermatoblasts of Strombus back to them.

In Crepidula the writer does not find an "indifferent germinal epithelium" of the nature described by other authors; but rather, two different categories of germ cells lying in what appears to be a follicular syncytium whose nuclei are small and stain characteristically. There are no large basal nuclei except the nuclei

of the primordial egg cells. If there are cells with a nutritive function they must be those of the syncytial layer. As has been said, the follicular cells have not been seen to divide by mitosis, at any period. The fact of amitotic division is hard to establish; but some indications of it have been seen, and it has been described in the follicle cells of the gonads of other and widely separated groups of animals.

Male development

Let us now return to a consideration of the general development of the young gonad. In the postlarval stage there may (fig. 3) or may not (fig. 2) be a small lumen in the rudimentary sex gland. The presence of a few spermatogonia in the lumen is not a sure sign that male development is about to take place. The free cells may remain inactive and degenerate, giving rise to homogeneously staining bodies such as appear in figure 1. The gonad shown in figure 1 is that of a more minute specimen than those shown in figures 2 and 3, yet spermatogonial cells have already been formed and cut off into the interior, and subsequently died instead of continuing their development, as their irregular shapes and homogeneous stain give evidence.

With further growth of the young Crepidula the gonad becomes enlarged and more irregular in shape. Processes are put out. These are not constant in number or direction; for the shape of the gonad depends upon the spaces which it may occupy between the lobes of the digestive gland or between the digestive gland and the intestine, and in different specimens the condition of the digestive gland and the amount of distention of the alimentary tract vary. One arm of the gonad usually grows to the left and downward, following the outline of the intestine. This seems to be the first direction of growth, and the process so formed sometimes becomes temporarily larger than the central part of the gonad. Growth also goes on posteriorly. The arm which reaches to the right and forward to join the goniduct becomes drawn out to considerable length, even into a tube or strand as small as the duct itself; yet readily distinguishable from it by the structure of its cells.

The gonad is now a small, ramifying body, lying between the alimentary tract and the digestive gland, and sometimes extending between the lobes of the latter. This condition may be retained for a long time. As has been said, 'sexually inactive' animals are often found in which the gonad is in an entirely undeveloped condition. Some animals have been kept in the aquaria for long periods without showing any signs of male development (e.g., outgrowth of penis), and when killed and sectioned they showed only a rudimentary gonad; while others during the same time became adult males.

Male development is inaugurated by the rapid division of the primordial male cells, cutting off spermatogonia which lie free in the lumen; and by the enlargement of the whole organ. The spermatogonial cells continue to divide rapidly, if male development goes on without interruption, and the lumen becomes solidly filled with them. The rapidly enlarging gonad, following the lines of least resistance, puts out new extensions which become swollen into follicles reaching in all directions. The ultimate shape of the gonad depends upon the position of the surrounding organs. The pressure of the latter affects the gonad differently in different animals; for the testis is of varying shape and extension. It sometimes reaches far forward, sometimes far backward toward the posterior end of the visceral sac, sometimes around the intestine to the left side of the body, and so on. In a large testis growth may have gone on in all these directions.

The cells which fill the lumen of the immature testis at this time (spermatogonia) are all of the same appearance. The cytoplasm is very meagre in amount compared to the size of the nucleus, and the outlines of the cells are somewhat irregular (fig. 24); in the case of cells lying close together the outlines are often hard to distinguish. The structure of the nucleus is exactly like that of the type B cells in the germinal layer, from which the spermatogonia have arisen.

Coincident with the rapid growth and frequent division of the spermatogonia, there occurs a certain increase in the number of the primordial egg cells. Their number in proportion to that of the spermatogonia of course becomes less and less during the

multiplication of the latter; for the primordial egg cells are found only in the germinal layer, while the spermatogonia fill the lumen as it grows larger and larger. Actually, however, the type A cells increase in number, and their presence is more noticeable than at any other time in the history of the gonad up to the initiation of the female phase. Their prominence is partly due to the fact that their chromatic bodies become more distinct and stain with greater intensity than at other times. There is not the slightest indication that these cells take part in the formation of any of the products of the testis, however, and it seems probable that they undergo a limited amount of division which is correlated with the enormous development of the gonad as a whole. Rarely one may find their large and characteristic division figures at this time (fig. 9).

The cells filling up the lumen of the gonad (fig. 3), all alike so far as visible characteristics go, follow two divergent paths of development after the close of the multiplication period; one of which leads to the development of the typical sperm capable of fertilizing the egg, while the other ends in the formation of the atypical sperm, which in Crepidula are of the 'apyrene' variety. Following the terminology of Meves ('03) the word 'apyrene' is used to describe atypical spermatozoa which in the adult condition contain no vestige of chromatic matter; 'oligopyrene' refers to those which contain a little chromatin; the true fertilizing elements are known as the 'eupyrene' spermatozoa.

Spermatogenesis. Historical. A. The typical series. In recent times the spermatogenesis of the true sperm of some Prosobranchs has been fully worked out (Meves, '03, on Paludina; Kuschakewitsch, '13, on Conus and Vermetus). The figures of the spermatocytes of the first and second orders, maturation divisions and spermiogenesis given by other authors have been compared with the conditions found in the testis of Crepidula, and so far as the observations have gone there are no striking peculiarities which would warrant a detailed description of the eupyrene spermatogenesis of Crepidula plana. The development of the spermatocytes will be carried only far enough to show their different behavior from the cells which are destined

to form the atypical sperm. Since there have been some conflicting accounts of the origin and development of the atypical elements it has been thought worth while to follow their development in C. plana.

A. The atypical series. The question of the atypical spermatozoa of Molluscs has had the attention of investigators from time to time since 1837, when they were discovered in Paludina by Von Siebold. Three of the latest articles on the subject, viz., those of Kuschakewitsch ('13), von Kemnitz ('14), and Reinke ('14) give historical reviews of the work of previous authors.

There has been the widest speculation as to the function of the atypical elements but no conclusive proof. Goldschmidt ('16) believes that these structures in Molluscs and probably similar ones in Insects are functionless by-products.

Two views have arisen in regard to the origin of the atypical sperm. Meves ('03), Stephan ('03), Kuschakewitsch and others find their origin similar to that of the true spermatozoa, their development divergent in various degrees from true spermatogenesis. Reinke on the other hand concludes that the apyrene sperm of Strombus do not come from spermatogonia at all, but from 'basal cells,' accessory elements of the testis; and that their development is not comparable to spermatogenesis. Reinke rejects the use of the terms 'apyrene spermatocyte' and 'apyrene spermatid' on account of the restricted meaning of 'spermatocyte' and 'spermatid' which refer to maturation phenomena. He designates the cell the 'apyrene spermatoblast' from its first appearance to the breaking down of the nucleus and scattering of the centrioles, and the 'apyrene spermatosome' during the subsequent changes up to the adult apyrene spermatozoon.

Although the origin of the apyrene sperm in Crepidula is not the same as that described for Strombus, and the end-product has an altogether different structure, the early developmental stages, at least, are so similar that it has been decided to retain Reinke's terms for the periods of differentiation, viz., 'spermatoblast' and 'spermatosome.' It is to be understood, however, that while the spermatoblast stage of Reinke begins with a cell

which is distinct from the spermatogonium and which is believed to have a different origin, the spermatoblast in Crepidula arises from a cell which cannot be distinguished from the spermatogonia of the true sperm; the cell will first be called a spermatoblast when it can be distinguished from those which are destined to undergo the maturation divisions.

Development of the apyrene sperm in Crepidula plana. After the spermatogonia which have been cut off from the peripheral layer of the gonad have undergone a number of divisions in the lumen, the progeny differentiate into primary spermatocytes of the eupyrene sperm, and spermatoblasts of the apyrene sperm. Figure 4 represents a section through an immature male gonad in which the apyrene spermatoblasts (*spb*) are just beginning to be distinguishable. They are about the same size as the cells which are to grow into the primary spermatocytes (*spcI*), but their relation of cytoplasm to nucleus is different. In the spermatogonia and spermatocytes of the true sperm, the nucleus takes up most of the body of the cell, the cytoplasm being restricted to a thin layer. In the spermatoblast the nucleus is smaller and lies eccentrically. The spermatoblast contains a cytoplasmic granule which is more prominent in the atypical cells than in the typical, though it occurs in all types of germ cells. This body, the nature of which is not clear, disappears during the later changes in the cell and leaves no trace. It is more fully discussed in the description of figure 4.

In the section through a young testis shown in figure 6 the differentiation of the typical and atypical series is more marked than in figure 4. Both the young eupyrene spermatocyte (*spcI*, at top of figure) and the apyrene spermatoblast (*spb*) experience a certain amount of growth at this time; but the growth of the spermatocyte is chiefly a nuclear growth, while that of the spermatoblast consists in an increase of cytoplasm, the nucleus remaining small. The chromatin of the spermatocyte begins to form the leptotene thread. The chromatin of the spermatoblast becomes aggregated in small masses, and the reticular condition of the nucleus, indistinct in the spermatogonial stage, can no longer be made out.

Figures 24 to 40 give successively the main steps of development of the apyrene spermatozoon. As the spermatoblast increases in size (fig. 25) it becomes distinctly different from the spermatogonium (fig. 24). Two centrioles (fig. 25, *co*) make their appearance in the cytoplasm and very near them a large capsular body to which will be applied the somewhat unsatisfactory term 'idiozome' (figs. 25 to 28, *i*). The proximity of this body to the centrioles, and the strands which have sometimes been seen reaching from it toward the centrioles, indicate that the centrioles have come out of its interior. A few doubtful cases have been seen where the centrioles seemed to be within the idiozome. There are no lines radiating from it into the cytoplasm as in the large 'centrosome' described by Reinke. It arises suddenly and cannot be connected with a previously existing centrosphere; it disappears and leaves no trace behind. During the spermatoblast stage the cytoplasmic granule referred to above is also very prominent (figs. 25, 26, 27, *x*) but later cannot be seen, or at least cannot be distinguished from chromatic matter which has been thrown free into the cell body from the nucleus.

The breaking up of the nucleus of the spermatoblast (figs. 26, 29, 30) and the division of the centrioles (figs. 27, 29, 31) constitute the change to the 'spermatosome.' The nuclear membrane dissolves and immediately the chromatic elements within the nucleus form into 'karyomerites,' or chromatic masses which come to lie free in the cytoplasm (figs. 30, 31, *ko*) and become scattered throughout the cell. The number, size and shape of these bodies is variable. The smallest of them are no larger than the chromosomes of the first maturation division of the eupyrene series, and, like the latter, are short and thick, and rather regular in shape. Others are much larger and very irregular. The clumps of chromatin are often so large that all the chromatic substance is contained in three or four of them. It appears that in such cases parts of the nucleus become constricted off and pass over directly into nuclear vesicles, i.e., the several lobes of chromatic material (fig. 29) do not break up into small karyomerites, but immediately form chromatic

vesicles as in figure 32. If the nucleus does form a large number of karyomerites (fig. 30) there is usually a subsequent fusion into fewer and larger elements (fig. 31).

The division and dispersal of the centrioles accompanies the disintegration of the nucleus. Previous to this time the number of centrioles most frequently seen is two (figs. 25, 26), occasionally four (fig. 27). They then usually become so dispersed or so much reduced in size by repeated division, they they cannot be identified at all (fig. 30) until they once more gather in a group at the periphery of the cell (fig. 32). There are occasional exceptions to this; one may, rarely, find the centrioles scattered in the cytoplasm during the stage of the karyomerites (figs. 29, 31).

In the mature testis, cells like that shown in figure 28 frequently occur. They resemble none of the stages of the eupyrene spermatogenesis, and they are probably apyrene spermatoblasts just previous to the disintegration of the nucleus; they appear to be forming indistinct pachytene threads in the nucleus which might be compared with those of a late stage of a eupyrene spermatocyte of the first order (fig. 6). Certain of the spermatoblasts in which the nuclear membrane has just been dissolved show their karyomerites gathered into a spherical mass as if they had been previously contained in a spherical nucleus (fig. 28) instead of an irregular one (fig. 26).

As stated above, where a large number of karyomerites have been formed they subsequently fuse together to make nuclear vesicles (figs. 31, 32). Several or many karyomerites group themselves together irregularly and begin to fuse in such a manner that their chromatic substance forms a shell, of irregular thickness, around a hollow interior, in which a coarse chromatic reticulum appears. There may be a few large nuclear vesicles, or many small ones.

The formation of the axial fibers now begins and the nuclear vesicles undergo degenerative changes. Neither of these two processes seems to be dependent on the other for either one may go on with greater speed than the other. In some cases the most of the chromatic matter has disappeared before the axial

fibers begin to grow (fig. 33); in others many nuclear vesicles remain when the axial bundle is much longer than the cell (fig. 36).

The outgrowth of the axial fibers takes place in a manner similar to that described for other atypical sperm. The centrioles gather at the periphery of the cell (fig. 32) and become grouped closely together. From each centriole a flagellum grows out of the cell, while at the same time each centriole divides and the migratory daughter elements move away through the cytoplasm, forming between them and the stationary daughter elements, the axial bundle. The migratory daughter centrioles grow straight through the body of the cell, apparently not through its greatest diameter, however; and before they reach a surface again they disappear (figs. 34, 35). The fibers which they have formed continue to grow and protrude from the cell. Before emerging from the cytoplasm the fibers are gathered into a very compact bundle (fig. 36) at their anterior ends. While the axial bundle is elongating the flagella at the posterior end of the cell experience some growth though much less than that of the axial bundle.

The axial bundle, as it grows out of what may be termed the anterior end of the cell, carries part of the cytoplasm with it; and the longer the bundle becomes, the more of the cell substance is disposed along it (fig. 38) until all the cytoplasm is used up and the spermatozoon reaches its adult form (figs. 39 and 61). When adult it is about one-third the length of the eupyrene sperm (fig. 60). The cross section of the apyrene sperm (fig. 39*c*) shows that the axial fibers are arranged about the periphery of the cytoplasm which has been drawn out from the cell body, and that there is a darkly staining core running through the center. This core begins to be evident at about the stages of figures 36 and 37, or even previous to that time. In the earlier stages it can be seen when looking down on the cell as in the above figures; but when looking at a cross section of the cell as in figure 40*a*, the axial bundle appears as a flat plate in which the individual fibers are not clearly separated and in which the core is not definitely shown. Sometimes a slight thickening can be

observed running through the middle of the axial plate (fig. 40*a*). A cross section through the cell body of a stage similar to figure 38 (fig. 40*b*) shows the core surrounded by axial fibers.

Nothing has so far been said concerning the number of the centrioles. It has not been found possible to count them in the early stages, because of the presence of other structures with which they may be confused. For instance the cytoplasmic granule seen in the early spermatoblast stage sometimes appears to persist after the disintegration of the nucleus; and there are often minute particles of chromatin free in the cytoplasm which are difficult to distinguish from the centrioles. In the adult apyrene spermatozoon, however, the fibers lie at the periphery of the cytoplasm, somewhat separated from one another, so that they can be observed in cross section. There appears to be either eight, nine or ten of them; it is possible that there is a constant number, some staining indistinctly and being overlooked; in which case the largest number counted (10) would be the correct one. The number of fibers would be (supposedly) the same as the total number of centrioles before the formation of the axial bundle. Actually the number of fibers in the adult apyrene spermatozoon seems to be less than the number of centrioles which congregate at the edge of the spermatosome, but this again is uncertain on account of the possible confusion of centrioles with chromatic elements, etc. The possibility is suggested that some of the fibers have taken a central position and become the core of the spermatozoon.

Returning now to the nuclear vesicles: in the young spermatosome these were composed of a shell of chromatic material surrounding a coarse chromatic reticulum. While the axial bundle is increasing in length the reticulum becomes gradually lost, leaving only a chromatic capsule containing a clear nuclear sap or karyolymph (figs. 34, 35, 36). From this point on there is a gradual dissolution of the nuclear vesicles, the thinner parts of the capsule disappearing first and leaving the thicker portions as crescentric bodies which themselves finally vanish. The chromatin has always disappeared before the cytoplasm of the cell is all disposed upon the axial bundle.

The foregoing development is the normal one and leads to the formation of the adult atypical sperm shown in figures 39 and 61; but this development does not always run through to completion in the manner described; for at any point in the development a peculiar sort of degeneration may set in. It results in a swelling of the cell body, a change in the staining reaction, and often the formation of large vacuoles. Some of the results of this digression from normal development are shown in figures 53 to 59 (on a smaller scale than figures 24 to 39). There is always a marked swelling of the cell body. The cytoplasm of these swollen and distorted cells is more homogeneous than the cytoplasm of the normal apyrene cells and takes a deeper eosin or light green stain. It is not true that the cells are dead; for when the contents of the testis of a live Crepidula are transferred to a slide, some of the distorted apyrene spermatosomes are seen to be in rapid movement. It is hard to escape the conclusion that this change is a purposeful one, and that the metamorphosed cells may have some nutritive function.

Some of the abnormal apyrene spermatosomes in Crepidula resemble the normal ones figured for other Molluscs. For instance, cells have been occasionally found on the slides which are extremely like the spermatosomes in Strombus (Reinke) when the albuminous bodies are in process of formation; and figure 58 has a very noticeable resemblance to Kuschakewitsch's figure 199 of the nearly adult apyrene spermatozoon of Vermetus. It is believed that this digressive method of development of the atypical cells in Crepidula is not a casual matter, a mere failure to complete the normal course, but that it is the expression of principles working out normally in other forms.

The abnormal apyrene spermatozoa are only occasionally found in the seminal vesicle with the normal apyrenes. The few that are present there show that degeneration has set in late in development.

The cells of the apyrene series in the testis are very much fewer than those of the eupyrene series. In the seminal vesicle there are about 6 eupyrenes to 1 apyrene. No apyrenes have been found in the seminal receptacles of the female. It is not

easy to see why they should not be found there, especially if the hypothesis of Giese is correct that the slender tip of the penis is inserted directly into the seminal receptacles; for in that case the ciliary action in the sperm groove of the male would probably carry the apyrenes along with the true sperm. The apyrenes are not so large nor so awkwardly shaped that they could not go into any aperture that the eupyrenes would be likely to pass. It is possible that the apyrene spermatozoa are passed into the seminal receptacles of the female, but that they speedily degenerate.

In smears taken alive from the seminal vesicle of the male, the apyrene sperm are seen to have a serpentine movement. They are not always in motion; some may be quiescent for a time, following which a wave-like movement proceeds rapidly from one end of the sperm to the other. After having been on the slide for some time the quiescent periods become longer and longer, and the movement less and less frequent. The eupyrene sperm, of the other hand, continue to move constantly, although more and more slowly until they cease entirely. The eupyrene sperm when left on the slide for some time will agglutinate; the apyrenes never do.

Giese ('15) has described the atypical spermatozoa of Crepidula unguiformis as 'oligopyrene,' i.e., with a small amount of chromatic material persisting in the adult structure (Meves). The three American species of Crepidula all have 'apyrene' sperm (without chromatin when adult). Giese's figure of the adult atypical sperm of C. unguiformis exactly resembles an immature stage in C. plana. Furthermore, Giese mentions a "fibrous secretion of unknown origin" in the seminal vesicle. It is suggested that he failed to recognize the adult apyrene element.

The development of the atypical spermatozoa in Crepidula differs from most of the other forms which have been described in that the spermatoblast does not outstrip the eupyrene spermatocyte during the growth period. On the contrary the spermatoblast and spermatosome remain about the same size as the fully grown spermatocyte, until the cytoplasm begins to apply itself to the axial bundle.

The adult apyrene sperm is shorter, in relation to the eupyrene, than in many other Molluscs where it has been reported. The apyrene sperm measures about 0.05 to 0.06 mm., the eupyrene about 0.16 to 0.17 mm.

As has been shown, the apyrene spermatoblasts are derived from cells which cannot be distinguished from the spermatogonia of the true sperm; yet their development is in no way parallel to that of the latter; there are no divisions of the spermatoblast after it has been differentiated. About the only suggestions of a relation with the spermatogenesis of the eupyrene series are, first, the occasional attempt by the spermatoblast nucleus to form a pachytene thread; and second, the slight resemblance of some of the karyomerites to the chromosomes of the first maturation division. The course of development in Crepidula, in the early stages at least, is more nearly like that of Strombus as described by Reinke, than like any other that has been described hitherto; yet Reinke has traced the apyrene spermatoblasts of Strombus to the 'basal nuclei' and does not consider them similar to the spermatogonia.

The present writer desires only to add the facts which have been observed in his material to the literature of this subject, in the belief that a sufficiently wide comparative study of atypical spermatozoa will eventually reveal their true significance. Whatever part they play in life processes, if any, they are interesting to one who would study the behavior of non-nucleated motile bodies of protoplasm. The writer feels also that the knowledge of atypical spermatozoa is not yet sufficiently inclusive to warrant dismissing them as mere 'functionless reaction-products.'

The adult testis

The testis, consisting of many lobules or follicles, shows great variation in size and in activity in different individuals. In the most active condition all the stages of spermatogenesis and of apyrene sperm development are found in a single specimen. The spermatogonia are more numerous at the periphery of the gonad, the later stages near the center. When new spermato-

gonia are forming in large numbers they make a complete germinal layer at the outside; but in less active testes the connective tissue sheath may be almost bare of cells, although all stages of spermatogenesis are present in the interior. At intervals along the sheath, however, there are always a few primordial male cells, a few follicle cells, and a few primordial female cells, attached to the connective tissue.

Follicle cells do not seem to be functional during the male phase, for they do not multiply in sufficient numbers to keep pace with the growth of the gonad during male development. In the very young specimen (figs. 1, 2, 3) and in the sexually inactive animals (fig. 50), the follicle nuclei are everywhere present in the germinal layer. The cytoplasm belonging to them is great in relation to the size of the nucleus, and is extended along the connective tissue sheath; in this layer of cytoplasm the true germ cells are embedded. No cell boundaries can be seen between the follicle nuclei; the latter often lie curved about the germ cells. It is evident that during the rapid extension of the gonad to form the testis, the follicle cells do not divide as fast as the others, since they are present in so few numbers in the adult male. Just what element of the testis does furnish the nourishment of the developing sperm cannot be stated. It might be suggested that the degenerating apyrene spermatosomes have some such function, in view of Reinke's ('12) comparison between the apyrene sperm of Strombus and the nurse cells of other Molluscs; but in Crepidula the spermatozoa are never gathered about any cell in the testis. They do not remain in the follicles long after they have become adult, but find their way at once into the seminal vesicle.

The primordial egg cells are also much less numerous, relatively, than they have been at any time previous to the adult male phase. They occur occasionally, either in the form previously described, or in a more faintly staining condition in which the chromatic bodies are hard to distinguish. In figure 41 are two which are so greatly changed that if it were not for transitional forms they could not be identified as primordial egg cells. The figure does not show how much they differ in stain-

ing reaction from spermatogonia and the young spermatocyte in the same region.

Similar stages of spermatogenesis are usually found grouped together; and when the spermatogonial or first and second maturation divisions take place, mitotic figures occur in groups. Many developmental stages of both kinds of sperm are found in a single follicle of the testis, however. There is very little difference in the amount of activity in widely separated regions of the gonad. Examination was made of sections through posterior follicles, middle part and anterior follicles in all phases of the sexual cycle; in immature males spermatogenesis was found to be slightly more advanced in the middle portion; but development or degeneration affects the whole organ.

History of the gonad from the male to the female phase

A. Regression of the testis. The first sign of lessened activity in the testis is the absence of some of the stages of spermatogenesis. The later primary spermatocytes are almost always the first to be missing. Many testes show spermatogonia, spermatids and sperm, but no spermatocytes. This seems to indicate that the cessation of activity consists in the failure of the young spermatocytes (which cannot be distinguished from spermatogonia) to undergo the growth period and acquire the leptotene nucleus. It does not prevent the fully grown spermatocytes from undergoing the maturation divisions and becoming spermatids, apparently, for in ordinary cases degenerating spermatocytes of the first and second orders are not seen.

Correlative with the disappearance of the spermatocytes there is a slight shrinkage of the testis away from the surrounding organs. Sometimes this shrinkage occurs even while the spermatocytes can yet be found; in fact shrinkage of the testis cannot be taken as a proof of impending loss of the male condition, though always accompanying it.

Later the number of spermatocytes becomes less and less, and some of the remaining ones begin to stain diffusely; more open spaces appear in the lumen of the gonad than formerly; the

spermatogonia no longer stain like living cells (fig. 42). It will be recalled (p. 17) that in the immature animal when male development does not immediately follow the production of spermatogonia which are set free in the lumen, they may degenerate and form amorphous bodies (fig. 1). The process during degeneration of the testis is the same.

The testis now contain only spermatids, sperm, and degenerate cells which are mostly spermatogonia. A few spermatocytes degenerate, but the majority complete the maturation divisions. The spermatoblasts and some of the spermatosomes of the atypical series suffer the same fate as the spermatogonia; but the majority of the later stages must complete their development, for only a few late spermatosomes are found degenerating.

In the meantime the transformation of the spermatids into spermatozoa, the passage of these into the seminal vesicle, and the shrinkage of the testis, continue. Eventually the gonad shrivels until nearly all the follicles are drawn in and the main sub-divisions of the organ remain as strands reaching here and there in the visceral sac, retaining their connection with one another. During the reduction, part of the connective tissue sheath is cast off into the perivisceral spaces.

During the shrinkage, marked changes have taken place around the periphery of the gonad. The reduction in size brings the few widely scattered cells found next the connective tissue sheath in the male phase, close together so that they form a continuous layer. This layer consists of primordial egg and sperm cells and follicle cells. Once more we have a follicular syncytium in which the germ cells are embedded. The chemical changes attendant upon the dissolution of the testis cells at this time seem to affect the primordial germ cells and make them harder to identify (figs. 44, 45). It has been said that during the male phase the primordial egg cells are changed in appearance, and this is true of the period of regression. The primordial sperm cells are also hard to distinguish. Their nuclei become distorted, as are those of the follicle cells, and conditions in general are hard to interpret. Some of the primordial germ cells of both types are, however, always distinguishable and there is no

doubt that they persist through the transition period without losing their real differentiation, though the form and staining reaction of some of them are affected by the chemical changes in the gonad.

The newly formed layer of cytoplasm all around the periphery of the gonad belongs, in all probability, to the follicle cells, although the cytoplasm of the germ cells which lie in it is not always distinguishable from the syncytium. It will be recalled that in immature and neuter animals there is also a syncytium around the periphery of the gonad containing follicle nuclei (figs. 1, 2, 3, 4, 50); and if regression of the testis takes place in small males, as it may do, the female condition does not immediately ensue, but the gonad returns to the neuter condition pending further growth of the animal. In such cases the gonad assumes the same appearance as in any neuter animal, the germ cells and follicular syncytium being present exactly as in a specimen which has never developed a testis. This has been observed in specimens which were brought into the laboratory as small males and later lost the male condition.

In the shrunken gonad the cytoplasm of the follicle cells is very loose and vacuolar (figs. 42, 43, 44, 45). The surface presented to the lumen is very irregular. The degenerating cells in the lumen becomes more or less fused into a mass. Even before this happens, however, processes from the peripheral cytoplasm reach out and join them (fig. 42), and the cells finally become enclosed within it (figs. 43, 46). The absorption and solution processes result in a thickening of the cytoplasmic layer. Many granules appear within it, which may be the result of coagulation of the cytoplasm itself, or the remains of the dissolving cells, or both. When all the remaining material in the lumen has been dissolved, typical germ cell nuclei again become evident in the periphery and a concentration of the cytoplasm, or a change in the staining quality of the cytoplasm, takes place about them (fig. 47). This results in the germ cells becoming more and more clearly distinguishable from the loose cytoplasm of the thick syncytial layer (fig. 48). Part of this thick layer itself seems to dissolve later, leaving faintly staining strands of a loose network, and

scattered granules. Part of it, however, containing the follicle nuclei, concentrates about the germ cells (fig. 49).

It is of interest at this point to refer to the observation of Buresch ('11) on the hermaphrodite Pulmonate, Helix arbustorum. This animal passes from a male to a female condition and during the transition period there is a series of marked degenerative changes in the gonad as there are in Crepidula plana. The process is very different, however, from that in Crepidula; in Helix arbustorum not only the male germ cells, but also the germinal epithelium and the follicle cells break up and are destroyed. The oocytes are then already in the resting or growth stages and the destruction of the germinal epithelium throws them into the lumen of the gonad, where they take up the dissolving cell elements as food and thereby grow rapidly. They then make their way to the uterus, meanwhile undergoing the maturation divisions.

In Crepidula plana the germinal epithelium, instead of being destroyed as in Helix, becomes thicker and more prominent than it has been during the male phase, and the follicle cells are more numerous than ever before.

B. Development of the ovary. In a large degenerate male, the beginning of female differentiation may begin immediately after the changes which we have been considering. As the primitive germ cells assume their characteristic appearance in the germinal layer, the primordial egg cells exceed the primordial sperm cells in number, and the nuclear pattern of the former becomes more distinct (fig. 49) than it has been at any time since the young testis was in the early stages of development. Follicular nuclei are present in large numbers. Whether the excess of primordial female over primordial male cells at this time is due to multiplication of the former or disappearance of some of the latter, or to both, is not certain; for since the number of eggs is very small compared to the number of sperm, the number of oogonial divisions is also very small and the mitotic figures are hard to find. It is certain, however, that from the stage of figure 49 on, periods of oogonial division may occur at intervals, and some of the material has fortunately been fixed while

division was at its height. Figure 51 is drawn from such material.

It is not intended to make a detailed report in this paper of the changes in the young oocytes leading up to the resting condition. An account of these processes is reserved for a later publication. Female development of the gonad will therefore be followed only briefly.

The various appearances of oogonial mitosis have already been summarized in figures 7 to 13. The last four of these figures were all taken from the same preparation as figure 51. The double chromatic bodies of the resting nucleus grow more distinct in preparation for division and elongate, forming the prophase chromosomes. It has not yet been made certain whether each chromatic body is concerned in the formation of a single chromosome, but probably this is not the case, for the chromatic bodies of the resting nucleus appear to be much more numerous than the prophase chromosomes. In contrast to the chromatic structures in the resting nucleus, which are duplex, the prophase and metaphase chromosomes appear, so far as they have been investigated, to be single; yet when the daughter elements separate in the anaphase they are sometimes double (fig. 10).

A certain phase of recent work deserves brief comment here. Kleinensteuber ('13) and Giese ('15) assume a very simple process of oogenesis in the Calyptraeidae which they have investigated, including Crepidula unguiformis. Giese's figures purport to show a direct transformation of epithelial cells into growing oocytes. In the three American species of Crepidula the present writer finds a complicated series of nuclear changes preceding the growth period of oocytes. Such a series has also been found in other Gastropods (Paludina, Popoff, '07; Helix, Demoll, '13). The question arises whether the material used by Giese and Kleinensteuber may not have been fixed at a time when the nuclear changes in the oocytes were already completed.

In figure 51, not only are the oogonial divisions going on, but the nuclear changes in the oocytes are under way. Synizesis is very common in this specimen. It will be observed that there are now no cells free in the lumen, which is indeed very small.

Figure 52 shows subsequent stages. The originally thin threads which went into synizesis become thickened after emerging and form the so-called 'pachytene' nucleus, which is not shown in figure 52, but which soon assumes the appearance of the nucleus in the extreme lower left-hand corner of the figure; i.e., the pachytene threads (which are plainly double) become shortened and thickened. Some of them are always oriented radially to the nucleolus, which at this time is undergoing marked growth. They then lose their sharp outline (fig. 52, upper left-hand corner) and their chromatin begins to distribute itself upon a reticulum, while the cytoplasm of the cell increases in volume. Thus the oocyte acquires the resting condition of the nucleus and enters upon the growth period.

During the formation of the resting oocyte the frequency of primordial male cells becomes less and less, and they are not seen dividing. The follicle nuclei become larger than before (figure 52 is drawn at the same magnification as figures 49, 50, and 51) so that they sometimes become hard to distinguish from the few primordial male cells which remain. The follicle nuclei can usually be identified by their darker stain and the disposition of their chromatin upon a coarser reticulum.

The oocytes continue to increase in volume until they take up nearly all the space of the gonad and cause it to enlarge. When ready for laying the mature ova are so large that the ovary takes up a large portion of the visceral sac. During the later growth stages of the large oocytes the nuclear figures characteristic of the younger stages (synizesis, etc.) become fewer and fewer. Primordial egg cells may always be found lying outside the oocytes next the connective tissue sheath; the primordial sperm cells disappear entirely.

History of the accessory reproductive organs

The undifferentiated goniduct. As has been stated previously, the primordium of the goniduct in postlarval specimens of Crepidula plana is a solid strand of cells which extends forward and to the right from a narrowed region of the gonad to the right

posterior angle of the mantle cavity, where the duct ends in a clump of cells touching the surface epithelium. The cells in this strand are easily distinguishable from the germ cells (fig. 62); they are more like the follicle cells of the gonad, and like them have no visible cell boundaries at this time. The cord is surrounded by a connective tissue sheath.

A lumen later appears in the middle of this strand (fig. 63) and the nuclei become definitely arranged around the hollow passage. A duct thus arises out of a solid cord. The walls subsequently become more flattened, as do also the nuclei within them, and the interior surface becomes more definite in outline (fig. 65). In specimens of C. plana up to 2 or 2.5 mm. in length the distal part of the duct cannot be seen actually to open into the mantle cavity; but where it reaches the epithelium of the latter it ends in a thickening of cells, by which the duct is fused with the epithelium, and in which no passage-way can be detected. In larger animals the duct does seem to be open to the exterior, but the aperture is very small.

Further differentiation of the duct depends entirely upon the sexual condition of the animal. In the neuter specimens the gonad is still found in this simple, rudimentary condition unless the individual is large enough for the first signs of female development to appear. The only change is a certain amount of thickening of the wall and the appearance of a few long cilia on the free inner surface of the cells. These long cilia are characteristic of the goniduct at all later stages up to the female phase. They are found throughout its whole length (though at certain periods they are lost in some regions). In fixed material they are not always regularly arranged, and while cilia in general do fix poorly, the irregularity of those in the goniduct cannot be entirely attributed to the action of the preparation fluids, for the intestinal cilia in the same slides are usually very well fixed, and their arrangement is as regular as the teeth of a comb. The length of the cilia in the goniduct is so great that they cannot be extended at full length except in an empty seminal vesicle, but reach to the middle of the passage from all sides, meet there and turn parallel to the walls, all extending in the same di-

rection. In sexually inactive animals the duct runs nearly straight from the gonad to the mantle cavity.

Development of the accessory male organs. a. Seminal vesicle and vas deferens. As soon as the male development of the gonad is well under way, the efferent duct differentiates into two regions: a proximal, widened, convoluted seminal vesicle, and a distal, narrower vas deferens. Figure 66 shows the proximal part just as it begins to widen. In this specimen the newly forming seminal vesicle was only just beginning to twist upon itself. A more advanced stage is seen in figure 67; the seminal vesicle has a number of turns, and the figure is an oblique section across one of them. The testis has produced some adult eupyrene and apyrene sperm, of which a few have made their way to the vesicle. Figure 68 shows the more distal part, or vas deferens, of the same goniduct. It has retained more of the primitive character.

b. The penis. In the meantime the penis has appeared as a small hump behind the right tentacle of the individual which is assuming the male phase. There is not the least sign of it so long as the gonad remains in an inactive condition, regardless of age or body size. Most of the stages of spermatogenesis are usually to be found in the testis before the penis becomes very prominent. Its growth is very rapid, however, and it may reach its full size within a few days, as will be shown later. When fully formed it is about three-fourths of the length of the entire body, a long, muscular organ with a slender tip. Its histological structure has been sufficiently described in forms nearly related to C. plana, by former authors (Haller, '92; Vayssiere, '93; Scheidig, '13; Kleinensteuber, '13; Giese, '15). During the outgrowth of the penis the seminal groove is formed along the right side of the neck by a sinking in of epithelial cells and the acquisition of cilia on their free surface. The position and structure of the sperm groove has also been described by the authors above cited.

Time of development of the male organs. The signs of male development always appear in the testis before they are evident in the accessory organs of reproduction. The development of the

latter does not lag far behind, however. Table 2 gives the relative conditions observed in the gonad, goniduct and penis of animals assuming the male phase.

TABLE 2

Relative conditions in gonad, goniduct and penis of specimens undergoing male development. Length of each specimen's shell given in millimeters. The penis has not reached its full development in any of the individuals, for by the time that takes place the testis is fully mature and the seminal vesicle has increased enormously and twisted upon itself many times. The great expansion of the seminal vesicle is due to the pressure of the mass of sperm within; for it never reaches a very large size until it is packed closely with sperm; and if it is emptied of sperm there is a subsequent shrinkage.

SPECIMEN	LENGTH IN MM.	GONAD	GONIDUCT	PENIS
31	3	A few spermatogonia	Proximal part slightly larger than distal, and beginning to bend. Distal end has extremely small lumen	Stump
32	4	Spermatogonia	Proximal part larger and convoluted	None
33	5	Rapid multiplication of spermatogonia	Proximal part larger and twisted into two or three loops; distal part, very small lumen	None
34	12	Spermatogonia	Proximal part slightly larger than distal, but not twisted	None
35	12.5	Spermatogonia	Proximal part slightly larger than distal, and just beginning to twist	None
36	13	Spermatogonia	About same size throughout	None
37	13	Spermatogonia and spermatocytes	Proximal part larger, but not twisted	None
38	8	All stages as far as spermatids	Proximal part larger and thicker wall and beginning to twist	Short
39	15	All stages to spermatids	Proximal part larger and thicker wall than distal; convoluted	Stump
40	10.5	All stages of spermatogenesis; and a few sperm	Proximal part a little larger than distal; convoluted; contains sperm	Short

Contents of the seminal vesicle. As stated previously, both eupyrene and apyrene sperm are found in the seminal vesicle

and in proportions of about 6 eupyrenes to 1 apyrene. In sections one may observe, however, that there are not always the same proportions in all parts of the goniduct. Near the distal end of the vesicle the proportions of apyrenes increases until they greatly outnumber the eupyrenes, and in the vas deferens beyond the sperm are almost all apyrene. It is probable that this is the result of a lesser motility on the part of the apyrene sperm; for the true spermatozoa which have got into the vas deferens have probably escaped to the exterior; while the more sluggish apyrenes are left in a group which fills up the passage of the vas deferens, even making a plug at the distal end of the vesicle.

As long as spermatogenesis goes on uninterruptedly the vesicle is kept packed full of spermatozoa. Relatively few specimens give evidence that the contents of the vesicle have been recently discharged in copulation. There are so many more males than females in the colonies that this is not surprising. When the testis begins to undergo the regressive changes which have been described in the history of the gonad, the majority of the male germ cells from the spermatocyte stage on complete their development and are passed into the seminal vesicle as adult spermatozoa. When the gonad has become inactive the vesicle may be still full of sperm and the individual able to function as a male if the penis has not begun to degenerate. The relative condition of the different organs during the period of regression is shown in table 3. The ten specimens in the table are selected out of about 75, to give the most representative conditions.

Degeneration of the accessory male organs. a. Seminal vesicle. The manner of the disposal of the spermatozoa which are left in the seminal vesicle after the testis is no longer active, is interesting (figs. 69 to 72). The enclosing wall of the vesicle becomes somewhat thicker and its tissue becomes looser and more vacuolar. The sperm then begin to penetrate into it (fig. 69). A part, at least, of the middle piece of the true spermatozoon may enter into the wall with the head, for it can be seen embedded in the cytoplasm after the head has gone in; it is uncertain

TABLE 3

Relative conditions of reproductive organs during regression of the testis. The column marked 'testis' gives the general appearance of the gonad; the columns marked 'spermatogonia,' 'spermatocytes,' 'spermatids,' 'sperm,' show the presence (+) or absence (0) of the said elements in the gonad of each specimen; 'f' few; 'm' many. The contents of the seminal vesicle and vas deferens, and the condition of the penis, are given under the appropriate columns.

SPECIMEN	LENGTH IN MILLIMETERS	TESTIS	SPERMATOGONIA	SPERMATOCYTES	SPERMATIDS	SPERM	SEMINAL VESICLE	VAS DEFERENS	PENIS
51	16	Somewhat reduced	+	+	+	+	Full of sperm	Apyrenes and some eupyrenes	Long
52	11	Somewhat reduced	+	f	+	m	Wall somewhat thickened; full of sperm	Apyrenes	Short, brown tip
53	9	Small	+	+	+	+	Full of sperm	Apyrenes	Long
54	6	Small, few cells; posterior part reduced to strands	+	0	f		Full of sperm	Not included in sections	Short
55	9	Not much reduced; much empty space inside	0	0	+	+	Full of sperm	Apyrenes	Long
56	11	Very small; some coagulation of cells	+	0	0	+	Thick wall; sperm coagulated in centre	Not included in sections	Long
57	8	Small	+	0	0	f	Thick wall; few sperm	Apyrenes	Stump, brown
58	12	Very small; inactive; tubular strands	0	0	0	0	Full of sperm	Not included in sections	Stump
59	sm	Very small; thick wall	0	0	0	+	Thick wall; partly filled with sperm	Not included in sections	Short
60	8	Very small; empty; thick wall	0	0	0	0	Thick wall; empty; ciliated	Not included in sections	Stump

whether the flagellum, which is very long in the eupyrene sperm of Crepidula, also penetrates or is left outside and dissolves in the lumen. Figure 70 shows a part of a vesicle filled with sperm of both kinds, where many eupyrene sperm heads have penetrated into the wall, and whatever part of the sperm body has also entered has dissolved. The sperm heads are often clumped together, and twisted about each other like the strands of a rope or like twisted synaptic threads. The clumps and the single sperm heads often though not always lie in clear vacuoles. The apyrene sperm as well as the eupyrenes get into the wall of the vesicle (figs. 71, 72).

There are indications that the entrance of the sperm into the wall is brought about partly by the motility of the sperm and partly by the amoeboid movements of the cytoplasm. The process is undoubtedly the same as we have already seen in the testis during its degeneration.

The sperm do not all remain alive till they are enclosed in the wall of the vesicle; some die and degenerate in the lumen (see specimen 56, table 3).

Of the sperm which are taken up by the wall of the vesicle, the apyrenes and the non-chromatic part of the eupyrenes are dissolved first; while the eupyrene sperm heads remain for some time longer, finally assuming a beaded and shrunken appearance (fig. 72) in the process of dissolution. The fact that some live spermatozoa remain in the lumen of the vesicle after most of the contents have been taken up and dissolved by the walls indicates that there is no substance liberated in the lumen which is fatal to the adult spermatozoa, but that there is a change in the cytoplasm of the vesicle cells, which allows the sperm to penetrate the wall more easily, and in fact assists the penetration; after which the cytoplasm may kill and dissolve the sperm. The same may be said in regard to the testis; but there is some change in the testis which affects the immature testicular elements in the lumen, particularly the spermatogonia and early spermatocytes, so that their development can no longer be completed.

Cilia are hard to distinguish on the inner surface of the vesicle as long as the lumen is filled with sperm, though they are prob-

ably present. During the passage of sperm into the wall and the shrinkage of the vesicle, the cilia seem to be in part destroyed. New ones probably appear later.

b. The penis and sperm groove. The loss of the male condition is accompanied by the degeneration of the penis (table 3). The latter process takes place within two or three weeks after the regression of the testis begins, but is not uniform enough in its occurrence to be used as an exact indication of the state of the gonad and vesicle. While degenerating the end of the penis becomes brown, and cells are evidently being sloughed off at this point, for the whole organ becomes shorter and shorter until only a brown stump is left; and this too finally disappears leaving no trace whatever. The sperm groove also becomes flattened out and loses its cilia.

Development of the accessory female organs. a. The oviduct. The further history of the efferent duct depends on the behavior of the gonad, as is the case at an earlier period of life. As has been made certain, the goniduct of a small male whose testis has degenerated may return to the rudimentary condition seen in the neuter animals, so that no evidence remains of the former male state unless the specimen has been marked and observed from the time when it bore the external genitalia of the male. In the following section we will consider the changes in the goniduct of a large degenerate male, in whose gonad young oocytes begin to appear.

The distal part of the goniduct (vas deferens in the male phase) does not change much during the degeneration of the testis, but remains a small ciliated tube. The wall thickens a little and lightly staining granules appear in the cytoplasm (figs. 73, 74). The proximal part, i.e., what was the seminal vesicle, is now an extremely thick-walled structure (figs. 75, 76), still retaining some of the twists and turns which it formerly had, though they are far less numerous than before. Where the growth of the body is rapid during this period (as shown by the nature of the shell) it is difficult to know how much the seminal vesicle has shrunken, for the loss of the convolutions might be attributed to the elongation of the visceral sac.

Two new conditions may now be observed in that part of the goniduct which was formerly the seminal vesicle. In the first place, cell outlines begin to appear between the nuclei, dividing the cytoplasmic wall into high, irregular cells which make up a columnar epithelium. Secondly the cytoplasm of these cells becomes less dense than was the cytoplasm of the vesicle wall, and in the most transparent regions are clusters of granules (fig. 76), indicating some change in the cytoplasm. The appearance of these granules recalls a somewhat similar condition in the wall of the gonad after the testis has degenerated (figs. 47, 48). Cilia begin to appear again in the proximal part of the goniduct (figs. 75, 76), the cell outlines become more and more distinct (fig. 79) and the cytoplasm more and more transparent (figs. 79, 80). The remains of the seminal vesicle no longer show any twisting, and the whole goniduct runs almost straight to the gonad. Cell outlines may be seen in the distal part of the duct (figs. 77, 78) as well as in the proximal.

In discussing accessory male organs it has been said that they never appear until the development of the testis is under way. Contrasted with this, certain accessory female organs begin to develop before the gonad exhibits any sign of development to an ovary. The formation of the uterus, for instance, is more or less independent of the sexual condition; it often begins in large sexually inactive animals, or even in very large males. The goniduct also sometimes assumes the appearance of an immature oviduct in large neuters. None of the accessory female organs take on their adult condition, however, until the oocytes are well advanced in the growth period.

During the growth of the oocytes, which lasts for a considerable time, the body of the animal is also growing rapidly, if not subjected to a dwarfing environment. The oviduct is increased enormously in size, though in comparison with the length of the animal it is shorter than the male goniduct. Figure 81 shows a cross section of the distal part of it at a time when the oocytes are in the process of yolk formation. Comparison with figure 77 gives an idea of the amount of growth since the stage last described. The walls have been thrown into longitudinal folds

and these folds are supported by connective tissue ingrowths from the surrounding sheath. The descendants of the high, transparent cells which made up the wall of the duct at an earlier period (fig. 78) have assumed a different character (fig. 82). They now form a cubical epithelium whose regularly disposed cilia are shorter than those of the goniduct at all previous stages. The proximal part of the duct, which was formerly the seminal vesicle, has not changed so markedly in appearance; it is still composed of transparent columnar cells, though they are now even more drawn out in length and have a clearer cytoplasm (fig. 84). The proximal part of the duct has not experienced the same relative growth in diameter as the distal (cf. figs. 81 and 83 with figs. 77 and 79).

From this point on, as the oocytes increase in size and in yolk content, the folded cubical epithelium of the more distal part of the oviduct is found nearer and nearer the ovary. The cells of the proximal portion undergo at this time an unequal growth into the lumen, nearly closing it up in places. A somewhat similar process has been figured by Giese in C. unguiformis.

Eventually the deep, transparent epithelium becomes limited to the extreme proximal end of the oviduct where the latter is connected with the ovary, and persists there when the animal is in the adult female phase. The rest of the oviduct is then composed of a cubical ciliated epithelium thrown into longitudinal folds.

The only further change in the oviduct, as the animal becomes a functional female, is the appearance of unicellular glands in its wall (fig. 85). They are first seen, here and there, in the more distal part of the oviduct a little later than the stage of figure 81. They become more and more numerous and are found more proximally; in the adult female they are found in all the folded epithelium of the oviduct. They contain a granular secretion which stains a deep blue with Delafield's haemotoxylin. Giese has described somewhat similar glands in C. unguiformis. In the case of the latter species they are more irregular and their enlargement seems to result in the partial destruction of the oviducal epithelium. Giese considers them pathologi-

cal. In C. plana there is no indication that they point to any abnormal condition, and it is more likely that they have something to do with the passage of the egg through the oviduct.

b. The uterus and seminal receptacles. The development of the uterus has not been taken up in detail. In its more general features it agrees with the account given by Giese ('15) for Calyptraea and Crepidula unguiformis, viz., it arises by a deepening and closing of the proximal part of the sperm groove at the opening of the goniduct near the shell muscle. Subsequently it extends both backward and forward from that point and its walls become transformed into a very high columnar epithelium with different kinds of glandular elements. Giese states that there are no cilia in the uterus of C. unguiformis. In C. plana the inner wall of the uterus is uniformly ciliated in all parts of the organ. In C. unguiformis the seminal receptacles are described as consisting of three pouches arising as evaginations of the uterus and opening separately into it near the oviduct. They are flask-shaped and not ciliated. In C. plana the seminal receptacles likewise arise as evaginations from the uterus; but there are at least nine of them, each with its own opening into the uterus, the openings being very close together. Each receptacle is composed of a thin-walled, non-ciliated sac connected to the uterus by a narrow tube whose wall is a cubical, ciliated epithelium.

c. The gonopericardial duct. No mention has been made thus far of the gonopericardial duct in Crepidula plana. This structure was discovered by Giese in Calyptraea and in Crepidula unguiformis, but found to be lacking in Capulus.

In Crepidula plana the gonopericardial duct is present. It arises, as in the two forms above mentioned, from a strand of mesenchyme cells which connects the most anterior extension of the pericardial chamber with the middle of the oviduct. It is never seen during the male phase regardless of the size of the animal, but becomes evident before the assumption of the female phase, during the period when the oocytes first reach the synaptic stages. The originally solid strand becomes hollow, and during the early growth period of the oocytes it begins to

take on the same structure which is being assumed by the oviduct at the same time; i.e., the walls, originally of thin flat cells like the pericardium, begin to form a cubical epithelium which acquires cilia and becomes thrown into longitudinal folds. Eventually it possesses unicellular glands of the same nature as those in the oviduct. It is of interest that the differentiation of the gonopericardial duct proceeds from the distal end, at its junction with the oviduct, and toward the pericardium. At a certain stage the distal part is composed of cubical ciliated epithelium, with ridges reaching into the lumen; the middle part has a thinner wall, but cells are dividing to form outgrowths into the lumen; in the proximal part (nearest the pericardium) the wall is composed of thin flat cells like the pericardium. In the adult stage the folded, glandular, cubical, ciliated epithelium extends the full length of the gonopericardial duct, and even projects somewhat into the pericardium.

Whatever the phylogenetic significance of this organ (Giese, '15) may be, one cannot escape the conviction that so highly differentiated a structure, occurring only in the female phase, must have a functional value connected with reproduction. The walls are so folded that they are capable of expansion like those of the oviduct, as though for the purpose of allowing eggs to enter; yet it is not clear what advantage could accrue from passing the eggs toward the pericardium before their entrance into the uterus, unless there is some action of the lymph in the pericardial chamber upon the ova. In this connection it will be recalled that the body fluids of animals so far as known inhibit, rather than facilitate, the fertilization of eggs by sperm (Lillie, '14).

GENERAL CONSIDERATIONS: ORIGIN OF GERM CELLS IN HERMAPHRODITE MOLLUSCS

In conclusion, it will be in place to consider what application can be made, from the observations, to the question of the origin of germ cells in hermaphrodites. The volume of work on this subject is very small and the conclusions of the various workers do not agree, even on the same material. The studies of

Ancel ('03) on Helix pomatia are among the best known and most fully developed investigations which have been made. Ancel believes that there is a true indifferent germinal epithelium in Helix, whose cells are capable of differentiation into indifferent sex cells or into nurse cells. The indifferent sex cells may develop into spermatogonia or oogonia according to the conditions of the environment. I quote the following from his conclusions:

. . . . une cellule progerminative indifférente devient mâle ou femelle suivant les conditions qu'elle rencontre dans la glande génitale au moment de son apparition, conditions réglées par la transformation d'un certain nombre de cellules épithéliales en éléments nourriciers élaborateurs d'un matérial spécial.

Demoll ('12 b), on the contrary, working on the same species as Ancel, finds the first differentiation of the male and female sex cells to take place at a time when a Nebenkern appears in the cytoplasm, more marked in the male cells than in the female. He concludes that the differentiation of the Nebenkern determines the character of the germ cell, which may be considered indifferent up to this time. He further concludes by analogy with dioecious animals in which the sex is determined by the accessory chromosome, that a chromosome difference is responsible for the differentiation of the Nebenkern.

Buresch ('11), working on Helix arbustorum, agrees with Ancel that the male and female germ cells are determined as such by the presence or absence of nurse cells. He does not find, as Ancel does, that there is a stage of spermatogonial development, then nurse cell development, then oogonial development; but that all the different elements of the gonad are being developed simultaneously.

In his "Germ Cell Cycle" Hegner has reviewed these and other works on the germ cells of hermaphrodites. It is not necessary to repeat Hegner's review of hermaphroditism in animals other than Molluscs, except possibly to call attention to the investigations of Boveri ('11) and Schleip ('11) on the germ cells of the hermaphrodite Nematode Rhabditis nigrovenosa, in

which it is shown that the male, female, and hermaphroditic individuals which occur have chromosomal differences.

In the plant kingdom two cases, at least, are known where one of the germ cells (the spermatozooid) of hermaphrodite plants is heterozygous for sex, while the other (the ovum) is homozygous. This was determined by crossing with dioecious forms (Shull, '14 and Correns, '07).

Now it is worthy of note that those authors (Ancel and Buresch) who have concluded that the female germ cell is differentiated out of the indeterminate sex cell as a result of a different nutrition from that of the spermatogonia have figured in their plates a rather simple method of differentiation of the oocyte. In their figures of the oogenesis there is no such complex series of changes in the nucleus as has been found in Paludina by Popoff ('07), as Demoll has described for Helix pomatia (the same form on which Ancel worked), and as the present writer has seen in Crepidula plana. Demoll, it will be recalled, does not hold to the nutrition theory of germ cell determination. The possibility occurs to the writer that the early stages of differentiation of the oocytes might be misinterpreted on account of their resemblance to the primary spermatocytes, in animals where the two existed side by side.

In every hermaphrodite animal it must be true that at some stage of development there is an indifferent sex cell; for the egg, and the developing embryo, carries within it the potentiality of developing two kinds of sex elements (Hegner, '14). The unsegmented egg, at least, is an 'indifferent germ cell' as well as an 'indifferent somatic cell.' The question with which we are concerned, then, is to find the point at which the male and female elements are separated. It does not seem necessary that this point must always be the same in the life cycle. It is not difficult to believe that in some species male and female elements are but separated during the early embryogeny, and in another that they are contained in the same cell through many cell-generations. It seems to the writer, however, that the now widely spread view that the germ cells of hermaphrodites arise from an actually undifferentiated germinal epithelium, even in

the adult life of the animal, is based upon insufficient evidence. The observations on Crepidula certainly do not bear this out. On the contrary they go to show that the male and female germ cells are recognizable as such very soon after the swimming larva takes up the creeping mode of life. When a method has been perfected for rearing the larvae through the free-swimming period, it is hoped that the still earlier history of the gonad may be investigated.

SUMMARY

Crepidula plana is a protandric hermaphrodite in which the male and female phases are completely separated from each other.

The assumption of the male condition does not always occur at the same stage in the life history, with respect to the age or size of the animal; and there is reason to believe that the male phase is sometimes entirely omitted.

The growth of the animal during the first part of its life, i.e. during the period in which male development may occur, is very variable and depends in part upon: 1) the amount of movement of the animal; 2) the amount of space available for the extension of the mantle; 3) the season of the year.

Primordial male and primordial female cells are both present in the gonad at all periods of life from the post-larval up to the adult female phase and are visibly different from each other.

Crepidula plana possesses, in common with other Prosobranchs, atypical as well as true spermatozoa. The former are of the 'apyrene' variety. They develop from cells which cannot at first be distinguished from spermatogonia. After the cells which are to form apyrene spermatozoa are differentiated there are no maturation or other divisions.

During the change from the male to the female condition part of the testicular cells complete their development and are passed into the seminal vesicle as adult spermatozoa. The rest are absorbed into the wall of the testis where they are dissolved; or else they degenerate in the lumen of the gonad. The gonad undergoes a temporary reduction in size; it again becomes large

when the oogonial cells, after a period of division, develop into oocytes and enter upon the growth period.

The goniduct serves for the transference of both spermatozoa and eggs, but is, structurally, entirely different in the male and the female phases. In the male phase it is differentiated into seminal vesicle and vas deferens.

The accessory male organs appear only when the testis develops. In the 'sexually inactive' animals there is no penis, seminal vesicle nor sperm groove. These develop very quickly, however, when male differentiation takes place in the gonad.

During the change from the male to the female condition or upon the premature loss of the male condition, the penis, seminal vesicle and sperm groove degenerate. Before its degeneration the seminal vesicle absorbs the contained spermatozoa into its wall, where they dissolve.

As the female phase is gradually assumed, the goniduct greatly increases in size, acquires longitudinal folds and glands in its wall, and becomes the oviduct. At this point in the life history the 'gonopericardial duct' is also developed, connecting the pericardial chamber with the oviduct.

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PLATES

ABBREVIATIONS

A, primordial egg cell
Aan, anaphase of oogonial division
asp, apyrene sperm
Asz, synizesis of oocyte
B, primordial sperm cell
C, core of apyrene sperm
co, centrioles
d, goniduct
esp, eupyrene sperm
f, follicle cell
fi, axial fibers
fl, flagella
i, idiozome
ko, karyomerites
nv, nuclear vesicles
ov, oocyte in growth period
spb, spermatoblast
spcI, primary spermatocyte (eupyrene)
spd., spermatid (eupyrene)
spg, spermatogonium
sps, spermatosome
x, cytoplasmic granules

PLATE 1

EXPLANATION OF FIGURES

1 to 4 and 6 Cross sections through parts of a gonad of immature specimens of Crepidula plana. × 681.

1 From a very small specimen, less than 1 mm. entire shell length. The primordial male (*B*) and female (*A*) cells are distinguishable and their differences are as marked as at any later time in the sexual cycle. A number of spermatogonial cells have been formed and deposited in the lumen, but have later degenerated forming irregular, homogeneously staining bodies.

2 From a specimen about 1.5 mm. length. Although slightly larger than the individual from which figure 1 was taken, this specimen has not developed any spermatogonia. It has only primordial male (*B*) and female (*A*) cells and follicle cells (*f*). The space marked '*d*' shows where the rudimentary goniduct joins the gonad. In many small specimens it has been observed that just at this point the wall is very thin, consisting only of the connective tissue sheath which surrounds both gonad and duct.

3 Specimen about the same size as that from which figure 2 was drawn. The gonad is further developed, however; a number of spermatogonia have been formed and lie in the lumen. This figure is drawn at the same magnification as figures 1 and 2; a comparison shows great differences in cell size in the different specimens. This may be partly accounted for by degree of activity in cell division.

4 Section through periphery of a gonad in which spermatogonial multiplication and a few divisions of primordial egg cells were going on. The specimen was 7 mm. long, with small outgrowth in the region where the penis will form.

5 Section through part of the gonad of a post-larval Crepidula convexa less than 1 mm. long. The stage of development represented is comparable to that of figure 1 in C. plana. As in the latter species, there are two types of primordial germ cells present.

6 Section through part of a nearly adult testis. All stages of spermatogenesis up to and including spermatids were found in this gonad, though no spermatids appear in this figure. Differentiation between the eupyrene spermatocyte (*spcI.* at top of figure) and apyrene spermatoblast (*spb*) is clear. A group of late primary spermatocytes (*spcI* at lower part of figure) and one spermatosome (*sps*), besides several spermatogonia (*spg*) are also shown. There is no very definite germinal layer around the periphery of the gonad. Length of specimen, 11 mm.

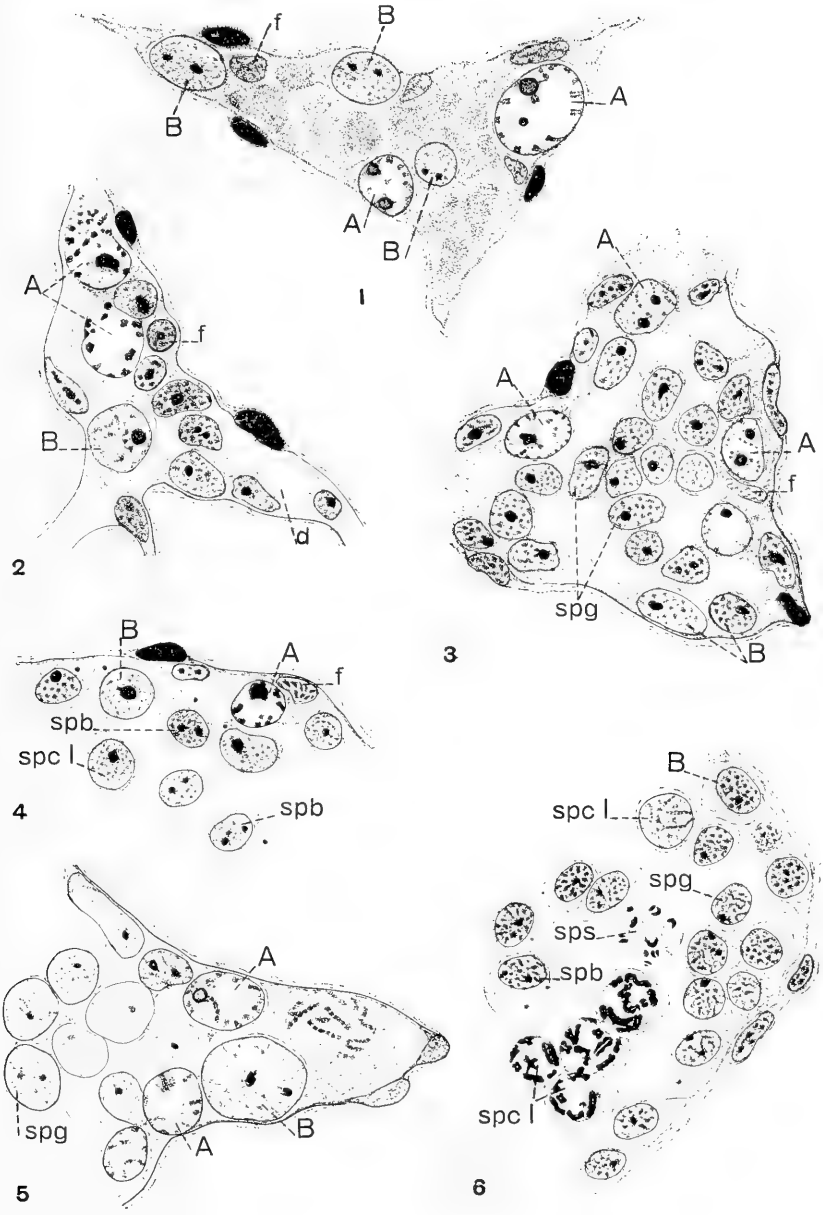
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PLATE 2

EXPLANATION OF FIGURES

7 to 13 'Type A' or primordial egg cells in various stages of rest and division. All × 1024. Figures 10 to 13 drawn from same preparation as figure 51.

7 Primordial egg cell in which the chromatic bodies are very distinct and clearly show duplex nature. From an immature male gonad developed as far as spermatocytes. Length of specimen, 8.5 mm.

8 Prophase of division of a primordial egg cell. Same preparation as figure 7.

9 Division of primordial egg cell, after splitting of metaphase chromosomes. From gonad containing spermatogonia and a few spermatocytes.

10 Anaphase of division of a primordial egg cell, or oogonium. Some anaphase chromosomes appear double. From specimen 10 mm. long, undergoing period of oogonial division. When collected, specimen was a male. During fifty-seven days it was kept under observation all traces of male character were lost and the shell grew 4 mm. in length (it was originally 6 mm. long).

11 Telophase of division of a primordial egg cell or oogonium. From same specimen as figure 10. Midbody prominent.

12 Young daughter elements resulting from division of a primordial egg cell or oogonium. From same specimen as figure 10.

13 Primordial egg cell or oogonium which has recently been formed by division of a preceding one, with chromatic bodies again becoming evident. From same specimen as figure 10.

14 to 20 Represent primordial male cells or spermatogonia in various stages of rest and division. All figures drawn from gonads of immature males. × 1024.

14 Primordial sperm cell or spermatogonium.

15 Prophase of spermatogonial division.

16 Metaphase of spermatogonial division.

17 Anaphase of spermatogonial division.

18 Late anaphase of spermatogonial division.

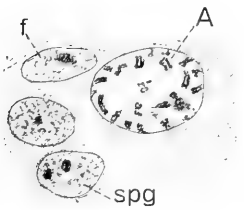
19 Telophase of spermatogonial division.

20 Daughter cells of spermatogonial division.

21 Unusual case of an oocyte undergoing partial development in an immature male gonad. The animal was quite a large one, however, (11.5 mm.) and it is quite possible that some of the female germ cells had begun to develop before the testis began to form. It will be shown in a separate paper that male development may interrupt and replace early stages of female development. In the cell figured here the synizesis stages have been completed.

22 Spermatogonial cell recently formed by division, showing prominent mid-body, which may be homologous with granule found at periphery of spermatoblast. Cells like this and figure 19 suggest that the eccentric position of the nucleus in apyrene spermatoblasts is due to failure to return to a central position after last division.

23 Primary spermatocyte showing prominent mid-body and spindle fibers remaining from last division.



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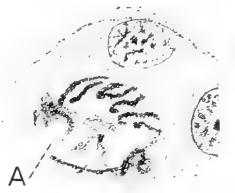

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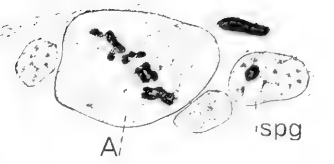

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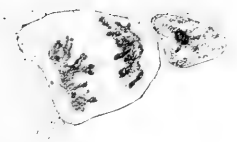

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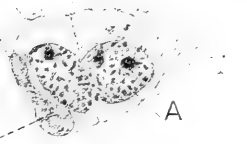

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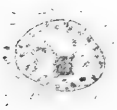

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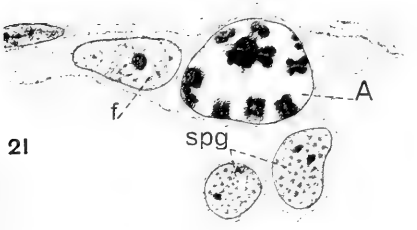

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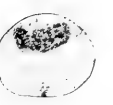

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23

PLATE 3

EXPLANATION OF FIGURES

24 to 40 Represent successive stages of development of the apyrene spermatozoon from the spermatoblast. All at a magnification of 3864 diameters.

It will be observed that in the figures representing the successive steps of the metamorphosis there is a great deal of variation in size. All figures were drawn with camera at the same magnification; as much variation was found as is represented. It is evident that these differences must be referred back to differences in volumes of the spermatogonia from which they arose, or to differences originating in the growth period.

24 Spermatogonium, from which arises the apyrene spermatoblast. It cannot be distinguished from spermatogonia which form eupyrene spermatocytes. The granule (*x*) seen in the cytoplasm is found in primordial male and female cells, spermatocytes and spermatoblasts, as well. It is most prominent in the spermatoblasts. The writer has not been able to determine the nature of this body. In some cases it is found at the very edge of the cell (fig. 25) and if it were always found there it might be considered a persisting mid-body (figs. 11, 22, 23); but the granule is often deep in the cytoplasm and sometimes there are two or three (fig. 6, *spb*). The centrosome or centrioles can usually be distinguished from the cytoplasmic granule.

25 Young apyrene spermatoblast. The nuclear membrane is heavy at this stage, and the chromatic pattern is coarser than in spermatogonia. The large capsular idiozome (*i*) has appeared and near it two centrioles (*co*). The nucleus is eccentric in position, which is characteristic of spermatoblasts.

26 Beginning of nuclear disintegration.

27 Optical section of part of spermatoblast to show centrioles divided into 4. Drawn at low focus; the nucleus lay over structures represented.

28 Cell showing what is probably a stage corresponding to that represented in figure 26, just before disintegration of the nucleus. The behavior of the nucleus is different, however; there is a suggestion of an imperfect thick spireme.

29 Transformation of spermatoblast into spermatosome. Nucleus very irregular and chromatin aggregating into karyomerites. One of the few cases where the centrioles can be seen after having divided several times.

30 Karyomerites (*ko*) lying free in cytoplasm. Centrioles are not to be seen and idiozome has disappeared.

31 Grouping and fusion of karyomerites.

32 Stage showing newly formed nuclear vesicles (*nv*) and the reappearance of the centrioles at one side of cell. Walls of nuclear vesicles formed out of chromatic matter of fused karyomerites.

33 Outgrowth of the flagella from the centrioles and ingrowths of axial fibres. Division of centrioles, and migration. Stationary centrioles stain more intensely than migratory ones, or are surrounded by more stainable substance.

34 and 35 Growth of axial fibres through cell body. Migratory centrioles not discernible. Largest nuclear vesicle in figure 34 still contains chromatic reticulum; in others, various stages of dissolution.

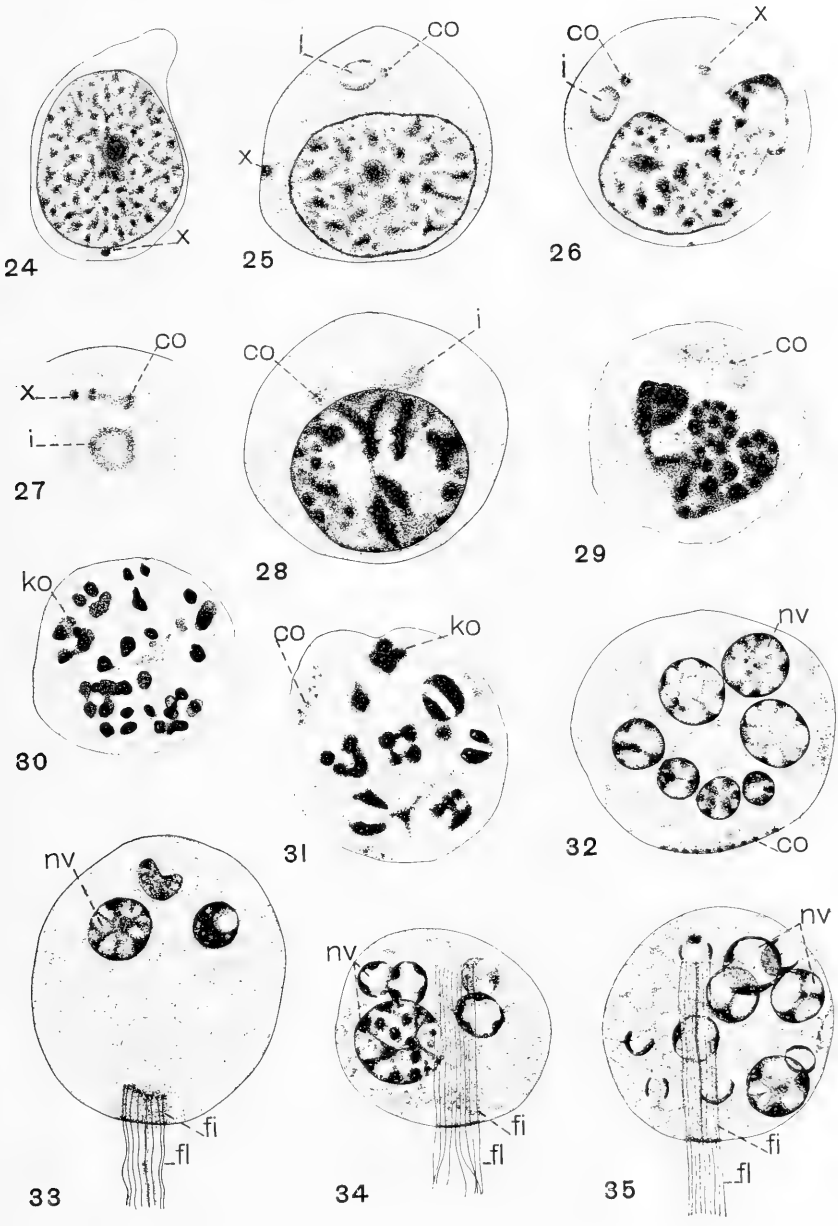
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PLATE 4

EXPLANATION OF FIGURES

36 Anterior end of axial bundle protrudes from spermatosome. Free flagella at posterior end have increased in length.

37 Further growth of axial bundle. As fibres elongate and project farther out of anterior end, they carry part of the cytoplasm of the cell body along with them.

38 Anterior ends of fibres remain close together in a dense bundle, while farther posteriorly considerable cytoplasm is deposited between them. Core becomes prominent in centre of axial bundle (*c*). Before the spermatosome has reached this stage it has already begun to show rapid movements.

39, *a* and *b* Adult apyrene spermatozoon, drawn in two sections to preserve same magnification as former figures. 39*a*, anterior; 39*b*, posterior. Cytoplasm of cell now entirely applied to axial bundle and flagella. An entire apyrene spermatozoon, on smaller scale, shown in figure 61, together with eupyrene spermatozoon for comparison.

39*c* Cross section through middle or posterior part of the adult apyrene spermatozoon. Axial fibres arranged around the periphery of the cytoplasm; in the centre, darkly staining core.

40*a* Optical cross section through body of spermatosome at about the stage of figure 37. Axial bundle flat in cross section.

40*b* Optical cross section through cell body of a spermatosome at about the stage of figure 38. Axial fibres no longer form a flat bundle, but are distributed about central core.

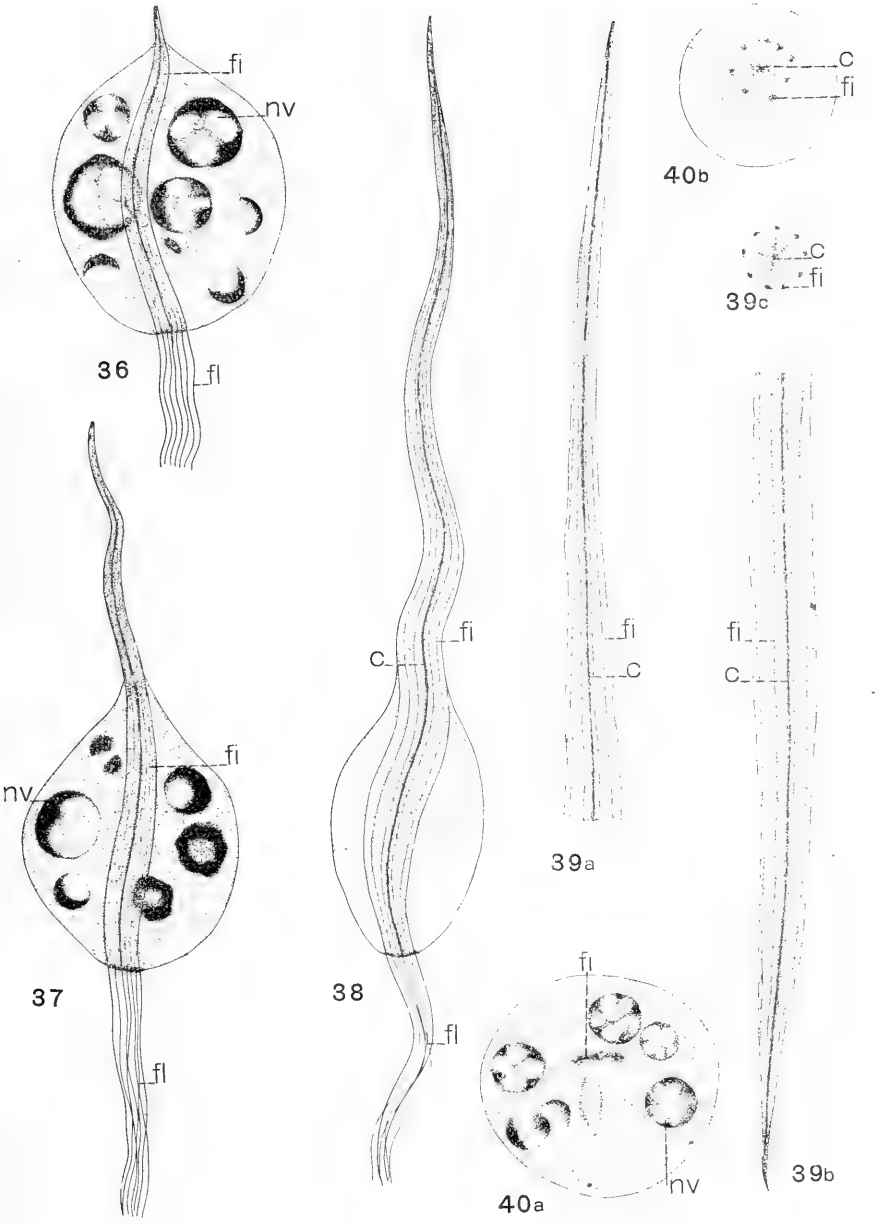
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PLATE 5

EXPLANATION OF FIGURES

All figures at a magnification of 1024, except figures 44 and 45, which are $\times$ 681.

41 Two primordial female cells (*A*) at periphery of adult testis. These cells often become very much changed in appearance during the male phase, as figure indicates. Spermatogonia (*spg*) and early spermatocyte (*spcI*) also shown. $\times$ 1024.

42 Section at periphery of testis during retrogression. Cytoplasm of peripheral syncytium reaching out to join to cells in lumen. $\times$ 1024 Length of specimen, 8 mm.

43 Inclusion of sperm heads (*esp*) and apyrene sperm (*asp*) in peripheral syncytium, which has become very much thicker than in former condition. While sperm, spermatids, etc., are being taken up into the wall of the gonad, some of the peripheral nuclei show the appearance of those in this figure. Apparently the tension of the nuclear membrane is relaxed in some regions and the contents of the nucleus becomes bulged out. This probably explains the distorted shape of most nuclei in figure 45 where the degenerate products have been dissolved. $\times$ 1024.

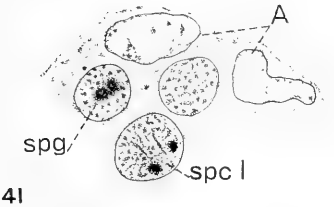
44 Section through part of degenerate male gonad from which all testis cells have absorbed. Peripheral syncytium deep and vacuolar. $\times$ 681. Length of specimen, 11 mm.

45 Section through strand of degenerate male gonad. Nuclei at periphery distorted. $\times$ 681. Length of specimen, 9 mm.

46 Section through different part of same gonad as figure 43. Sperm heads embedded in wall. $\times$ 1024.

47 Section through peripheral wall of gonad during transition period. Specimen from which figure was drawn was taken as adult male (15 mm.) from a colony; during thirty-seven days after transferring to a second hermit shell, penis entirely degenerated, and gonad was found inactive, when sectioned. $\times$ 1024.

48 Later stage. Part of syncytial wall becomes dissolved, while part condenses about follicles nuclei. Cytoplasm of germ cells becomes more clearly defined. $\times$ 1024. Length of specimen, 13 mm.



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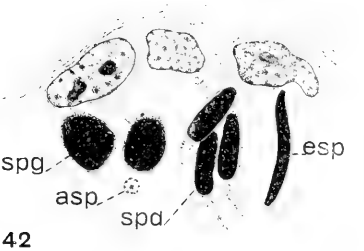

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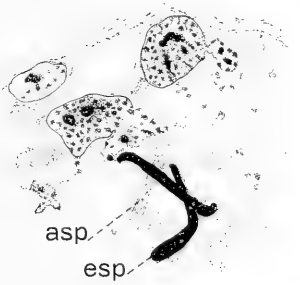

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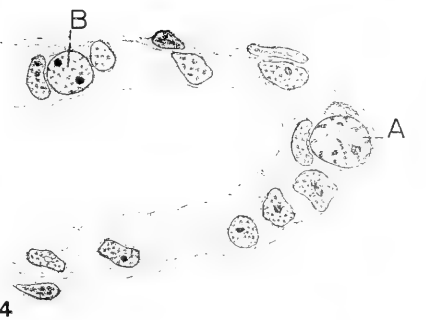

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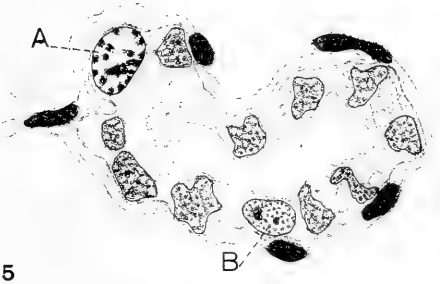

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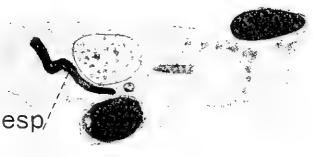

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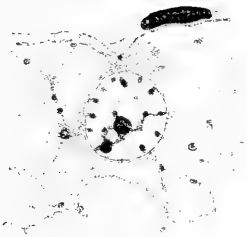

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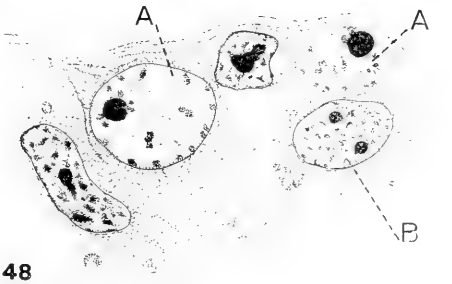

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PLATE 6

EXPLANATION OF FIGURES

49 Section through part of gonad showing first indications of female development, i.e., presence of many primordial egg cells surrounded by follicle cells. Gonad consists of almost solid strands. × 681. Length of specimen, 10 mm.

50 Section through wall of gonad in neuter specimen 10 mm. long with thin, smooth shell. 50*a* and 50*b* drawn at same place, but at different foci.

51 Section through an immature female gonad during later oogonial division and early nuclear changes of oocytes. × 681.

52 Section through immature female gonad during later nuclear changes and formation of growing oocytes. Follicle nuclei become very much enlarged as oocytes develop, and in adult ovary many more are present than in this figure.

In the larger of the two resting oocytes in the figure, there is very little matter in the nucleus which takes a chromatic stain. The basichromatin at least is limited to a few granules disposed here and there on the linin threads. Some of these granules lie at the very periphery of the nucleus, and just outside in the cytoplasm there are many granules which have a similar appearance. Whether chromatin is escaping into the cytoplasm, or whether chromidia are appearing in the cytoplasm, must be determined by specific stains for chromidia. × 681. Length of specimen, 17 mm.

53 to 59 Apyrene spermatosomes which are degenerating during metamorphosis. All × 1024.

53 Beginning of degeneration shortly after formation of nuclear vesicles. The cell has several large vacuoles. Two centrioles appeared at extreme edge.

54 Nuclear vesicles breaking up. Cell outlines irregular.

55 Degeneration while the axial fibres are growing out. The cytoplasm is full of large vacuoles.

56 Spermatosome in which all the nuclear matter has disappeared, but no fibres have grown out.

57 Degeneration during outgrowth of the axial fibres.

58 Degeneration during outgrowth of the axial fibres. This spermatosome resembles Kuschakewitsch's figure of the nearly adult apyrene spermatozoon of Vermetus.

59 Degeneration after the apyrene spermatozoon has become nearly adult. The cytoplasm is in small globules and the axial fibres are thus left close together in a strand.

60 Adult eupyrene spermatozoon.

61 Adult apyrene spermatozoon.

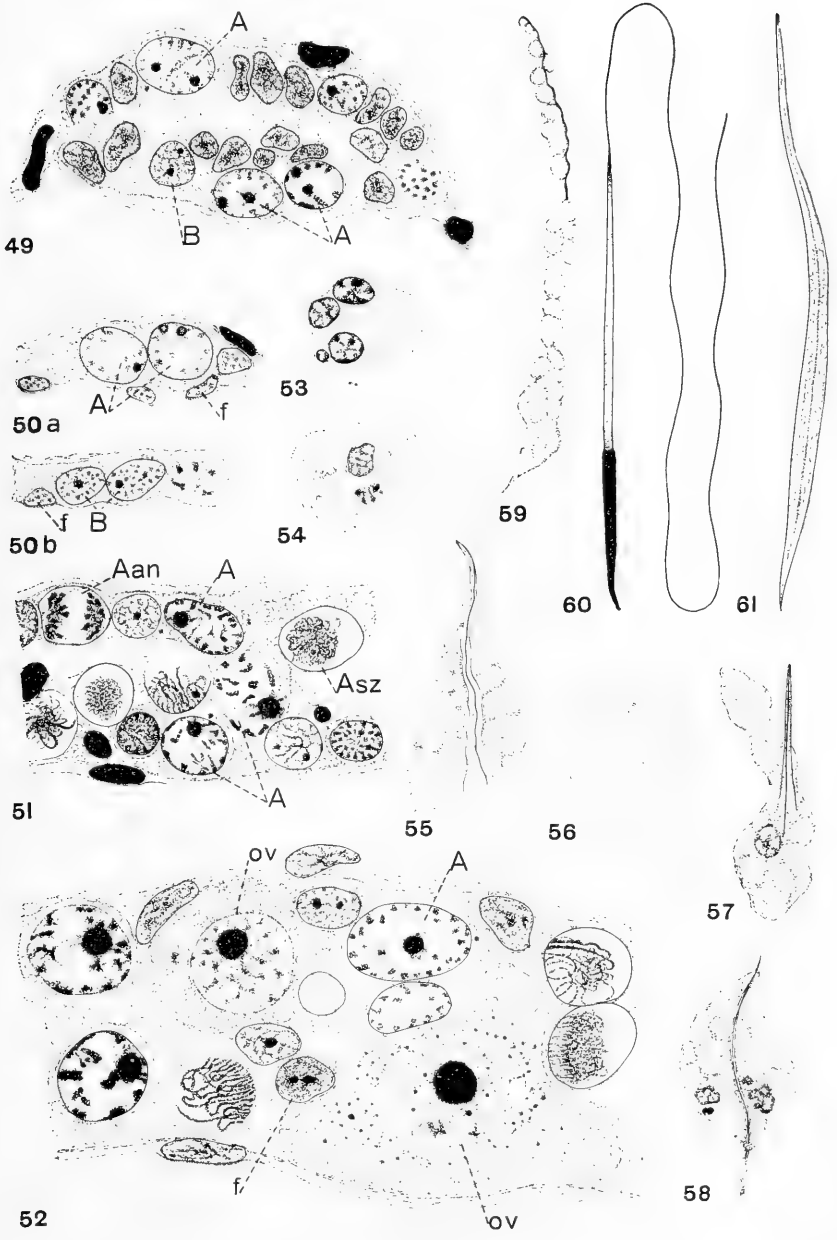
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PLATE 7

EXPLANATION OF FIGURES

All figures × 681

62 Primordium of goniduct in specimen $1\frac{1}{4}$ to $1\frac{1}{2}$ mm. long. Figure drawn at proximal end of strand, where latter joins gonad. Optical section.

63 Slightly later stage, showing appearance of lumen in middle of strand. Optical section.

64 Higher focus at same place as figure 63, showing surface of duct.

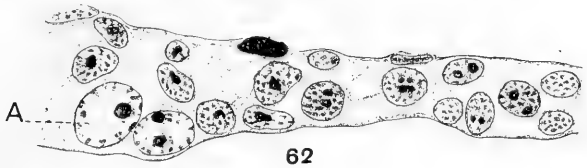
65 Later stage, showing the flattening of the walls and the nuclei within them. Gonad of specimen contained spermatogonia, some of which have got into the goniduct.

66 Proximal part of the goniduct of specimen where rapid spermatogonial multiplication was taking place in gonad. Duct enlarged and slightly twisted upon itself. Cilia appearing. Length of specimen, 8 mm. Penis partly developed.

67 Section through one turn of seminal vesicle in specimen having nearly mature testis. A few sperm have been formed and reached the seminal vesicle. Length of specimen, 5 mm.

68 Distal part of goniduct (vas deferens) in same specimen as above.

69 Section of small part of wall of seminal vesicle in specimen with degenerate testis, showing entrance of sperm head into wall. Length of specimen, 11 mm.



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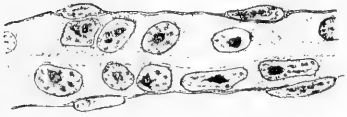

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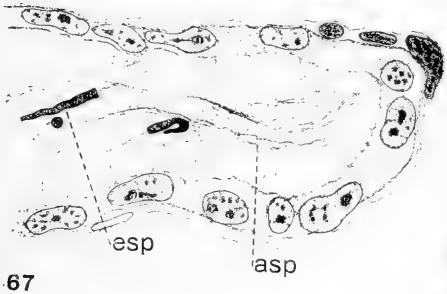

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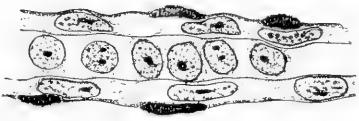

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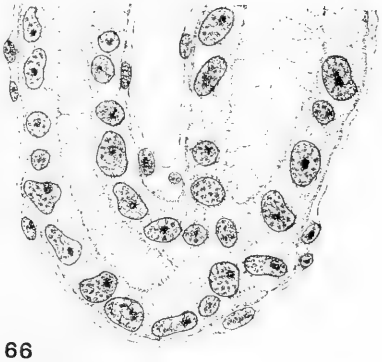

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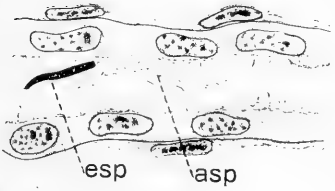

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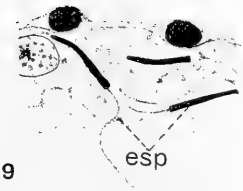

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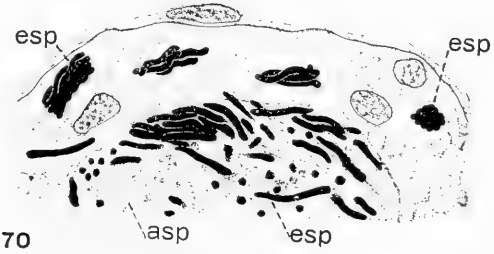

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PLATE 8

EXPLANATION OF FIGURES

71 Showing an apyrene spermatosome, which has abnormally developed (see text) entering wall of seminal vesicle. × 681. Length of specimen, 14 mm.

72 Showing eupyrene sperm heads (*esp*) and apyrene sperm (*asp*) in wall of vesicle. Cytoplasm has extended out along apyrene sperm. One eupyrene sperm head has begun to break up, assuming beaded appearance. Length of specimen, 11 mm. Gonad reduced to small strands. × 681.

73 to 76 From a single specimen a large animal (17 mm.) which had been a male when first collected, but which during 48 days under experiment had lost the male condition. All sperm in seminal vesicle had been absorbed.

73 Section through distal part of goniduct; somewhat oblique section. × 85.

74 Enlargement of small part of same. × 681.

75 Section through degenerate seminal vesicle. × 85.

76 Enlargement of small part of same. × 681.

77 to 80 Sections through goniduct of large sexually inactive specimen, formerly male. No oocytes yet present in gonad.

77 Section through distal part of goniduct. × 85.

78 Enlargement of small part of same. × 681.

79 Section through proximal part of goniduct (former semial vesicle). × 85.

80 Enlargement of small part of same. × 681.

81 to 84 Drawn from the goniduct of an immature female 20 mm. long, with yolked oocytes in ovary (fig. 83).

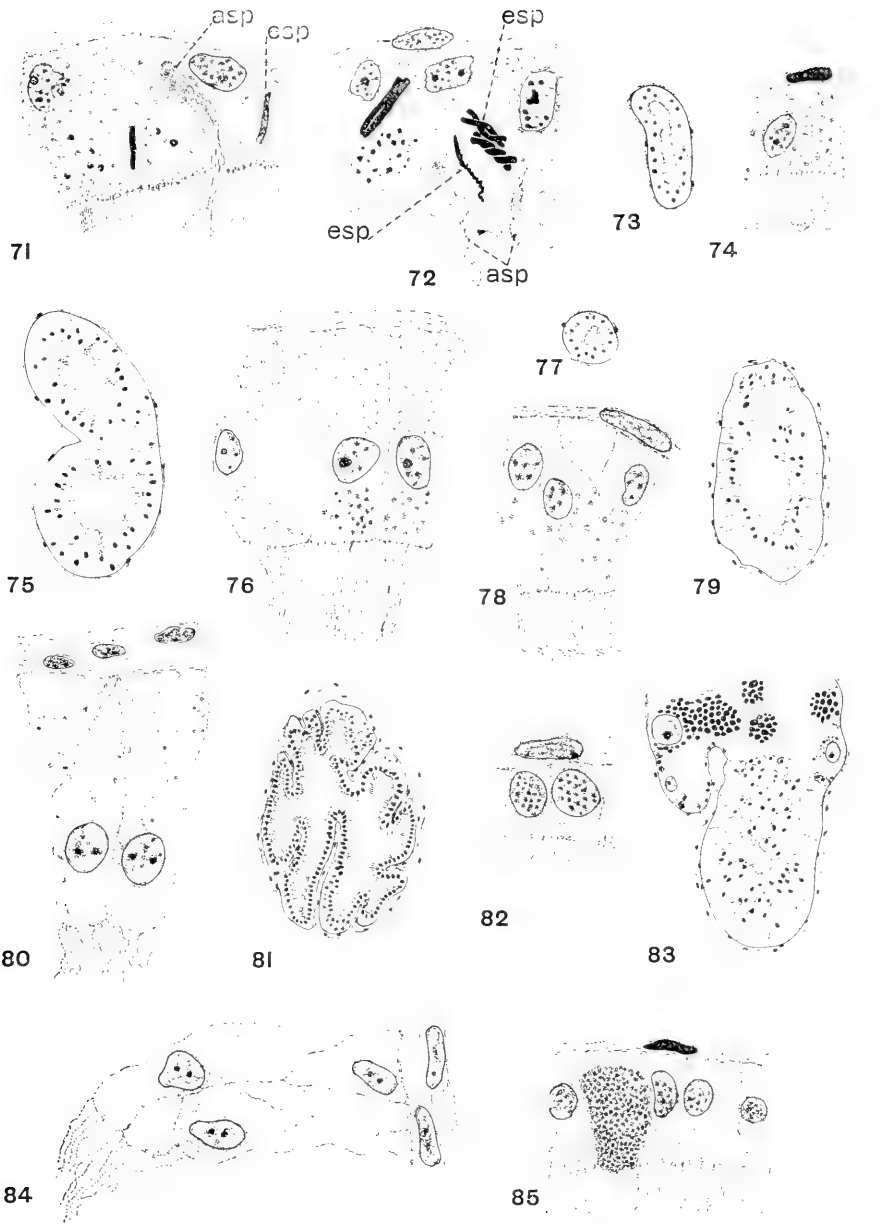
81 Distal part of goniduct (now oviduct) showing great growth and infolding of walls. × 85.

82 Enlargement of small part of epithelium of same. × 681.

83 Proximal part of oviduct in same animal. Somewhat oblique section. × 85.

84 Enlargement of small part of epithelium of same. × 681.

85 Section through epithelium of oviduct of adult female, to show unicellular gland.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PHOTOMECHANICAL CHANGES IN THE RETINA OF NORMAL AND TRANSPLANTED EYES OF AMBLYSTOMA LARVAE

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THREE TEXT FIGURES AND ONE PLATE

INTRODUCTION

The changes in form and position of the visual cells and their nuclei and of the pigment in the retinal epithelium is a subject concerning which a vast literature has been accumulated. There are, however, points here and there concerning which our knowledge is incomplete. One of these is the influence of the central nervous system upon the photomechanical changes. The work which has been done has been recently reviewed by Detwiler ('16) and by Arey ('16 b). From this experimental evidence it appears that photomechanical changes may take place after the optic nerve has been cut, i.e., independently of central control.

Amblystoma larvae, normal, eyeless, and individuals with transplanted eyes, were being used extensively in experiments on the physiology of the melanophores and it was decided to make a study of the reactions of the retinal pigment and of the visual cells of these animals to light and darkness. It occurred to us that the investigation of the effects of light and darkness on transplanted eyes would be a method devoid of certain objections that might be raised against the sectioning of the optic nerve which involves shock and degeneration. Such a transplanted eye is not only under no normal nervous control but has never been so. There is a chance that nerve fibers, both spinal and autonomic, may grow into the eyeball, but the chance is indeed slight, and still less that they could exert any influence on the movements of the retinal elements.

Eyes were transplanted in frog tadpoles (Rana palustris) as well as in Amblystoma larvae, the stage of development used for the operations being that when the tail bud is just beginning to be perceptible (Laurens '14). The optic vesicle was removed and transferred to the slightly enlarged cavity made by removing the auditory vesicle. All transplants were made on the left side, the right eye being left to serve as a control. For some reasons all but two of the tadpoles died, before they reached a length of 30 mm. Sections of the transplanted eyes of the two survivors, killed just before metamorphosis, showed essentially normal conditions. It is planned to repeat the experiments on tadpoles for the reason that they will in all probability show even greater reactions and more extensive changes, as indicated by the results on normal eyes, than are to be reported now on the eye of Amblystoma.

It is known from the work of Lewis and others that the transplanted optic vesicle of the Amphibian will develop into essentially a normal eye. Uhlenhuth ('13, '13 a and '13 b) has shown that the same thing is true of the eye when it is transplanted in larval stages after its differentiation is far advanced. We have found in Amblystoma that the eye developed from the transplanted optic vesicle is essentially normal, all the elements being present with the same relative number of rods and cones. There are certain differences, however, to be noted. In the first place the characteristic form (to be described later) of the rod nuclei is lost, and secondly the number of ganglion cells is much less than in the normal eye. The optic nerve also has never been observed to be present. Moreover the orderly arrangement in two separate rows of the rod and cone nuclei is sometimes, though not always, disturbed.

In figure A a view of the anterior end of an Amblystoma larva, 31 mm. long, is given to show the general position and appearance of the transplanted eyes. In figure B a section of such an eye is shown.

HISTORICAL

Before proceeding to a description of results a brief review of the literature so far as it concerns photomechanical changes in the Urodele retina will be given.

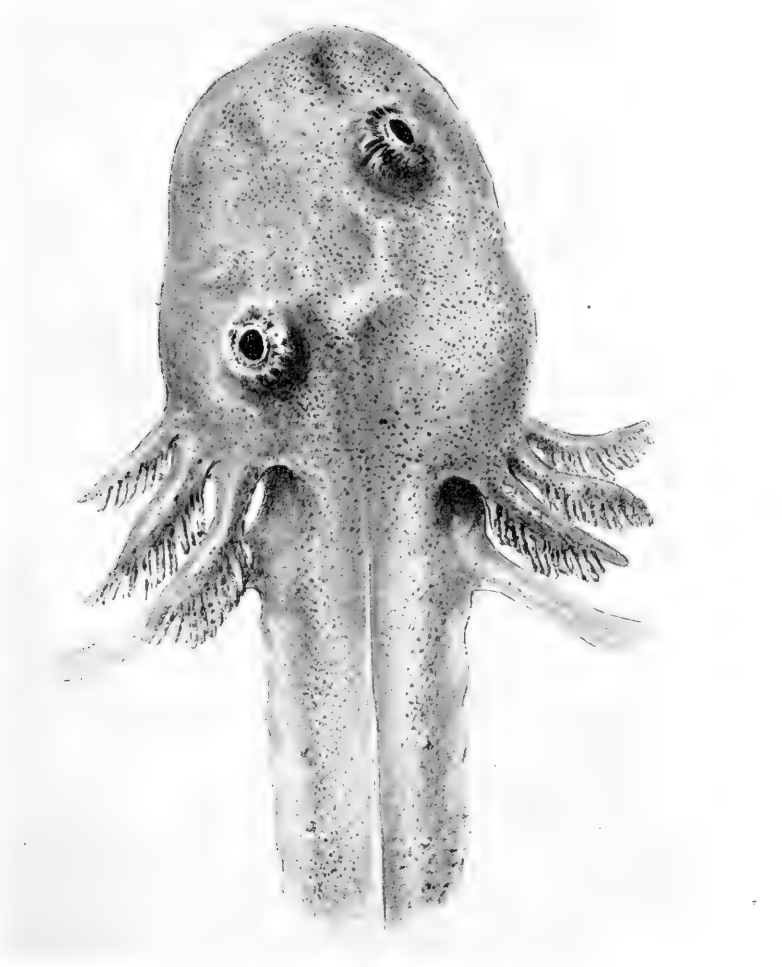

Fig. A The anterior end of a 31 mm. larva to show the general appearance and position of the transplanted eyes.

First as regards changes in the position of the pigment in the epithelial cells. Angelucci ('78) and van Genderen Stort ('86 and '87 b) both describe it as taking place in the eye of Triton. Garten ('07, p. 20, figs. 7 and 8) shows quite a decided difference in the position of the pigment in light and dark eyes, although he states that it is much less extensive than in many other vertebrates, and that the pigment, even in the dark eye

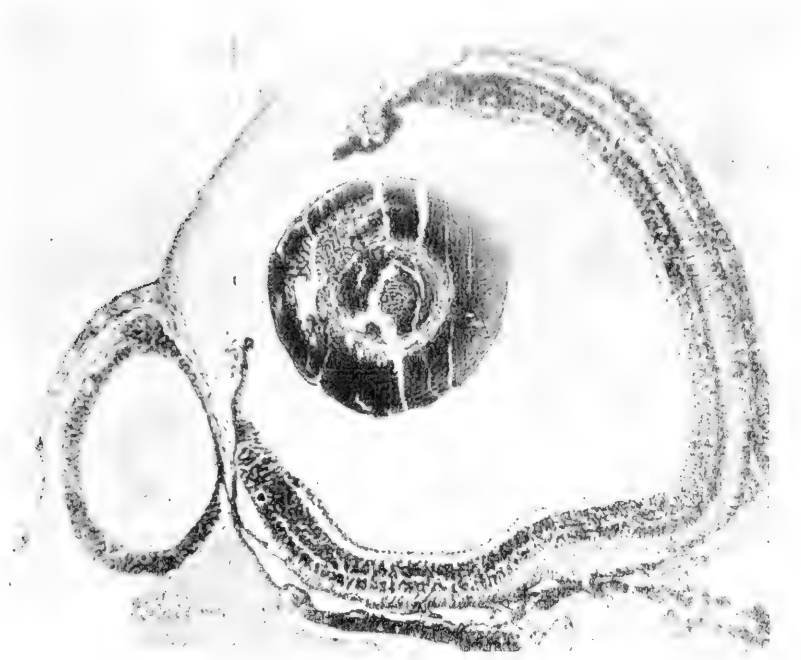

Fig. B A section of a transplanted eye. Note the small number of ganglion cells.

reaches down to the ellipsoids (p. 70). Howard ('08) observed pigment migration in the eye of Necturus which Arey ('16 a) found to amount to about 8 μ.

As regards the inner segment of the cones van Genderen Stort ('87 a) described and figured a shortening caused by light in the eye of Triton, and Angelucci ('94) claimed that in the eye of the salamander the cones stretched in darkness, an observation which Garten ('07, p. 32) was not able to substantiate.

Changes in form and position of the rods have also been reported. Angelucci ('94) found that the very large rods of Salamandra clearly show a decrease in the length of the outer segment after illumination. Garten ('07, p. 49) also observed that the rods of the illuminated retina of the Salamander were a little thicker and shorter. Van Genderen Stort ('87 a) has further reported that in the dark eye of Triton the nuclei of the rods extend over the external limiting membrane, resulting in making the rod longer. In the light eye the rod nuclei are described as all lying below the external limiting membrane. This change in the position of the nuclei of the rods is supposed to be brought about by the contractility of the connection between the rod nucleus and the nuclei of the granular layer. Angelucci ('94) reports the same thing as happening in the eye of Salamandra maculata. Garten ('07, p. 21 and 50) was able to confirm these observations for Triton (see his figs. 7 and 8) but owing to variations in his results on Salamandra he leaves the matter in doubt.

ANATOMICAL FEATURES

The larvae were exposed to light or darkness for varying lengths of time. The entire animal was killed by throwing it into sublimate-acetic. After being hardened in alcohol, the upper jaw, containing the eye balls was cut off, and sectioned after imbedding in paraffin or the piece containing the eyes was cut into two, right and left, parts and imbedded and cut separately. The sections were 10 μ in thickness, and were stained in eosin and toluidin blue, or in Ehrlich's hematoxylin and eosin.

The visual cells of Amblystoma consist of both rods and cones in the approximate proportion of 4 : 3. The most striking thing about the rods is the short length of the 'myoid' and the peculiar shape of the nuclei (see figs.). Instead of being round or oval these nuclei are angular, and show an extension on the internal side by means of the continuation of which the connection between the nuclei and the internal granular layer is effected. The external limiting membrane lies just below the lower (internal) angles of the rod nuclei, so that the greater part

of the nuclei is outside the membrane (fig. C). In young adults the general shape of the nucleus is the same as in the larvae, though the corners are slightly more rounded. The large oval nuclei in the second row belong to the cones.

The cones are of three kinds. On the edges of the retina particularly, but scattered here and there through it, one finds large, stout cones, with a very short myoid and a bellied out

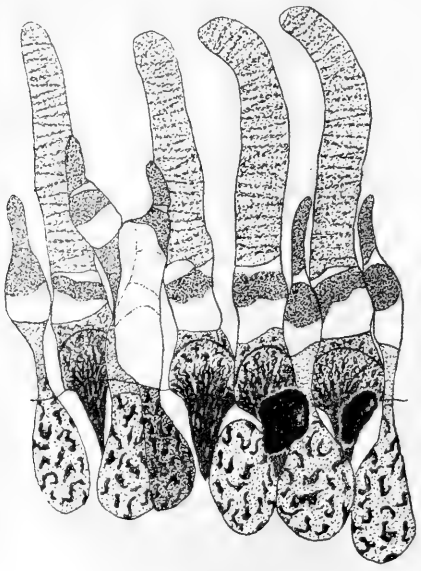

Fig. C A portion of the retina of a light eye, showing the visual cells with their nuclei, four rods, three single small cones, and one double cone.

inner segment with a clear ellipsoid and an oil drop. The outer segment is short and conical. The typical cone is much smaller, and has a longer, more slender myoid, a clear ellipsoid and an oil-drop in the inner segment. This type of cone is always single. The third type is one that is always, as far as can be ascertained, united with one of the first type to form a double cone. It differs from the small cone described in having a much onger myoid. The nuclei of the larger element of the double

cones are slender and long and tapering, instead of roundly oval, and stain more intensively. They are to be seen in several of the figures.

EXPERIMENTAL

Pigment migration. In a preliminary communication (Laurens and Williams '16) it was stated that although there was a decided forward movement of pigment when the eye was illuminated, it was impossible to measure the extent of this movement because of the fact that the distance from the external limiting membrane to the nearest pigment needle, or from the choroid edge of the epithelial cells to the farthest pigment needle, is practically the same in both light and dark eyes. In darkness, however, most of the pigment was found to be massed near the base of the epithelial cells, while only a comparatively small number of needles extended into the protoplasmic processes between the visual cells. In light, on the other hand, a decidedly greater amount of pigment was found toward the external limiting membrane resulting in the basal layer being thinner.

As a result of further study involving the examination of additional material it was found that this statement did not altogether fit the case. In figures 1 and 2 a light and dark eye are illustrated. A glance will show that these two figures, as far as pigment position is concerned, do not represent a typical light and dark eye respectively. For in the light eye the pigment is actually more retracted than in the dark eye. Nevertheless more pigment is forward in figure 1 (light eye) than in figure 2 (dark eye) although the basal layer of pigment in figure 2 (dark eye) is hardly any thicker than in figure 1 (light eye). These two figures represent extreme cases such as led us to the conclusions stated in our preliminary communication concerning the inability of making measurements of the amount of migration. That figures 1 and 2 are really light and dark eyes respectively is shown by the comparative lengths of the cone myoids.

Figures 3 and 4 are two other figures of a light and dark eye respectively. These are, in contradistinction to figures 1 and 2, characteristic of light and dark eyes in general as far as the

position of pigment is concerned. In figure 3 the pigment is further forward than in figure 4, and in addition the basal layer of pigment is thinner. When figure 3 (light eye) is compared with figure 2 (dark eye) the condition mentioned in our preliminary communication is very apparent. If it were not for the relative thicknesses of the basal layer of pigment it would be very difficult (disregarding the cones) to say whether the one or the other was a ight or a dark eye.

In a few cases the pigment in light eyes has been observed to occupy an extreme position, the pigment needles being as far forward as the ellipsoids of the rods. The average of measurements, however, of light and dark eyes give a distance of 36 μ from the choroidal edge of the pigment epithelial cells to the farthest pigment needle in light eyes, and of 29 μ in dark eyes, so that we have an average pigment migration of about 7 μ.

In all these cases, whether the pigment showed the expected light or dark position or not, the characteristic difference in the length of the cones was present. Why pigment migration did not take place although cone contraction did must remain unanswered. It may have been due to the fact that optimal conditions were not offered in the length of time of exposure, etc.

In the transplanted eyes the pigment migration is even greater in extent than in the normal eye. Figures 5 and 6 are of a light and dark transplanted eye respectively. The average of all measurements made of transplanted light and dark eyes, gives a distance of 12 μ as the extent of the migration of pigment. Why the extent of the migration should be greater in transplanted eyes than in normal is not clear. Detwiler ('16) found that in the eye of the turtle there was what he called a loss of tone when the optic nerve was cut. Both the pigment cells and the cones seemed to relax in that the pigment extended down further (partial light position) and the cones stretched (partial dark position) though there was no great difference in the amount of movement occasioned by light and darkness as compared with that in normal eyes. Arey ('16 b) has obtained some very interesting results with the retina of Ameiurus. He found that when the optic nerve only is severed that the char-

acteristic photomechanical responses fail to take place, also that after hemisection of the nerve, movements occur only in that region of the retina adjacent to its intact side. When the eye was left attached to the body by the optic nerve alone, or when it was excised, essentially normal responses take place. After further experimentation involving the cutting of muscles, etc., he concludes that in association with the muscles innervated by the oculomotor nerve there is an inhibiting mechanism the effect of which is evident when the optic nerve is cut, and that there are functional efferent nerve fibers in the optic nerve of Ameiurus, the impulses in these fibers blocking the tonic inhibition exerted by the inhibiting system. The retinae of Abramis and Fundulus were found not to show this condition.

Contraction of the cones. Regarding the influence of light and darkness on the myoid of the cones it is hardly necessary to say more than a word since the figures speak very clearly for themselves. Figures 1 and 3 are of light eyes, 2 and 4 of dark eyes. The differences between them are evident. The transplanted eyes show the same condition of affairs (figs. 5 and 6). Measurement of normal light and dark eyes gives an average of 7.4 μ as the extent to which the length of the cone can be changed by light and darkness. The length of the cone inner segment in the dark eye, that is extended condition, is about 25 μ (not the entire length of the cone as was erroneously stated in our preliminary communication). The extent of movement of the cones of the transplanted eyes is about 14 μ.

Movements of the rods. No differences in the diameter or in the length of the outer segments of the rods of light and dark eyes could be clearly demonstrated. Nor could any very constant differences be found in the length of the rod myoid. The rod myoid in Amblystoma larva is very short, never longer than 2 μ. Figure 1 shows them particularly clearly. In most of the rods of light eyes they are of maximum length, though in some cases the myoid is so short that the ellipsoids of the rod seems to be in contact with the nucleus. On the other hand in dark eyes the myoid is in most rods of minimal length, between $\frac{1}{2}$ and 1 μ long, though here again in the dark eyes the length of the rod

myoid may measure the maximum 2 μ. All that can with certainty be said is that there seems to be a slight shortening of the rods in darkness, a shortening, however, which is only between 1 and $1\frac{1}{2}$ μ in extent.

There is, however, a very evident difference in the shape of the rod nuclei, according as to whether the eye is a light or a dark one. In the light eye there is a distinct rounding off of the angular corners of the nuclei particularly of the external ones, so that the boundary of the nuclei on the myoid side is a curve of regular outline, instead of running for a time almost parallel with the internal boundary of the ellipsoid as it does in the dark eyes. This is a result which might happen if the nuclei were subjected to a pull on the internal side (see figs. 1 and 3 as compared with figs. 2 and 4). The changes in the position of the rod nuclei described by van Genderen Stort and Garten in Triton are supposed to be due to the contractility of the connections between the rod nuclei and the granular layer. Perhaps contractility is not so highly developed in the eyes of Amblystoma so that when the pull takes place all that can happen is for the nuclei to change their shape in response. Certainly they do not change their relative position with regard to that of the cone nuclei or of the external limiting membrane, which passes, as mentioned above, just below the lower angles of the rod nuclei (fig. C).

SUMMARY

1. Pigment migration and cone contraction take place in the transplanted eyes of Amblystoma larvae as well as in normal eyes, and to a greater extent.

2. The extent of pigment migration in the normal eye averages 7 μ. In the transplanted eye 12 μ.

3. The extent to which the myoid of the inner segment of the cone contracts in the normal eye is 7.7 μ. In the transplanted eye 14 μ.

4. The rod myoid may lengthen in the light, but if it does so, it is only to the extent of between 1 and 1.5 μ.

5. There are no differences in the length or diameter of the outer segments of the rods of light and dark eyes, nor do the rod nuclei change their position.

6. The rod nuclei, however, do change in form. The angles of the nuclei in the light becoming rounder as if in response to a pull.

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PLATE 1

EXPLANATION OF FIGURES

All figures on this plate were photographed at a magnification of 1000. They have been reduced one-half in reproduction.

1 A light eye of a 45 mm. larva showing the typical light or contracted condition of the cone myoid. The pigment, however, is in the retracted or dark position.

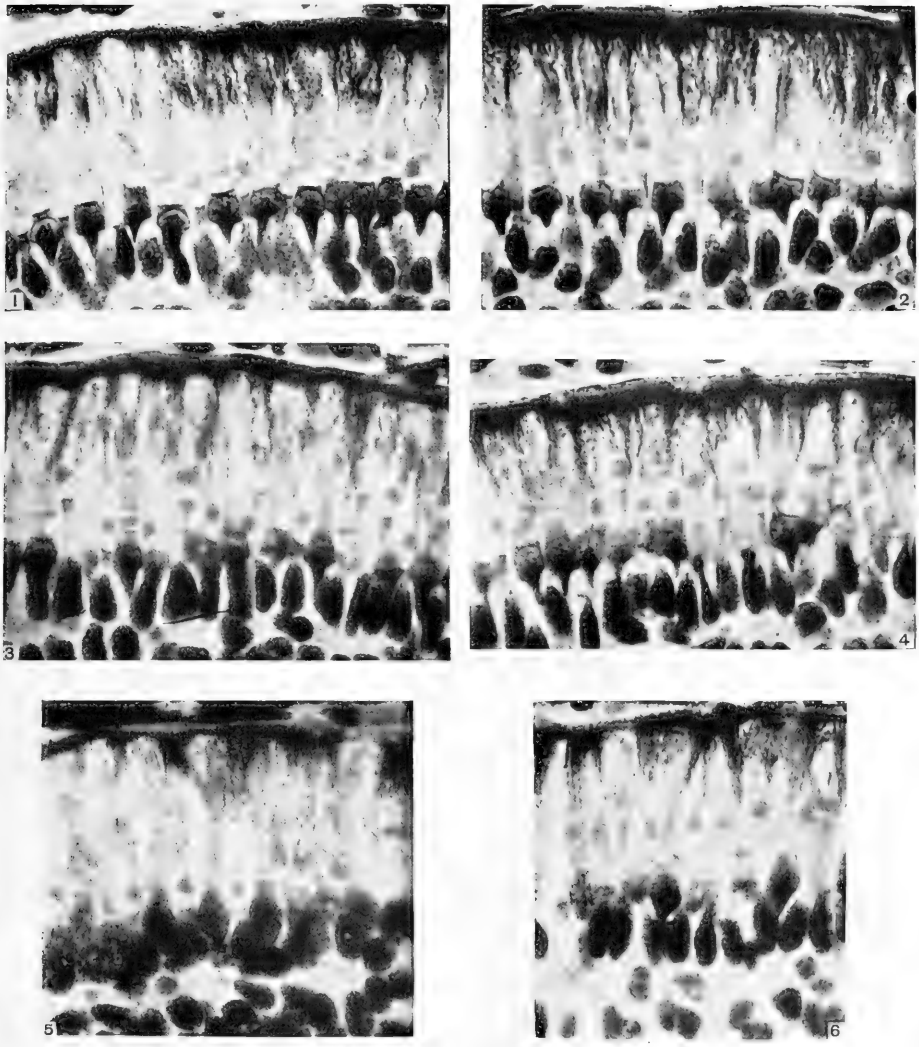
2 A dark eye of a 42 mm. larva, showing the typical dark or extended condition of the cone myoids. The pigment, is again, however, not typical but shows the forward or light position.

3 A light eye of a 39 mm. larva showing the typical condition of both cones and pigment.

4 A dark eye of a 41 mm. larva showing the typical condition of both cones and pigment.

5 A transplanted light eye of a 43 mm. larva showing the typical light condition of contracted myoids and forward pigment.

6 A transplanted dark eye of a 41 mm. larva showing the typical dark condition of extended cone myoids and retracted pigment.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THE RÔLE OF THE AUDITORY SENSORY EPITHELIUM IN THE FORMATION OF THE STAPEDIAL PLATE

FRANKLIN PEARCE REAGAN

Department of Comparative Anatomy, Princeton University

TEN FIGURES

Certain structures which seem to have undergone transformation in the process of transition in vertebrate life from aquatic to terrestrial conditions have always been of great morphological interest. Structures of this sort are abundant in the pharyngeal region, which, especially in earlier times, furnished a most productive field of study. Such study was often concerned with the homologies among the nervous, muscular, vascular, skeletal and epithelial derivatives of this pharyngeal region.

Between the intermittent communications of the pharynx with the exterior there are developed mesenchymatous visceral arches in which chondrification takes place, forming the so-called 'visceral ribs;' these may ossify and retain their original position as components of the pharyngeal skeleton, or they may become greatly modified in the higher forms, acquiring a special function very unlike that of their more primitive homologues. In the lower vertebrates the pharyngeal ribs or components of the visceral skeleton tend to preserve their original resemblances to each other. Particularly striking here, also, are the great independence and the wide separation of the visceral skeleton from the skeleton of the central nervous system—the cranial skeleton. In some of the primitive elasmobranchs, for instance, the mere cutting of three ligaments may be sufficient for the complete separation of these two skeletal complexes (i.e., visceral and cranial). But even in forms as low as the holocephali, the visceral skeleton has become immovably articu-

lated with the brain-case. Here the palatoquadrate is firmly fused with the cranial wall, and the upper end of the hyoid arch (hyomandibula) takes no part in this union; this is a distinct advance towards the differentiation of a definite quadrate.

The skeletal complex of the first visceral arch forms the cartilage of the jaws. The antero-dorsal cartilage is known as pterygoquadrate. The ventral element comprises Meckel's cartilage. In selachians these two are suspended from the skull by the hyomandibular cartilage, the latter having been derived from the dorsal portion of the second visceral arch. During the ontogeny of the higher vertebrates the pterygoquadrate and hyomandibular homologues become very closely associated with the developing cranial skeleton, either losing entirely their identities, or becoming greatly transformed. Certain it is that the jaw articulations of the lower forms are not homologous with those of higher ones.

The hyomandibular, or at least the dorsal portion of the second visceral cartilage, is believed by many to be represented by that columnar bone in the series of ear-ossicles which becomes the most intimately associated with the otic capsule. In amphibia, sauropsida, and monotreme-mammalia, this homologue has been called by various names such as 'columella,' 'columella auris,' and 'columella cranii.' The cartilaginous plate fitting into the fenestra ovalis has been designated in amphibia as the 'operculum.' In higher forms the cartilaginous plate occupying the fenestra ovalis is known as the stapedial plate.

The terms 'columella' and 'columella cranii' have been employed with a great deal of confusion, as, for instance in the writings of Gegenbaur and Schimkewitsch. Gegenbaur (Vergl. Anat. der Wirb., p. 374) interprets the columella of amphibia as follows:

> In urodeles there is a ligamentous process stretching from the operculum to the cartilaginous quadrate. In the anura the operculum is continued as an elongate ossified staff, the columella, which is to be regarded as a part of the auditory apparatus. These are two skeletal units which have taken the place of the hyomandibulare.

He regards (p. 380) the columella of reptiles as a homologue of the upper end of the hyoid arch, but derives the operculum from the chondrocranium. On p. 386 he states:

> To the labyrinth region belongs still another bone which springs up from the pterygoid to the parietal—the columella. It has a cartilaginous Grundlage (Leydig) which is laid down on a process of the chondrocranium (Gaupp) and which is also distinguishable in amphibia and evolves itself in lacertilia into a columnar form which is characteristic of it.

It is difficult to see by what line of reasoning this latter columella could be regarded as belonging to the labyrinth region. It is much farther removed from the labyrinth region than a great deal of the pterygoid itself from which it is derived. This evident error would be of minor interest if it had no counterpart in the writings of more recent observers. Schimkewitsch, (Lehrb. d. vergl. Anat., p. 121) describing the conditions in Sphenodon, states: "From each pterygoid there reaches up a 'platten förmig' bone (anti-epipterygoideum or columella cranii) which represents a process of the palatoquadrate which has already existed before in anuran amphibians." Although he deals with the homologies of this bone in almost all groups of reptiles, he neglects its description in the skull of the snake (figures from Boas) where (figs. 134 and 135) the term columella cranii is applied to the ear-ossicle whose internal end fits into the fenestra ovalis. If these two columellae are homologous it is puzzling that both should exist in the same reptilian form.

In higher mammals the auditory 'columella' seems to be represented by the staff-like portion of the stapes. There seems at present to be little doubt that the latter arises as a chondrification in the mesenchyme of the second visceral arch; our observations concerning this point are not, however, in complete unison. Concerning the origin of the plate-like cartilage which forms the distal (distal from the point of view of the visceral skeleton) portion of the stapes homologue which closes the fenestra ovalis there is very great diversity of opinion. According to one view, the entire stapes including the stapedial plate arises as a chondrification in the second visceral arch. Among

the many observations supporting this view may be mentioned those of Baumgarten, Broman, Huxley, Keibel, Kingsley, Peters, Rabl, Reichert, Schenk, Schafer, Zittel, and Zondek; closely allied to this view we have phylogenetically the hyomandibular origin of the stapes, supported by Baraldi, Claus, Gegenbaur, Hasse, R. Hertwig, Kükenthal, and Wiedersheim, and Parker. On the other hand, there are observations which tend to show that the entire stapes arises from the otic capsule, constituting then, a portion of the cranium and in no way related to the visceral skeleton. Among these observations may be mentioned those of Fuchs, Kölliker, Marshall, Parker and Bettany, and Wiedersheim. Minot derived the stapedial plate as an independent chondrification in the fenestra ovalis. These interpretations have been combined into a third view according to which the stapes is formed partly from the otic capsule (i.e., the stapedial plate), and partly from the hyoid arch. This view of the mixed origin of the stapes has been supported by Gradenigo, van Norden, and Schultze. Günther described the stapes as developing from the mandibular arch. Dreyfuss thought it developed from either the first or second arch. Cope and Frazer described the origin of the stapes as 'peri-arterial.' O. Hertwig regards its origin as uncertain. Finally one might mention the view of Siebenmann who would dismiss as immaterial the possibility of a definite relation of embryonic visceral cartilage and adult ear ossicles.

One must admit with Keibel (1912, p. 281) that "there is great difficulty in tracing back a skeletal structure to the early pre-chondral stages, and it carries with it the danger of subjective interpretation." It seems desirable temporarily to set aside considerations of phylogeny and attack the problem from the point of view of the mechanics of development involved in a single ontogeny.

It is a well known fact that the crania of all vertebrates pass through a common phase of development which may be regarded as a ground-plan of vertebrate cranial formation. Surrounding the anterior end of the notochord there is found a parachordal cartilage, and anterior to this are located the trabeculae. In the

meantime there have arisen three pairs of bilaterally symmetrical sensory epithelia—those of the nostrils, eyes, and ears. Each of these sensory epithelia becomes more or less completely surrounded by a prechondral cytoblastema which subsequently becomes cartilaginous. So striking is the intimacy with which each cartilaginous capsule adjusts itself to the contour of its respective epithelium, that it seems not unreasonable to suppose that each epithelium in some way furnishes the stimulus which effects the chondrification of the surrounding mesenchyme. If this inference be correct, it is evident that the removal of a given sensory epithelium would inhibit the development of the corresponding cartilage. If this also be true, the early removal of the auditory epithelium would furnish a means of testing to what extent the stapes homologue together with its stapedial plate can develop in the absence of a cartilaginous otic capsule, or in the absence of the stimulus to which the latter owes its formation.

The work of W. H. Lewis ('04) is of great interest and importance in this connection. Lewis found that if the otocyst of an anuran be transplanted to the mesenchyme of a urodele, there develops around the transplanted otocyst a cartilaginous capsule which is typically urodelan in character. Unfortunately, experimentation of this sort is at present impossible so far as mammalian development is concerned. Avian development does, however, lend itself to this sort of procedure.

REMOVAL OF THE OTOCYST

Chick embryos of from thirty-five to sixty hours constituted the material for experiment. The experiments were of two types: 1) one of the otocysts was completely or incompletely removed by insertion into it of a very warm fine-pointed platinum needle, for a sufficient length of time to coagulate the liquid contents of the otocyst, whereupon the sensory epithelium would adhere to the needle when it was removed; 2) the otocyst was transplanted.

After being subjected to this sort of treatment the eggs were sealed and allowed to incubate for various lengths of time—

generally to an age at which the normal stapes on the uninjured side was well differentiated. A distinct advantage of this method is that the normal side serves as a control.

In general the results of the removal of the otocyst have been made known through a previous communication (Reagan, '14).

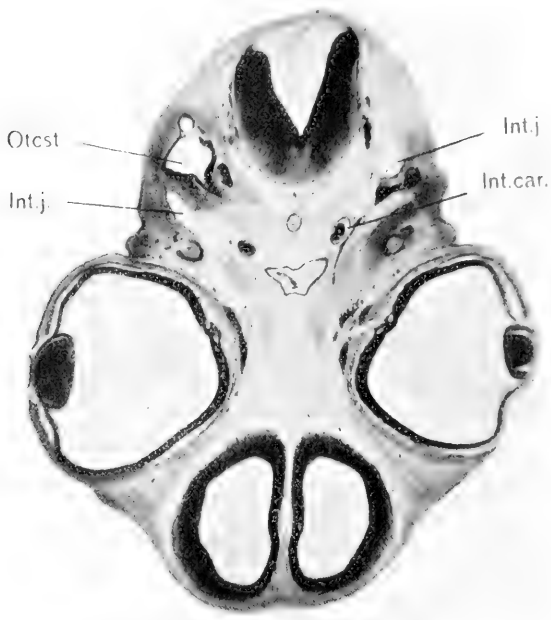


Fig. 1 Frontal section through the otic region of a five-day chick. On the left side an otocyst is present. On the right none is seen. The section is otherwise almost symmetrical. All sections figured in this account are viewed from their posterior faces so that the embryo's left corresponds to the reader's left. P. E. C.[1] No. 1120. *Int car.*, internal carotid; *Int. j.*, internal jugular; *Otcst.*, otocyst.

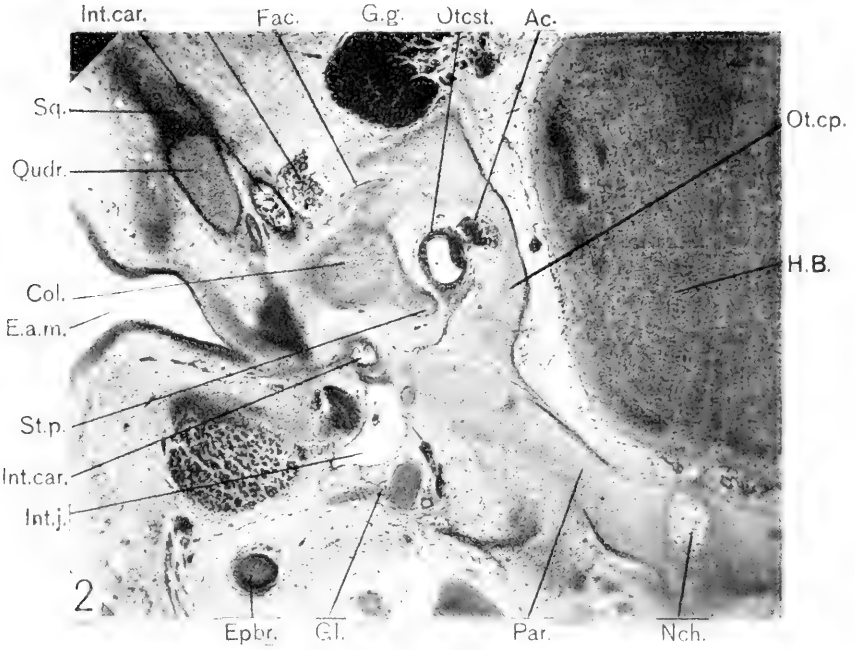
A preliminary study was made of embryos at an age in which the mesenchymatous tissue surrounding the normal otocyst was still in a prechondral or membranous stage. Figure 1 shows a section through the otic region of a chick embryo which had been incubated for five days. On the unoperated side there is a

[1] Princeton Embryological Collection.

typically developed otocyst, around which the mesenchyme has begun to condense, staining rather deeply. On the operated side of the same embryo (right side of figure 1) it will be noticed that the otocyst is entirely lacking. The mesenchyme occupying the former region of the otocyst resembles in every way the other surrounding, lightly staining mesenchyme, and shows no evidence of condensation into an otic capsule. There was no attempt at regeneration of the removed auditory epithelium. This embryo had not yet developed visceral cartilages.

We may now consider cases in which the embryos which had been operated upon were allowed in each case to live until the stapes of the normal side was well developed. Eight and nine-day embryos were found to be most favorable for study.

Figure 2 represents a frontal section through the otic region on the normal side of an eight-day embryo from which the right otocyst was almost completely removed at the forty-fifth hour of incubation. The columella is seen lying between the external auditory meatus and the fenestra ovalis. Fused to the inner end of the columella is a flange-like ring of cartilage; but the two are everywhere separated by a distinct perichondrium which seems to be shared in common by the periphery of the mesial end of the columella and by the lateral internal surface of this flange of cartilage, the stapedial plate. A large part of the stapedial plate, or more correctly, the stapedial flange—projects freely into the mesenchyme. A portion of it, however, is continuous with the cartilage of the otic capsule, and seems always to have been a part of that cartilage. Situated inside the otic capsule is the auditory epithelium. The plane of section lies transverse to the transitional portion of the epithelium between sacculus and lagena. Mesial and dorsal to this epithelium is a portion of the acoustic nerve. A small branch of the internal jugular vein projects into the otic capsule. Dorsal to the capsular region, the facial nerve is seen emerging from its foramen. The plane of section is too far ventral to show the proximal connection of this nerve. A section in such a plane is shown in another embryo in figure 8. Dorso-anterior to the facial nerve will be noticed the large Gasserian ganglion, fibers from which are seen,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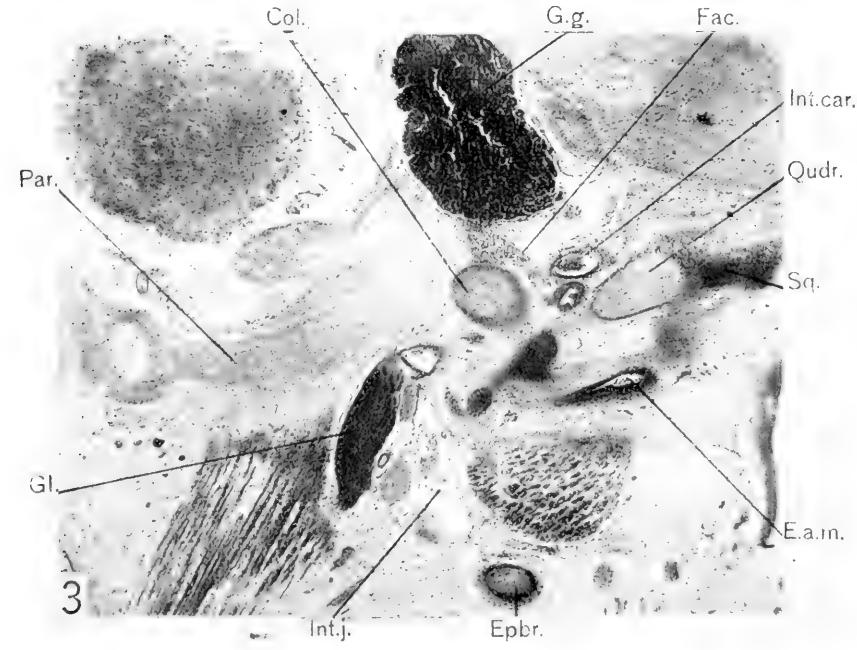
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in neighboring sections, to pass under the internal carotid. Mesial to the latter is the large internal jugular. Lateral to the carotid is the cartilaginous quadrate. Ventral to it is the parachordal cartilage which surrounds the notochord and is continuous laterally with the otic capsule. Ventral to the hind brain is the parachordal cartilage which surrounds the notochord and is continuous laterally with the otic capsule.

Figure 3 represents a frontal section through the operated side of the same embryo. This side presents striking contrast to the conditions just described for the normal side, inasmuch as certain structures are absent. The auditory epithelium has been removed. The otic capsule has failed to develop. The columella, similar to that of the normal side, projects mesially from the external auditory meatus. There is no stapedial plate. The inner end of the columella abuts against (but is not fused with) the parachordal cartilage. Some of the region which would normally have been occupied by the otic capsule has been invaded by the Gasserian ganglion, which seems to have migrated postero-ventrally and completely imbibed or fused with the facial ganglion. Thus it happens that in neighboring sections the large Gasserian ganglion appears to give rise to the facial nerve, which sends a branch to the anlage of the stapedius muscle or at least of a muscle in close relation to the columella. In most other respects the conditions are similar to those on the normal side.

Conditions similar to those in this embryo have been obtained in a large number of cases. It seems highly probable that the stapedial plate owes its formation to the same general stimulus which initiates the cartilage-formation that constitutes the otic

Figs. 2 and 3 Frontal sections of the otic regions of an eight-day chick from which the right otocyst was removed at a time at which the embryo was forty-five hours old. Figure 2 shows the normal side while figure 3 shows the operated side in which a stapedial plate has failed to develop. P. E. C. No. 1117. *Ac.*, acoustic nerve; *Col.*, columella; *E.a.m.*, external auditory meatus; *Epbr.*, epibranchial cartilage; *Fac.*, facial nerve; *G.g.*, trigeminal ganglion; *Gl.*, glossopharyngeal nerve; *Int. cor.*, internal carotid; *Int. j.*, internal jugular; *Nch.*, notochord; *Ot. cp.*, otic capsule; *Otcst.*, otocyst; *Par.*, parachordal; *Qudr.*, quadrate; *Rh.*, hind brain; *Sq.*, squamosal; *St. p.*, stapedial plate.

capsule. Whether the stapedial plate is to be considered a part of the otic capsule, or as an independent formation in the fenestra ovalis is purely a matter of interpretation. The greater part of the stapedial plate seems to be structurally independent of the otic capsule, while a portion of it is fused with the latter probably from a very early time, seemingly having arisen from a part of the otic capsule. It seems reasonable to assume that the avian stapedial plate is not a part of the visceral skeleton. At any rate, if its constituent mesenchyme is a derivative of the second visceral arch, that mesenchyme is powerless to form a stapedial plate unless stimulated to do so by the auditory epithelium.

It has often happened that after the seventh day the columella became much flattened or irregular on its inner end. On the eighth day, this internal end was often found to have fused with some neighboring cartilage, sometimes with the parachordal, and sometime even with the quadrate. This may conceivably be an expression of its normal habit of fusing with a stapedial plate. Such abnormal procedure generally necessitates a bending of the columella from its normal direction. This tendency to fuse with neighboring cartilages seems to become even more marked in later development. In one case, for which I offer no explanation, the columella is bifurcated, one branch being fused with the parachordal, while the other branch (fig. 10) is curved dorso-laterally, its free end being connected with the external auditory meatus by a column of dense prechondral mesenchyme.

The plane of section of figure 10 on the operated side presents a picture quite similar to that in figure 168 in Lillie's "Development of the Chick." Owing to the great similarity of the quadrate cartilage on the right side of my figure 10 to that cartilage which Lillie had labeled 'Meckel's' cartilage, I feel convinced that Lillie may have misinterpreted the section of quadrate in his figure. On the left side of my figure the otic process of the quadrate extends dorso-posteriorly to unite with the otic capsule. External to this is the anlage of the squamosal.

It is of interest to note that if a very small portion of the otocyst be left in the mesenchyme, the latter will chondrify in

the region of the small abandoned sensory epithelium and follow its contour very closely. If, for example, a small ventral portion of the otocyst be left in the embryo, it will take on the form of a hollow sphere which will generally be found in the region normally occupied by the lower portion of the lagena. The cartilage surrounding it will likewise be found to be a sphere. It may be of interest to state that in chemically treated teleost embryos it sometimes happens that both otocysts fuse more or less completely in the median plane. Whatever may be the configuration of the sensory epithelium, the developing otic capsule always conforms to the shape of the former. It seems well established that the cartilages forming about the three main pairs of sensory epithelia in the head are formed in response to the presence of those epithelia.

DISPLACEMENT OF THE OTOCYST

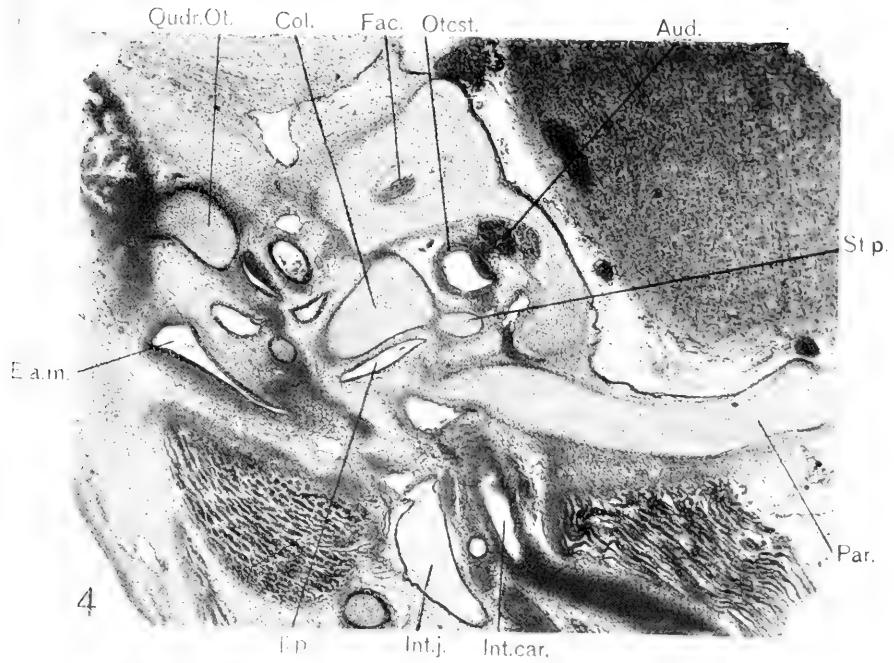
It was found that the otocyst, when displaced into foreign mesenchyme, caused cartilage-formation. It was hoped that both distinct otic capsule and stapedial plate might be induced to form from mesenchyme far removed from the head-region. The results of this sort of experiment are not yet sufficiently clear-cut to warrant their discussion. One case of displacement may, however, be profitably considered, namely, one in which the upper portion of the otocyst was transferred to a region which afterwards proved to be the foramen through which the facial nerve passed.

The embryo to be described was fifty hours old at the time of operation. A warm needle was introduced into the otocyst, the liquid contents of which became sufficiently coagulated that a pull of the needle was able to move the otocyst in the loose mesenchyme. The needle was pulled outwards and dorso-anteriorly and held in that position as steadily as possible for a considerable time. A small amount of warm Ringer's solution was allowed to run down the needle, drop by drop, until the needle could be pulled out of the coagulum. In this instance the entire otocyst did not follow the needle but pulled asunder, a small portion remaining in its original position; this fragment

which was left behind assumed a spherical form. When the embryo was sectioned it was found that this hollow sphere of epithelium occupied a position symmetrical with the distal extremity of the lagena of the uninjured side, possessing like the latter, a cartilaginous capsule which appeared as a rounded projection of the parachordal cartilage. The displaced portion of the otocyst (figs. 5 and 9) may be interpreted as utriculus with perhaps a portion of the sacculus. The anlage of the endolymphatic duct, and in all probability a portion of the utriculus were destroyed. The remainder of the displaced epithelium, together with the intimately related acoustico-facialis ganglion are found to have occupied and dilated the foramen in the cartilage which is normally occupied by the geniculate ganglion and traversed by the facial nerve.

Figure 4 is a photograph of the normal side of a frontal section through the columellae of the embryo just described. The figure requires no explanation beyond that given for figure 2. Its chief value lies in the fact that it affords a comparison of the normal stapes with that of the operated side. The conditions of the latter are shown in figure 5, which is from the opposite side of the same section from which figure 4 is taken. The columella on the operated side resembles in size, shape, and position, that of the normal side. Auditory epithelium does not appear in this plane of section on the noral side. There is no otic capsule in this section. Coincident with this fact is that of the absence of a stapedial plate. It will be noted that two cartilages are present around the exit of the facial nerve. The more ventral one has a dorso-ventral direction, its lower end

Figs. 4 and 5 Photographs of the otic region of a nine-day chick embryo in which the right otocyst was early displaced into the region of the future exit of the facial nerve. Both figures lie in the same transverse plane. Figure 4 is through the normal side. Figure 5 is through the operated side. It shows the ventral-most portion of the displaced otocyst together with the facial nerve. These are frontal sections. P. E. C. No. 1118. *Aud.*, auditory nerve; *Col.*, columella; *E.a.m.*, external auditory meatus: *E. Col.*, extra-columella; *Fac.*, facial nerve; *G.g.*, trigeminal ganglion; *Gl.*,glossopharyngeal; *Int. car.*, internal carotid; *Otcst.*, otocyst; *Par.*, parachordal; *Qudr.*, quadrate; *Sq.*, squamosal; *St. p.*, stapedial plate.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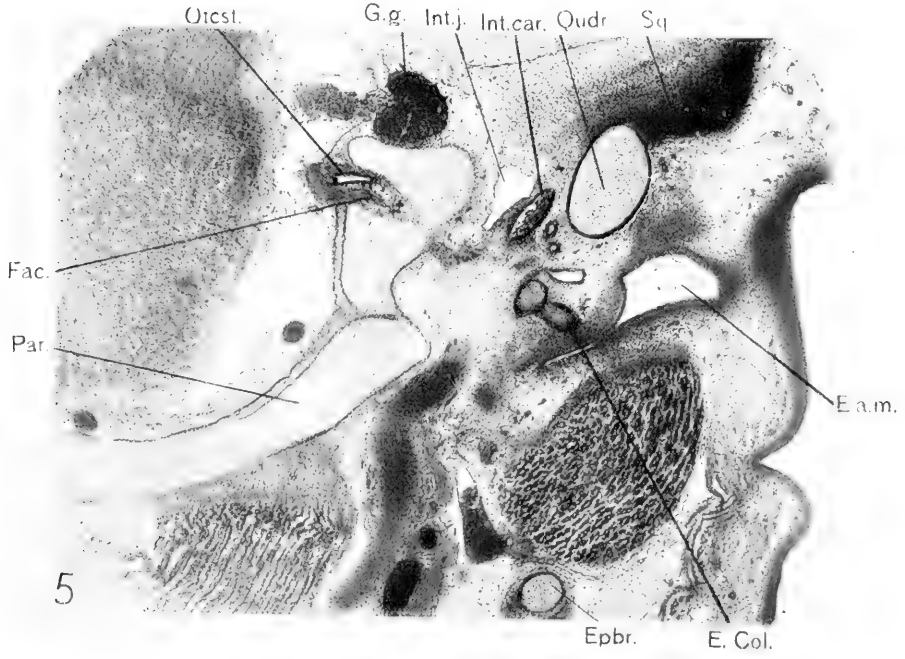
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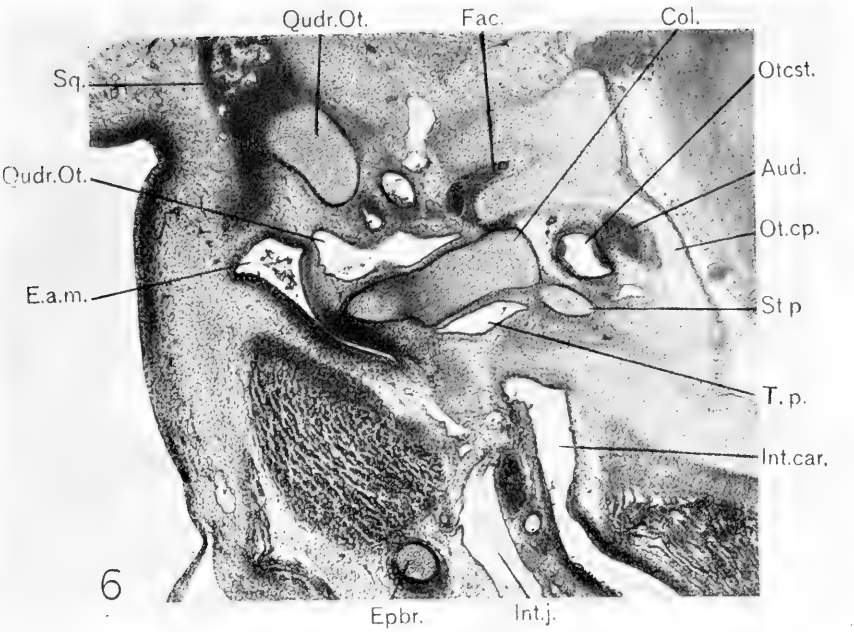
having as a base the lateral extent of the parachordal. This cartilage seems to be comparable to that which is found mesial to the auditory epithelium on the normal side. Dorsal to the facial nerve will be noticed a section of the cartilage which forms the roof of the capsule which surrounds that nerve. It is evident that the cartilages surrounding the facial nerves on the two sides are in every way comparable. There is reason to believe that these two cartilages, portions of the otic capsule, perhaps, have arisen in response to the presence of the facial nerve, acoustico-facialis ganglion, and the portion of the otocyst which was moved into the potential region of the foramen for the facial nerve. In some cases it was observed that the acoustico-facialis ganglion, by reason of its intimate connection with the otocyst, was removed with the latter, a cartilaginous covering for the nerve failed to develop. Examination of figure 3 might seem at first to afford evidence against this view, for we have here a facial nerve which is not surrounded by a cartilage. In figure 3, mesial and internal to the distal extent of the parachordal cartilage will be noticed an L-shaped cartilage which might conceivably represent the cartilage described as the capsule of the facial nerve in figure 5, having become displaced through pressure. The conditions might easily be correlated with the displacement of the trigeminal ganglion. There is perhaps another alternative. It might be assumed that the L-shaped cartilage or its normal homologue represents a portion of the parachordal complex which arises independently of the sensory epithelial tissue. In other words it might be assumed that not all of the otic capsule arises in response to the sensory epithelium, but perhaps in part to a stimulus from other nervous tissue. In figures 5, 6, and 10, it will be seen that a portion of the capsule surrounding the lagena on the uninjured

Figs. 6 and 7 Photographs from a section which passed through the greatest extent of the columellae of the same nine-day embryo from which figures 4 and 5 are taken. Figure 6 is from the normal side. Figure 7 is from the operated side. Note that the columellae are about equal in length and that there is no stapedial plate in figure 7. P. E. C. No. 1118. *Qudr. ot.*, otic process of the quadrate; *T.p.*, tympanic pouch. (Other abbreviations as in figures 1 to 5). The upper portion of the tympanic pouch in figure 6 was unfortunately labeled 'Qudr. ot.'[1]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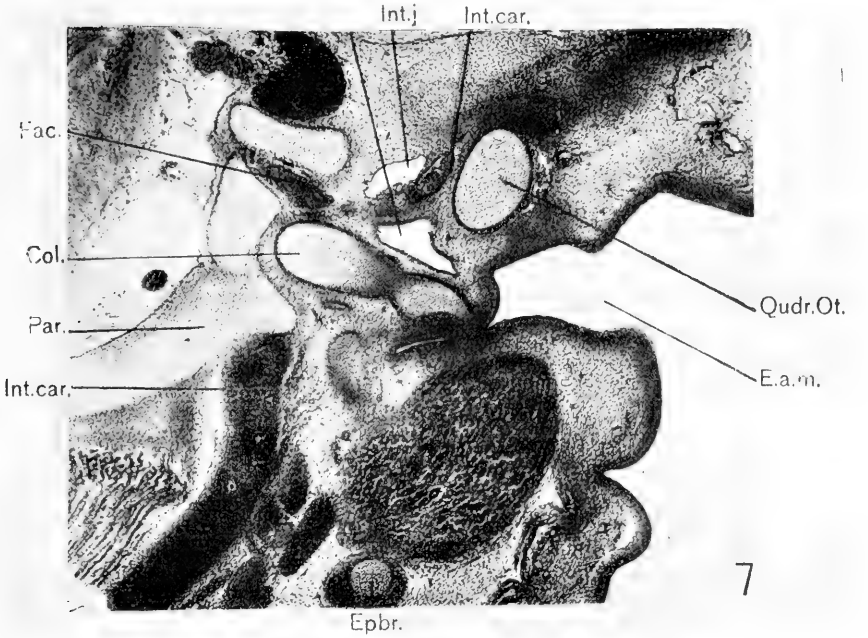
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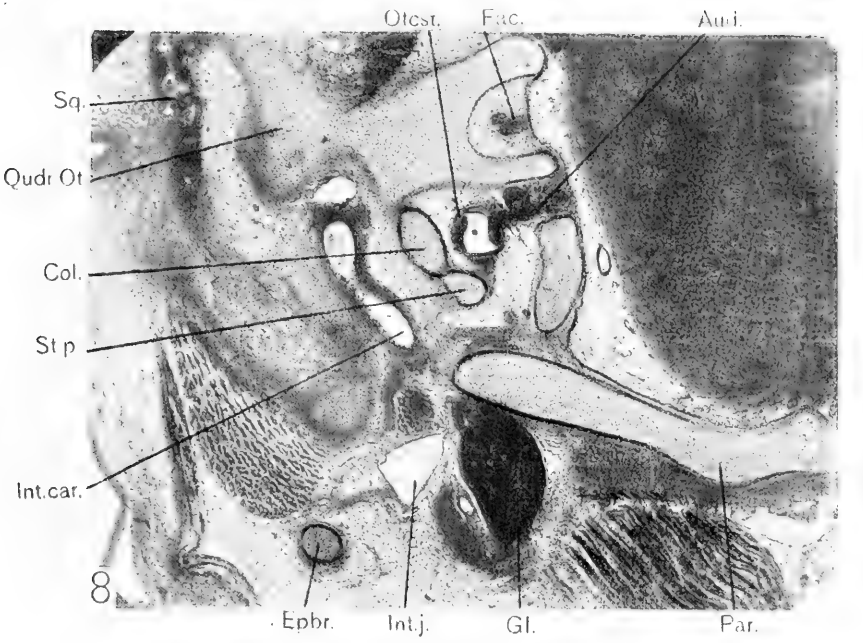
side is represented on the operated side by a narrower parachordal.

Figure 6 represents the normal region in a section of the same embryo, through the head of the columnella. It shows the relation of the facial nerve and its capsule. Figure 7 represents the operated side in the same plane of section as that of figure 6; the acoustico-facialis ganglion lies against the lower extremity of the otocyst. Sections in this region studied progressively towards the dorso-posterior region reveal the cavity of the transplanted portion of the otocyst. It is not difficult to see that the otocyst here occupies the foramen for the facial nerve, and that the acoustic and facial ganglia still preserve their intimacy of connection.

Figure 8 shows the normal side of a section somewhat dorsal to the plane of section of figures 6 and 7. The proximal portion of the facial nerve is seen entering its capsule on the mesial surface of the latter. Laterally this capsule is joined by the otic process of the quadrate. In the abnormal portion of this same section (fig. 9) one sees the capsule of the facial ganglion and nerve occupied by the sac-like otocyst; the latter more than fills the cavity at the inner entrance to the capsule and projects out into the scant mesenchyme between the cartilage and the brain-wall. Inside the capsule the sensory epithelium is surrounded by a vascular plexus. Where the auditory epithelium projects mesially towards the brain wall, it is internally covered by a perichondrium-like condensation of the adjacent mesenchyme which is continuous with the perichondrium of the cartilaginous capsule. Lateral to this capsule will be seen the otic process of the quadrate together with the overlying squamosal. In sections still more dorsad, the otic process of the quadrate is fused with the cartilaginous capsule surrounding the epithelial sac indicating the comparability of this cartilage to that of the unoperated side through which the facial nerve courses.

Figs. 8 and 9 Frontal sections through the region of the facial ganglion of this same nine-day embryo. On the normal side the facial ganglion (fig. 8) is shown. Figure 9 is the abnormal side of the same section in which the cartilage which would normally have surrounded the facial ganglion surrounds the entire otocyst. (Abbreviations as in previous figures. P. E. C. No. 1118.)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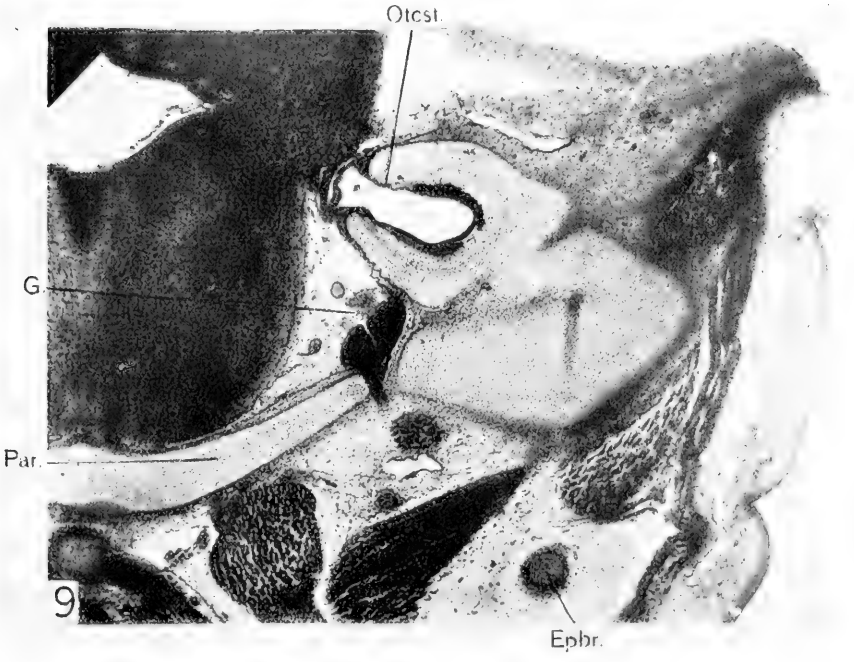
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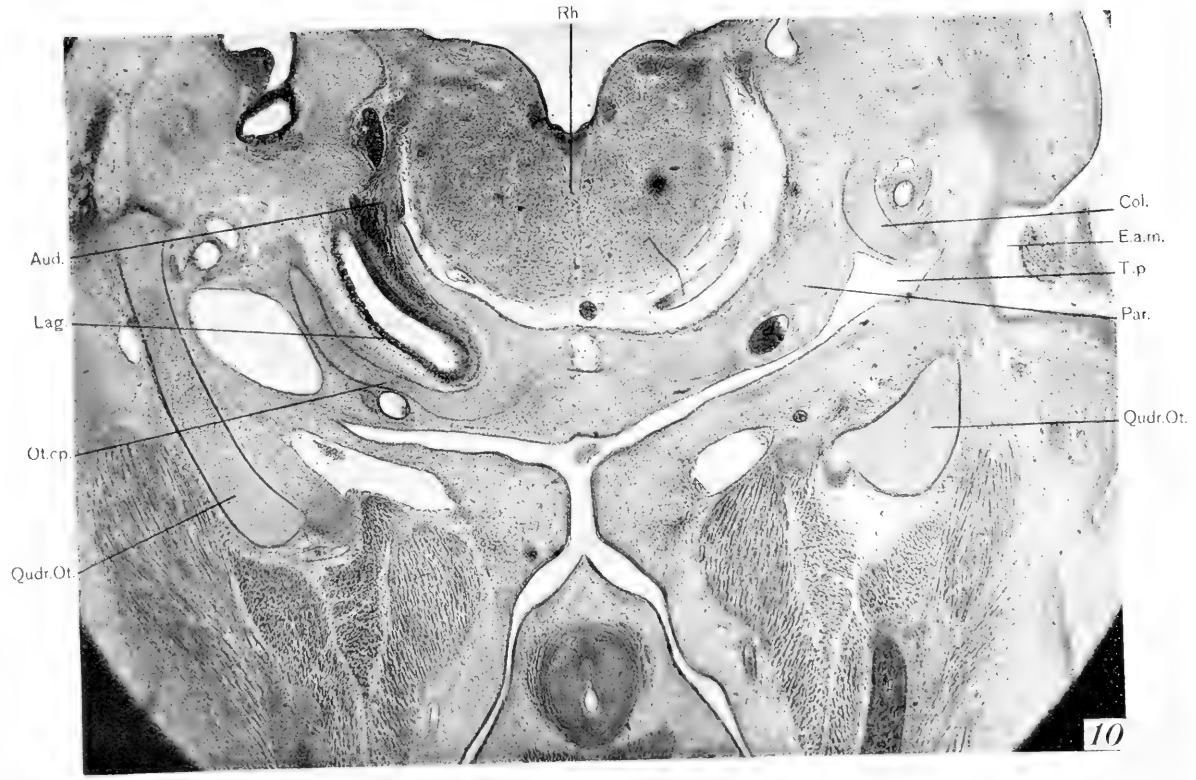

Fig. 10 Cross section of the otic region of a nine-day chick from which the right otocyst was removed at the age of 50 hours. The right columella is much curved dorso-laterally. There is no stapedial plate on this side. Note the absence of otocyst and otic capsule. P. E. C. No. 1119. *Lag.*, lagena. (Other abbreviations as in previous figures).

If there exists a necessary nexus between the conditions above described and the deductions which have been made, it may be profitable to emphasize the following points:

The removal of the otocyst from young chick embryos seems entirely to do away with the stimulus to the later development of the otic capsule.

Embryos devoid of otic capsules fail to develop stapedial plates.

If it be objected that the removal of the otocyst produces the apparent inhibition by injury or removal of the mesenchyme surrounding the otocyst, it can only be urged that in case of mere transplantation it is unlikely that there was sufficient heat to injure the outlying mesenchyme when the auditory epithelium itself was not seriously injured. If it be objected that such formative mesenchyme was moved bodily in case of complete removal, it might be urged that such displaced mesenchyme failed to produce a stapedial plate in case of displacement of the otocysts. Furthermore, it seems improbable that the injury of the mesenchyme at the site of first invagination of the sensory epithelium should affect very profoundly the mesenchyme lying ventral to the hind brain in the potential region of the extremity of the lagena.

The columellar portion of the stapes of birds seems to penetrate the center of the stapedial plate (stapedial 'flange' or stapedial 'ring'), so that the central mesial surface of the columella actually forms a portion of the internal surface of the stapedial plate at least in embryos of eight to ten days. The present study has not determined the permanency of these conditions.

Whether the results here outlined demonstrate the stapedial plate to arise from the otic capsule, they certainly point to a possible kinship of the two structures, in that the same exciting stimulus is a factor in the production of each. The columellar portion of the stapes attains its full length and normal proportions in the absence of a stapedial plate; this fact offers a significant contrast in the nature of the latter structures. The stapes-homologue in birds seems to be of mixed origin, cranial and visceral.

It is evident that the development of the otic capsule and of the stapedial plate belongs in that class of phenomena which have been designated as the 'interactions of parts' and again as 'dependent development.' Hertwig ('94) was one of the first to attach great importance to those developmental process which are due only indirectly[2] to the original constitution of the fertilized ovum itself and likewise those due only indirectly to the external environment. These he designated as the "perpetually changing mutual relations in which cells of an organism are placed to one another." According to Herbst, all movements, tropic or tactic, and many processes of differentiation are responses of a formative, as well as a directive nature. Thus far the clearly demonstrated cases of interaction of parts are relatively few. Loeb has shown that the position occupied by the pigment cells on the yolk-sac of Fundulus is an oxygenotatic reaction. Several cases have been reported in which early displaced embryonic cells have resumed their original position.

The development of certain parts of the vertebrate eye has been shown to be of a 'dependent' sort; there is, however, a certain amount of disagreement over the experimental results obtained by various observers. Spemann showed that if the formation of the optic vesicle of the frog be inhibited by injury to the medullary plate, or that if its approximation to the ectoderm be prevented, a lens will not form. Lewis obtained similar results on Amblystoma. He showed that ectoderm from other regions transplanted to that region approximated by the optic vesicles would give rise to a lens. Schaper removed the entire central nervous system from a tadpole except the fore-brain and optic vesicles. He found that a lens developed in situ in the ectoderm and not as an infolding from it; he believed the lens was self-differentiating. On the other hand, King found that lenses might develop in the entire absence of an optic cup in the tadpole, while Mencl found the same to be true in an abnormal embryo of Salmo salar. In these two latter cases it is possible that stimuli exerted by the fore-brain itself caused lens-forma-

[2] Clark ('15) wrongly regards such phenomena as those in which hereditary constitution plays no part.

tion. Werber has shown that numerous isolated lenses may be developed in chemically treated teleost embryos in the absence of optic vesicles. Even here we may have a response to stimuli exerted by disrupted diencephalic tissue. The evidence in favor of self-differentiation of the lens seems to be entirely outweighed by the production of lenses in foreign ectoderm in the experiments of Lewis. Spemann and Lewis have also shown that the formation of the cornea is a response to stimuli from the optic cup, and will take place even though the lens be removed; that the cornea, once formed, degenerates with the removal of the optic cup. The work of Lewis on the formation of the amphibian otic capsule has already been mentioned. In a recent communication, I believe I have demonstrated that myocardial concrescence is a tactic response to the presence of endocardial tissue. Finally the stapedial plate is of interest in this same connection. If these several observations be confirmed, investigations of this sort may prove to be of extreme interest. It is probable that the interaction of parts is of greater importance than we are generally aware. In many cases we have reason to believe that in the earliest stages in the formation of many organs, a stage of equipotentiality of parts is not widely departed from, but that as development proceeds, totipotence is lost and powers of self-differentiation are more strongly asserted. In general one should expect from this that 'dependent development' would be a phenomenon confined to the earlier stages of ontogeny; there seem, however, to be certain exceptions to this. Of these exceptions, two sorts seem to be well established. First, it is a striking fact that many of the cases of 'dependent development' above discussed have to do with relatively late organ-formation. In fact it seems reasonable to suppose that the action of a differentiated part upon an undifferentiated one would be greater, the farther the activity of the former had advanced. The cases cited have to do with organ-formation in which the process is one of synthesis or 'composition' of rather diverse sorts of tissue. A second exception is that of non-differential cleavage or non-differential development in which an apparently indifferent tissue sometimes maintains itself until relatively late in ontogeny, re-

taining its embryonic character and possessing several or many diverse potentialities. In case of an individual cell of such a tissue, one of these potentialities becomes realized while the others do not; this probably takes place in response to a stimulus. It seems highly probable that mesenchyme is a tissue of this sort. At any rate chondrification can be artificially produced in mesenchyme to form structures whose existence was most certainly not predetermined in the fertilized ovum; conversely, mesenchyme which would normally have chondrified can be prevented from so doing by the removal of the exciting stimulus to chondrification. It may well be that the diverse sorts of connective tissue and vascular tissue afford cases parallel to this in their development.

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BRISTLE INHERITANCE IN DROSOPHILA

II. SELECTION

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TEN FIGURES

CONTENTS

PREVIOUS RESULTS

The general scheme of the inheritance of the extra bristles that appeared on the thorax in a race of Drosophila ampelophila has been demonstrated in a previous account.[1] It was shown that the extra-bristled condition behaves as a Mendelian reces-

[1] MacDowell: Bristle inheritance in Drosophila. I. Extra bristles. Jour. Exp. Zoöl., vol. 19, no. 1, 1915.

sive character in crosses with normal wild flies; that, in the second generations of such crosses, the distribution of the numbers of extra bristles as a whole is lowered in comparison with the distributions in the uncrossed race; that, in general, small flies have fewer extra bristles than large ones; that the selection of high grade parents effected an immediate increase in the means of the offspring; and that the continuous selection of flies with the highest numbers of extra bristles as parents, failed to be accompanied by a continuous increase in the means of the offspring, after the 8th generation. The following hypothesis was found to be in accord with all the facts discovered at that time:

There are units of inheritance that influence the numbers of extra bristles; these units are effective only in the absence of a certain other unit that prevents the appearance of all extra bristles.

In the present paper experiments are presented that give unquestionable support to this hypothesis. Four main experiments have been carried out as tests for its correctness.

1. The continuation of the selection of high grade parents till there could be no possible doubt that the means were no longer being raised as they were in the early generations of selection.

2. The selection of low grade flies at the beginning of the extra bristled race, (low selection) to establish a low race. The success of this experiment would show that the germ plasm of the original extra flies was not all uniform.

3. The establishment of a low grade race from the high selected race by selecting the lowest instead of the highest grade flies as parents (return selection). The success of such an experiment would demonstrate that there were genetic differences between the high and low grade flies in the high selected race; the failure of this experiment would show that there are no longer present such genetic differences as were present in the earlier generations, causing the rapid rise in the means.

4. The establishment of a low grade race from flies with extra bristles that appear in the F_2 of a cross with normals (extracted

low selection). The success of this experiment would demonstrate that the germ plasm in such a race had been altered by the cross.

HIGH SELECTED RACE

Frequency distributions by generations

The most intimate view of the course of the main selection experiment is given by the frequency distributions given in figure 1. This figure presents two parallel series of curves; those at the right represent the distributions of the totals of each generation from the 12th to the 49th, on a percentage basis; those at the left, the distributions, based on actual numbers, of an inbred line from a single pair of parents in each generation. The grades and numbers of the parents selected are indicated by small frequency curves between the curves of the totals of the generations; the grades of the parents selected in the line of single families are shown by small squares and triangles.

Besides showing the results of selecting high grade parents, both in single families with few individuals and in total generations with many individuals, one of the most important points to be brought out by these curves is, that the distributions from single pairs of parents tell in general the same story as the total generations that were raised from parents of different grades. The comparison of the means in these two series will make this point still more clear. This comparison is made in figure 2, *A* and *B*.

Since the flies in the single families are also included in the total generations, a certain amount of similarity would necessarily be occasioned; yet the differences in the numbers of families and of offspring (indicated in the figure) appear to give the close similarity in the two sets of means a special significance. That the curve of the means of the single families follows that of the total generations, indicates that the latter is not merely the chance result of a collection of families with independently varying means, but fairly describes the condition of the race in different generations. Attention must be

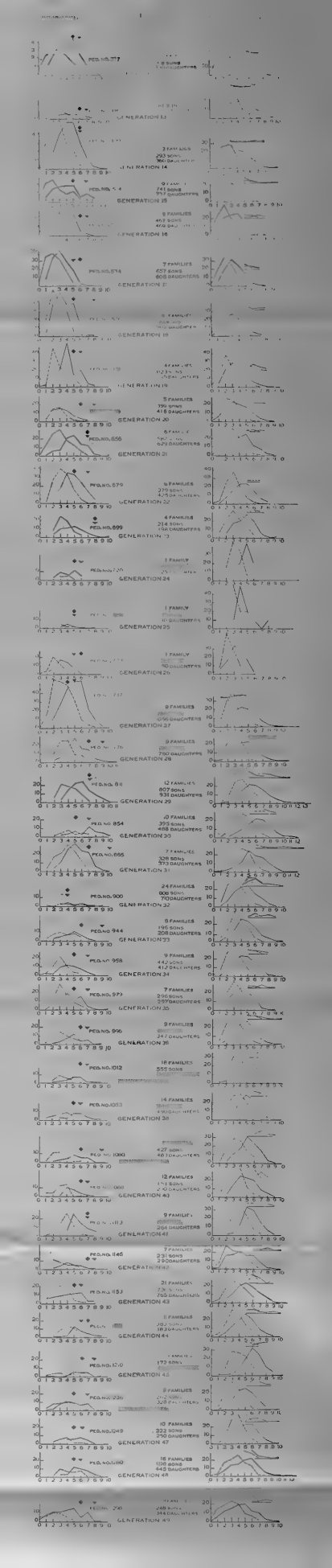
called to the fact that this line of single families has not been chosen with any regard to the grades of the flies. This is the only line that can be continuously followed to the end of the experiment.

Means

Before any interpretation of the means of the high selected race can be attempted, certain facts must be made clear. In generations 24, 25 and 26 there hatched only one family each. In generation 26 unfavorable conditions nearly caused the loss of the whole race. In order to continue the experiment parents with fewer bristles than the standard for selection were bred in producing generation 27. Generations 29 to 40 inclusive were raised in a room automatically maintained at 90° F. and 50 per cent relative humidity. In generations following the 32d, pressure of other work precluded the examination of all the flies that hatched in a bottle, so that no more flies were examined in one generation than were required to find suitable matings for the production of the next generation. Since the first flies hatching in a bottle tend to have more bristles than flies hatching later[2] one could be reasonably sure of finding the highest grade flies in the first few days of hatching. The result of thus not counting all the flies that hatched, was to raise the

Fig. 1 Frequency distributions of generations 12 to 49 in the high selected race. The units on the base lines show the classes of extra bristles, the ordinates represent frequencies. The series of curves at the right show the distributions of all the flies in each generation; these frequencies are plotted on a percentage basis so that the equal areas included by the curves in each generation do not equal numbers of individuals. The curves at the left show the distributions of a single line of families with only one pair of parents in each generation; the ordinates for these curves are the actual numbers of flies. This single line is the continuation of one called *E* in figure 6, page 81, MacDowell '15. The distributions of the parents chosen in the total generations are shown by small curves between the larger ones; the parents chosen in the line of single families are shown by squares (males), and triangles (females): solid lines—daughters broken lines—sons. The pedigree numbers of the families in the single line, and the numbers of families and flies in the different total generations are given on the figure.

[2] MacDowell: loc. cit., p. 86.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means. This fact, together with the warmth of the constant temperature room, renders the means in this period (generations 32 to 49) obviously uncomparable with the means in earlier

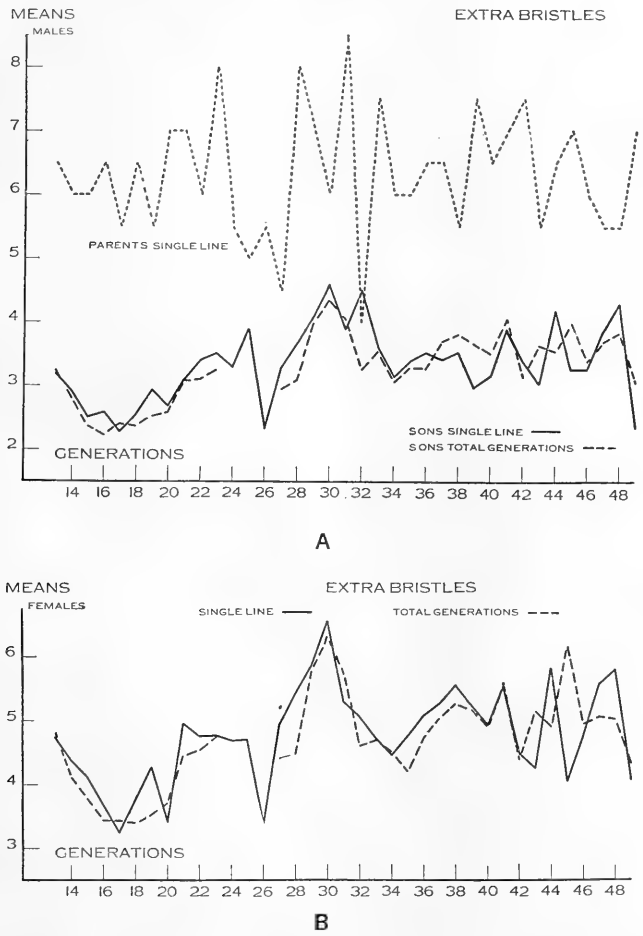


Fig. 2 Means in the line of single families compared with means in the total generations. Since there was only one family each in generations 24, 25 and 26 there is only one curve through these generations. In 'A' the sons are compared in 'B' the daughters are compared. The mean grade of the two parents in each generation of the single line is shown with the 'A' curves. When single families are examined it is found that fluctuations in the parental grades are not accompanied by corresponding changes in the average grades of the offspring.

periods. The progress of selection must be sought within this period and within the earlier period.

With these facts in mind the curves showing the means of the sons and daughters in the high selected race are to be examined (fig. 3). The means for generations 2 to 11 differ somewhat from those given previously. In order to increase the rigidity of selection, families with certain lower grade parents were

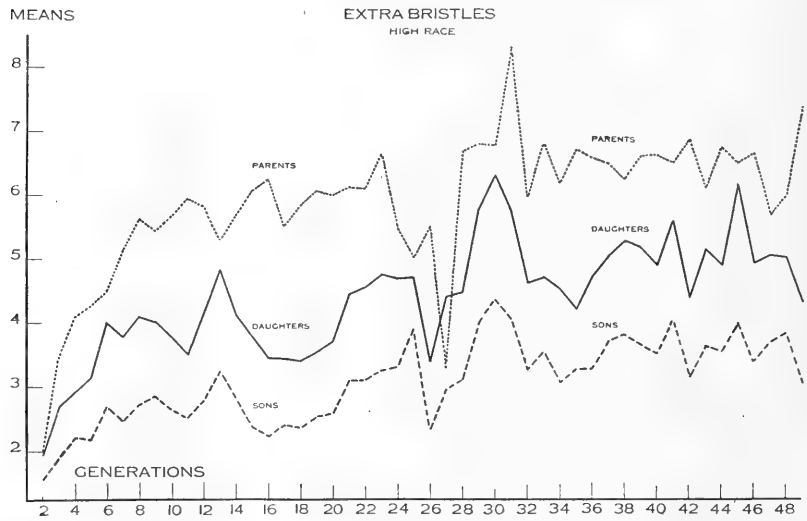


Fig. 3 Means of the parents, sons and daughters in the total generations of the high selected race. The means of the parents have been weighted according to the numbers of their offspring. The curves of the sons and daughters are much alike in their fluctuations; except in the early generations the curves of the parents and offspring do not show parallel fluctuations.

excluded together with all their descendants. As here given, the means include all such families. The resulting changes in the means are insignificant from the standpoint of a general conclusion, since they do not modify the general form of the curve.

In the earlier report, the initial rise in the means was emphasized. It would be a difficult matter to determine just where this initial advance was stopped. Were certain striking

and evident responses to environment not present, such as are found in generations 26 to 30, the curves might be treated mathematically, and a theoretical curve fitted, but since such clear evidence of the irregular influence of environment is present, this mathematical treatment seems out of place. The critical period is between generations 11 and 23. In this period it seems possible that the ideal condition is represented by the series of high points at grade $4\frac{3}{4}$ for the females, that the long gradual decline is due to less and less favorable conditions, which later are improved till the high point is again reached. It may be somewhat problematical whether the high or the low points (at $3\frac{1}{2}$) in this period should be considered ideal, yet it does seem probable that an intermediate position would not be so considered. Moreover there can be found no tendency for the later generations in this period to be higher than the earlier ones. The irregularities in generations 24, 25 and 26 may be discounted, since, as explained, these include very small families. Although the high peak in generations 29 to 31 may not be directly due to the conditions of the constant temperature room, yet its occurrence immediately upon the introduction of the flies into that room, and the absence of any comparable high region in all the twenty months of the experiment, strongly suggest a causal connection between the two. However it is evident that whatever stimulus the constant humidity and temperature may have been at first, the effectiveness of this stimulus became weakened long before the flies were removed from this room. The portions of the curves of the sons and daughters following this high region show general fluctuations, but one can not detect a tendency for the earlier generations to have lower means than the later ones. Between generations 36 and 38 there is a continuous rise, but this rise does not exceed the limits of fluctuation shown by the later generations.

To conclude, it may be said that there is a rise at the beginning of selection; that this is followed by a period in which no rise is discernible; that the irregularities in generations 24, 25 and 26, and the sudden rise in generations 29 to 31, are not due to the

selection; and finally that in the last period (generations 32 to 49) no permanent rise in the means can be found.

Extremes of variation

The range of variation gives further light on the nature of the changes during selection. If the race as a whole is being changed, upper and lower extremes should change as well as the means. In this race the lower limits show very slight variation. In all the generations (excepting the 24th and 25th) the low limit for the male is either grade 0 or 1; in 33 generations it is 0; in 12 generations it is 1. The females are a little higher;

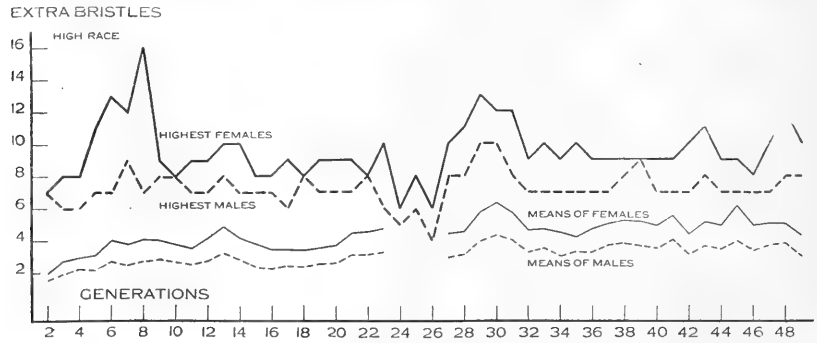


Fig. 4 Highest grade son and daughter in each of the generations of the high selected race, compared with the means of all the sons and daughters. Selection has not resulted in the raising of the high limit of variation.

in 20 generations the low limit is 0; in 18 generations it is 1; in 7 generations it is 2. From this one may say that the low extreme is a relatively fixed point. The high extremes are more variable. These are shown in figure 4. The highest grade recorded (16) was found in generation 8; the highest grade recorded (13) in the 41 generations of selecting that followed this, appeared in the first generation raised in the constant temperature room. Throughout the whole series there appears a tendency for the highest female to be grade 9 and the highest male, grade 7. Selection then has not called into being any new grades that were not obtainable very near the beginning of the experiment.

Standard deviations

If the extremes in a frequency distribution are stationary, the movement of the mean will tend to modify the standard deviation. If the mean and the mode of a curve are close to one end of the distribution, their movement towards the middle of the range will have the tendency to raise the standard deviations. There is a fairly close approximation to these conditions in the frequencies of the different generations. The early generations show means and modes close to the lower end of the scale of grades; in later generations the means rise toward the middle of the scale, but the extremes are not greatly changed. This leads one to expect the increase in the standard deviations that actually has been found to follow the increase in the means. Moreover the standard deviations fall when the means fall.

In figure 5 *A* and *B* the standard deviations for the sons and daughters are separately compared with their respective means. Attention must be called to the fact that the means and the standard deviations are plotted in these curves on different vertical scales, and that the coincidence of the lines does not indicate fluctuations of equal magnitude, but rather, fluctuations in like directions. The close parallelism between these two sets of constants disappears soon after the 32d generation. This is apparently due to the fact that, as explained, the complete yield from each bottle was not recorded. This incomplete sampling slighted the lower part of the distributions so that the means were raised, but the standard deviations were reduced and rendered irregular. Besides rising and falling with the means in each sex, the standard deviations of the sons are lower than those of the daughters, in the same way as the means of the sons are lower.

The general conclusions to be drawn from the study of the standard deviations may be stated as follows: The standard deviations of the frequencies with lower means are lower than the standard deviations of the frequencies with higher means. The generations between the 32d and the 49th of course form an exception to this statement, but in accord with the expla-

nation of this exception is the claim that the higher averages in this period are due to incomplete counts and not to a modification due to selection.

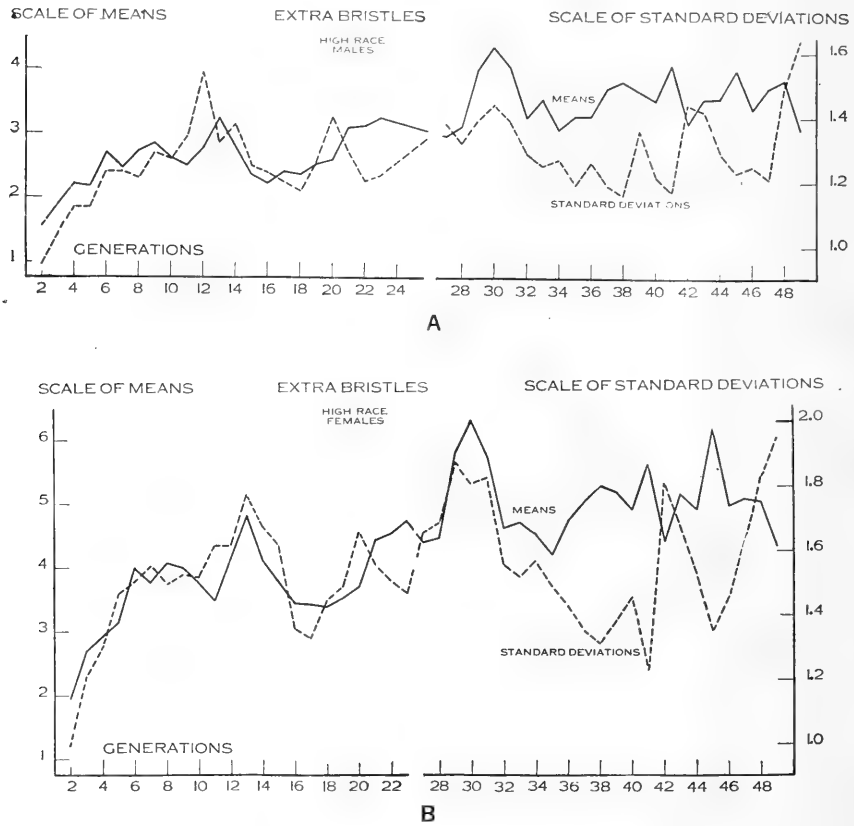


Fig. 5 Standard deviations in the high selected race compared with the corresponding means. It must be noted that the standard deviations are plotted on a scale five times as large as that of the means. Generations 24, 25 and 26 have been omitted on account of their small numbers. 'A' is based on the sons, 'B,' on the daughters. A close parallelism is demonstrated between these constants. All flies from each bottle were not counted in generations 33–49 inclusive; this probably accounts for the break in this relationship that appears in those generations.

Discussion of the distributions

The study of the frequencies of the 49 high selected generations, whether by the means, the extremes, or the standard deviations, has failed, except in the first few generations, to indicate any advance that may be attributed to selection. It seems that breeding only high grade parents has not succeeded in producing increasingly higher distributions, that new extreme grades have not appeared after the continued selection of the highest flies as parents. Other than genetic factors seem to influence the character of the distributions, yet it remains possible that a part at least of the fluctuations in the means may be due to corresponding variations in the parents selected. This possibility is considered in the following section.

Comparison of parents and offspring

The point of primary theoretical interest is not the ability, or inability, to advance the means and raise the whole distribution of a race by selection. The crux of the selection problem is whether abmodal parents have abmodal children. Specifically it is, do parents with higher bristle grades produce children with higher bristle grades?

When the means of the offspring, which have already been considered by themselves are compared with the means of the parents that produced them, important evidence is forthcoming. Referring again to figure 3, this comparison will be considered. The dotted line in this figure shows the means of the parents selected. These have been weighted according to the numbers of offspring produced by the various grades of parents included in each mean. The means of the parents in each generation are plotted on the same vertical line as the means of their sons and daughters. The basic numbers for these curves will be found in table 1.

One finds on making this comparison that, excepting the first few generations, the means of the parents and their children vary with great independence. The high peaks of the parents curve are not accompanied by high peaks in the curves of their

TABLE 1

Data from the high selected race arranged to show the relationships between the parents selected in the different generations and their sons and daughters.

	PARENTS															OFFSPRING							
	Distribution according to numbers of extra bristles												Means			Numbers of individuals		Modes		Means		Standard deviations	
Generation	1	2	3	4	5	6	7	8	9	10	11	12	Males	Females	Weighted means; males and females	Males	Females	Males	Females	Males	Females	Males	Females
12					6	2		1	1				5.20	6.60	5.8252±0.036	268	300	2	4	2.783±0.063	4.154±0.060	1.540±0.045	1.623±0.043
13			4	1		6	5						5.37	5.50	5.2802±0.145	81	117	3	4	3.247±0.099	4.838±0.111	1.329±0.070	1.783±0.079
14			1			2	2	1					5.66	6.66	5.6983±0.027	293	360	3	4	2.805±0.054	4.122±0.060	1.380±0.038	1.689±0.042
15					10	5	1		1	1			5.22	6.55	6.0632±0.039	741	737	2	3	2.372±0.031	3.784±0.040	1.255±0.022	1.626±0.026
16					5	6	5	2					5.66	6.77	6.2473±0.027	467	469	2	4	2.233±0.039	3.446±0.042	1.239±0.024	1.365±0.030
17					9	4	1						5.57	5.28	5.5035±0.015	657	606	2	3	2.412±0.032	3.437±0.036	1.205±0.022	1.330±0.026
18					13	4	2	2	1				5.27	6.36	5.8207±0.024	845	907	2	3	2.376±0.027	3.405±0.032	1.179±0.019	1.454±0.023
19					9	11	5	3					5.64	6.35	6.0540±0.016	1123	1115	2	3	2.534±0.025	3.530±0.030	1.266±0.018	1.499±0.021
20					2	5	2	1					5.60	6.80	5.9878±0.046	399	418	2	3	2.672±0.046	3.782±0.054	1.384±0.030	1.663±0.038
21					4	4	3	1					5.83	6.33	6.1101±0.031	582	629	3	4	3.100±0.036	4.442±0.042	1.291±0.025	1.569±0.030
22					2	5	5						6.00	6.50	6.0870±0.019	379	425	3	4	3.111±0.041	4.548±0.049	1.200±0.029	1.515±0.035
23					1	3	2	2					6.50	6.75	6.6288±0.039	214	198	3	5	3.257±0.056	4.742±0.070	1.224±0.040	1.470±0.050
24					1	1							5.00	6.00	5.5000	20	25	3	5	3.300±0.118	4.680±0.130	0.781±0.083	0.968±0.092
25					2								5.00	5.00	5.0000	10	10	3	4	3.900±0.201	4.700±0.098	0.943±0.142	0.458±0.069
26					1	1							6.00	5.00	5.5000	42	50	2	3	2.333±0.080	3.380±0.108	0.867±0.064	1.129±0.076
27	2	4	5	5	2								2.66	3.44	3.2842±0.030	988	1056	2	4	2.963±0.030	4.404±0.034	1.380±0.021	1.664±0.024
28					4	3	7	2	2				6.11	7.33	6.6785±0.037	606	780	3	5	3.114±0.036	4.473±0.041	1.323±0.026	1.693±0.029
29					7	3	7	4	2		1		5.58	8.00	6.7969±0.025	867	931	4	5	3.988±0.033	5.778±0.042	1.396±0.023	1.889±0.029

30					3	3	9	4	1				6.20	7.50	6.7627 ±0.033	395	488	4	7	4.342 ±0.049	6.309 ±0.055	1.440 ±0.034	1.815 ±0.039
31						4	3	2	1	2	1	1	6.57	9.71	8.2845 ±0.050	328	373	4	5	4.036 ±0.052	5.721 ±0.064	1.396 ±0.037	1.833 ±0.045
32					16	19	7	3	2	1			5.75	6.54	5.9558 ±0.033	808	710	3	5	3.250 ±0.031	4.617 ±0.039	1.290 ±0.022	1.566 ±0.028
33					1	6	3	3	1				6.00	7.57	6.8106 ±0.034	196	208	3	4	3.541 ±0.061	4.692 ±0.071	1.259 ±0.043	1.520 ±0.053
34					5	7	4	2					5.55	6.76	6.1615 ±0.023	442	412	3	4	3.061 ±0.041	4.512 ±0.052	1.270 ±0.029	1.577 ±0.037
35					4	3	4	1	2				6.00	7.14	6.6972 ±0.048	296	297	3	4	3.277 ±0.049	4.202 ±0.058	1.196 ±0.033	1.490 ±0.041
36					4	8	4	1		1			5.66	7.00	6.4652 ±0.040	343	347	3	5	3.277 ±0.046	4.723 ±0.059	1.265 ±0.032	1.432 ±0.037
37					7	12	11	5	1				5.66	7.27	6.4727 ±0.025	555	637	3	5	3.710 ±0.034	5.033 ±0.036	1.193 ±0.024	1.358 ±0.026
38					9	8	7	4					5.57	6.85	6.2369 ±0.025	489	490	4	5	3.816 ±0.035	5.269 ±0.040	1.161 ±0.025	1.314 ±0.028
39					3	12	10	3	2				6.00	7.26	6.5862 ±0.029	427	483	4	5	3.656 ±0.044	5.174 ±0.042	1.366 ±0.031	1.387 ±0.030
40					13	9	2						6.08	7.00	6.6167 ±0.026	151	200	4	4	3.516 ±0.067	4.895 ±0.070	1.228 ±0.048	1.468 ±0.049
41					4	8	2	3	1				5.66	7.11	6.4857 ±0.043	296	264	4	5	4.061 ±0.046	5.591 ±0.051	1.172 ±0.032	1.237 ±0.036
42					2	3	6	2	1				6.28	7.28	6.8542 ±0.046	231	290	2	4	3.147 ±0.064	4.386 ±0.072	1.446 ±0.045	1.813 ±0.051
43					10	19	8	2	3				5.66	6.85	6.0825 ±0.020	730	765	3	5	3.640 ±0.035	5.148 ±0.041	1.421 ±0.025	1.677 ±0.030
44					4	10	4	1	2	1			5.90	7.18	6.7363 ±0.056	389	383	3	5	3.542 ±0.044	4.890 ±0.053	1.291 ±0.031	1.534 ±0.037
45					5	6	6	5					5.72	7.27	6.4876 ±0.041	172	192	4	6	3.982 ±0.063	6.156 ±0.066	1.232 ±0.045	1.353 ±0.046
46					3	6	5	2	2				5.66	7.66	6.6271 ±0.027	262	328	3	5	3.389 ±0.052	4.945 ±0.054	1.251 ±0.037	1.464 ±0.026
47					10	5	4	1					5.10	6.50	5.6631 ±0.028	222	250	4	6	3.707 ±0.055	5.060 ±0.070	1.212 ±0.039	1.637 ±0.049
48					9	16	4	2		1			5.50	6.68	5.9537 ±0.022	398	445	4	6	3.844 ±0.051	5.029 ±0.059	1.497 ±0.038	1.834 ±0.041
49						9	2	3	2	1	1		6.11	8.44	7.3656 ±0.038	248	344	3	5	3.056 ±0.070	4.325 ±0.071	1.648 ±0.050	1.951 ±0.050

sons and daughters; nor are low points in the parents' curve accompanied by low points in the curves of their children. When the parallelism of the curves of the sons and daughters is noted, the lack of such parallelism between the curves of the parents and children is very striking.

High points

Parents' curve	Generations 11, 16, 23, 31, 49
Children's curve	Generations 13, 30, 41, 45

Low points

Parents' curve	Generations 13, 17, 27, 47
Children's curve	Generations 11, 26, 35, 42

There is a sort of similarity between the curves of the parents and children that results from the fact that the flies selected as parents for one generation are averaged with the children in the preceding generation. If the mean of a generation is very low, there will be found few high grade flies to select as parents for the next generation, and accordingly the means of the flies selected will be low. In this way low points in the children's curves will be followed by low points in the parents' curve in the next generation; and in like manner high points in the children's curves will be followed in the next generation by high points in the parents' curve. In generation 11 the means of the offspring are low, in generation 12 the parents are low; in generation 26 the offspring are low, in generation 27 the parents are low; in generation 30 the offspring are high, the parents are high in generation 31; Such relationships, of course have no significance in the question of the genetic differences between high and low grades of extra bristles. However, the following facts do have significance for this question: there is a difference of nearly $3\frac{1}{2}$ bristles between the means of the parents in generations 27 and 28, while their offspring differ by less than one-fifth of a bristle; in generations 28, 29 and 30 the parents' means are nearly alike, while the means of the offspring rise $1\frac{3}{4}$ bristles.

A similar comparison is offered in figure 2A. In this figure the means of the sons in individual families is compared with the

grades of the parents. The wide differences in the parents in different generations is due to the fact that only one pair of parents is included in each mean. The same story of independence is told by these curves as by those including total generations (Fig. 3). As the curves demonstrate this point very clearly, it will not be necessary to cite them in detail.

Considering again the comparison between the means of single families and of the total generations, one finds further evidence of this independence of the grades of the parents and their offspring. Single pairs of parents produce offspring whose averages are close to the averages of offspring from parents of various grades in the same generation. The distribution of the parents selected in the whole race, given in table 1, should be studied in comparison with the parents in the single line.

So, besides finding that in successive generations high parental averages are not accompanied by high filial averages, it is found that in the same generation a single pair of parents produces offspring with an average similar to that of the offspring from parents of different grades. The significance of this point will be discussed in a later section.

Conclusions from the high selected race

From the above presentation of the facts concerning the high selected race, the following conclusions are drawn: Through the breeding of flies with the highest numbers of extra bristles, the means of the race were raised for a series of generations; after this no evidence was found of any further influence of selection upon the means of the race. Since the lower limit of bristle variation remains practically fixed, and the variations in the upper limit are slight with no relation to the selected parents, the changes in the variation of the race, as measured by the standard deviations, have no genetic significance; the standard deviations rise and fall with the means. The initial success and subsequent failure of selection, as indicated by the means, is also shown by the relationship between the grades of the parents and their offspring. In the early generations an in-

crease in the means of the parents produced an increase in the means of their offspring; in all the other generations an increase or decrease in the means of the parents had no influence on the means of the offspring. Higher parents produced higher offspring in the early generations; higher parents did not produce higher offspring in the following generations.

LOW SELECTED RACE

Already substantial evidence has been presented that shows that in the earlier generations the lower grade parents produce children with lower mean grade.[3]

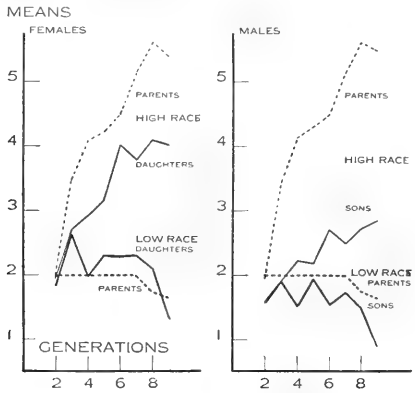


Fig. 6 Low race, low selected from the beginning, before the high selected race was differentiated. The means of the parents and offspring in this race are compared with the means of the parents and offspring in the high selected race. Means of the parents in the high race are weighted according to the numbers of their sons and their daughters.

But perhaps clearer than any other evidence in support of this conclusion is found in the establishment of a low race as the result of selecting low grade flies at the beginning of the experiment. One pair of flies, grade 2, from the second generation of the high race, gave rise to a line that was carried on for eight generations by mating only the flies of grade 2 or under. As in the high race, all matings were made between brothers and sisters in pairs. Figure 6 shows the means of this low

[3] MacDowell: loc. cit., p. 70.

grade race in comparison with the means of the high selected race. The flies selected as parents in the low race are shown, as well as the flies selected as parents in the high race. The clear cut divergence of the curves of the two sets of means, whether males or females are considered, leaves no question as to the interpretation to be accepted. The means of the low race are lower than those of the high race because the germ plasm of the low-selected flies determined lower bristle numbers than did the germ plasm of the high-selected flies. The establishment of this race has considerable importance; it proves that there are differences in the germ plasm that differently influence the numbers of extra bristles; it proves that inheritance plays a part in the variation of the numbers of extra bristles.

RETURN SELECTED RACE

If the continuous appearance of germinal variations be assumed to be the cause of the progress of selection that was demonstrated in the early generations, it would be expected that the reverse movement of the means could be effected at any time by selecting the low grade flies. Whether or not the advance found in the early generations can be reversed becomes a test of the hypothesis that variations in the germ plasm are continuously taking place. If it be found that the return selection does not lower the means as fast as the advance selection raised them it would seem that the germ plasm, having passed through a series of selections, had become changed in regard to its properties of variation. In other words, this would mean that selection, in reducing the variability of the germ plasm, had limited the possibilities of further successful selection; that certain specific properties being sorted out of the germ plasm, one could not again obtain from such a ine the complete array of somatic variations previously obtainable.

Means

From the 15th generation of the high race, four pairs of low grade flies (averaging 1.12) were mated to start an inbred race

that was carried on for 8 generations before it was lost. A definite limit had been set above which no flies should be used as parents. It happened there were such a few flies available below this limit that the number of matings was small. Since there is a tendency for the low bristle grades to appear on small flies, the selection of low grades accomplished the sorting out of the smallest and weakest flies as progenitors of the race. These two explanations seem to account for the fact that no flies at all appeared in the matings made from the 8th generation of this line. A second series of return selections was started from the 26th generation of the high race, but this series also came to a premature termination. In summarizing these data, the flies have been grouped according to the time their parents were mated, instead of according to the generation. This has been done so that, in studying different selected lines, the flies compared would have more nearly similar environmental conditions. It frequently happened that the matings for one generation would be made during several weeks so that families in different generations would be hatching at the same time. This would often result in families in different generations being raised under more similar conditions than families from the first and last matings in a single generation. All the progeny of flies mated in the same half month have been grouped together and corresponding groupings have been made of the families in the high selected race. These classes are called 'groups.' All the families mated in the first half of June, 1915, are averaged and plotted in the first point of the curve in figure 7; all the matings made in the same time in the high race are averaged as the first point in the curve so marked. There are also shown in figure 7 the averages of the parents selected (dotted lines) that produced the offspring averaged in the solid lines. The data are given in table 2 *A* and *B*. The averages of the parents have been weighted according to the numbers of their sons in '*A*' and according to the numbers of their daughters in '*B*.' Although the low and the high selected parents average about four bristles apart, the averages or their offspring are very close together. In both the return and high selected races

the means of the sons in the successive groups, as well as the means of the daughters, follow along closely together. The one exception to this close similarity of the two sets of groups lies in group 18 in the second return series. Yet this is probably

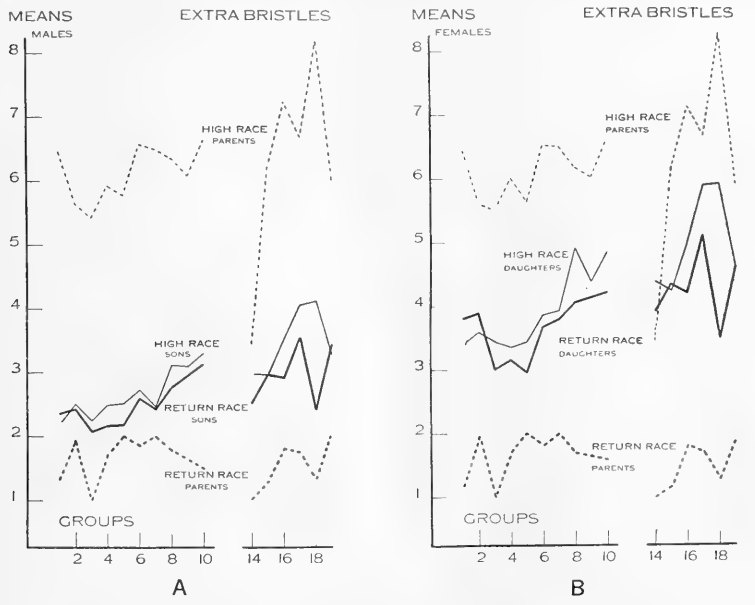


Fig. 7 Return race, selected for low bristle grades, starting from the 15th generation of high selection. Means of offspring and their parents are compared with means of offspring and their parents in the high selected race. All the flies have been grouped according to the half month in which the parents were mated. All the parents are weighted according to the numbers of their sons or daughters. 'A' based on son, 'B,' on daughters. The offspring from high and low grade parents average very nearly the same. The means of the return race fall slightly lower than those of the main high race, but this difference does not increase with continued selection and is probably due to the weakening effect of breeding the lowest grade flies, since these are the smallest and the weakest.

not significant, as there happen to be only a few flies included in this mean. The low productivity in these groups is one of the signs of the weakened condition of the return race at this time. Even though the means of the return and the high race are close together, there appears a tendency for the return means to hang just a little lower than those of the high race. Such a differ-

TABLE 2A

Data from the three races high, return and extracted, grouped according to the half month in which the parents were mated, and arranged so that flies raised at approximately the same times in the different races may be compared. 'A' gives the records for the sons

GROUP	PARENTS						SONS								
	Means (weighted by sons)						Means						Standard deviations		
	Nature of selection						Nature of selection						Nature of selection		
	High	Number of families	Return	Number of families	Extracted	Number of families	High	Number of flies	Return	Number of flies	Extracted	Number of flies	High	Return	Extracted
1	6.467	7	1.312	4			2.191±0.035	366	2.357±0.060	154			1.244±0.031	1.103±0.042	
2	5.645	8	1.949	5			2.504±0.032	617	2.422±0.041	237			1.194±0.023	0.941±0.029	
3	5.429	10	1.000	1			2.240±0.032	512	2.077±0.136	26			1.077±0.023	1.035±0.096	
4	5.924	4	1.703	5	2.000	4	2.487±0.037	489	2.168±0.039	441	1.754±0.034	395	1.212±0.026	1.234±0.028	0.969±0.024
5	5.767	10	2.000	6	1.707	11	2.517±0.030	798	2.170±0.040	323	1.581±0.023	693	1.258±0.021	1.075±0.028	0.899±0.016
6	6.562	6	1.852	5	1.935	6	2.729±0.041	461	2.587±0.060	206	1.662±0.036	343	1.320±0.029	1.272±0.042	0.982±0.025
7	6.471	3	2.000	2	1.669	7	2.452±0.056	263	2.426±0.137	54	1.371±0.034	383	1.350±0.040	1.498±0.097	0.984±0.024
8	6.337	2	1.779	4	0.941	3	3.116±0.074	163	2.764±0.087	127	1.060±0.063	86	1.394±0.052	1.450±0.061	0.873±0.045
9	6.061	8			1.026	7	3.086±0.030	775			1.645±0.044	262	1.227±0.021		1.052±0.031
10	6.620	6	1.477	6	0.366	5	3.291±0.053	237	3.125±0.053	264	1.733±0.042	225	1.213±0.037	1.283±0.038	0.947±0.030
11					1.033	8					1.613±0.044	181			0.882±0.031
12	5.500	1			1.008	3	4.300±0.117	20			2.368±0.120	57	0.781±0.083		1.346±0.085
13	5.403	2			1.884	8	2.634±0.102	52			1.688±0.054	173	1.092±0.072		1.057±0.038
14	3.448	8	1.000	2	1.224	8	2.962±0.031	920	2.526±0.065	135	1.995±0.027	799	1.393±0.022	1.121±0.046	1.128±0.019
15	6.224	6	1.260	3	0.912	5	2.944±0.041	498	2.947±0.059	169	1.730±0.039	315	1.360±0.029	1.134±0.042	1.028±0.028
16	7.223	5	1.791	5	1.759	16	3.500±0.048	270	2.904±0.048	542	1.875±0.016	1531	1.163±0.034	1.651±0.034	0.935±0.011

17	6.666	19	1.733	7	1.248	12	4.042±0.029	1128	3.538±0.051	435	2.464±0.022	952	1.462±0.021	1.586±0.036	1.017±0.016
18	8.163	10	1.319	4	1.765	11	4.102±0.046	401	2.406±0.085	69	2.189±0.027	655	1.370±0.033	1.054±0.060	1.010±0.019
19	5.983	23	2.075	5	1.634	12	3.255±0.031	808	3.438±0.100	73	2.120±0.036	350	1.297±0.022	1.271±0.071	0.998±0.025
20	6.426	14			1.745	11	3.246±0.036	574			1.825±0.045	234	1.295±0.026		1.037±0.032
21	6.506	10			1.974	12	3.214±0.043	355			1.499±0.035	277	1.210±0.031		0.860±0.025
22	6.476	27			1.948	15	3.549±0.027	913			1.676±0.033	401	1.237±0.019		0.968±0.023
23	6.225	14			2.000	8	3.816±0.035	489			2.197±0.035	364	1.161±0.025		0.992±0.024
24	6.606	15			2.000	7	3.656±0.044	427			1.987±0.051	157	1.366±0.031		0.951±0.036
25	6.656	12			1.879	7	3.516±0.067	151			1.850±0.044	207	1.228±0.048		0.949±0.031
26	6.515	9			2.000	11	4.061±0.046	296			2.824±0.039	296	1.172±0.032		1.002±0.027
27	6.885	7			2.000	5	3.147±0.064	231			1.555±0.055	126	1.446±0.045		0.931±0.039
28	6.083	21			1.747	11	3.640±0.035	730			2.072±0.039	293	1.421±0.025		1.014±0.028
29	6.749	11			1.738	8	3.542±0.044	389			2.161±0.044	180	1.291±0.031		0.883±0.031
30	6.497	11			1.777	12	3.982±0.063	172			1.886±0.053	193	1.232±0.045		1.100±0.037
31	6.662	9			1.761	6	3.389±0.052	262			1.783±0.082	92	1.251±0.037		1.178±0.058

TABLE 2B

Data from the three races high, return and extracted, grouped according to the half month in which the parents were mated, and arranged so that flies raised at approximately the same times in the different races may be compared. 'B' gives the records for the daughters.

GROUP	PARENTS						DAUGHTERS								
	Means (weighted by daughters)						Means						Standard deviations		
	Nature of selection						Nature of selection						Nature of selections		
	High	Number of families	Return	Number of families	Extracted	Number of families	High	Number of flies	Return	Number of flies	Extracted	Number of flies	High	Return	Extracted
1	6.446	7	1.175	4			3.407±0.049	373	3.800±0.058	200			1.396±0.034	1.218±0.041	
2	5.619	8	1.962	5			3.586±0.038	558	3.885±0.063	236			1.329±0.027	1.455±0.045	
3	5.508	10	1.000	1			3.431±0.041	522	3.000±0.198	28			1.394±0.029	1.558±0.140	
4	6.003	4	1.709	5	2.000	4	3.353±0.042	536	3.156±0.044	442	2.239±0.037	410	1.452±0.030	1.382±0.031	1.120±0.026
5	5.631	10	2.000	6	1.746	11	3.445±0.034	842	2.967±0.050	303	2.108±0.025	732	1.472±0.024	1.294±0.035	1.039±0.018
6	6.519	6	1.816	5	1.915	6	3.857±0.052	420	3.670±0.087	188	2.095±0.046	306	1.579±0.037	1.771±0.061	1.189±0.032
7	6.476	3	2.000	2	1.675	7	3.675±0.068	271	3.782±0.161	55	1.787±0.042	382	1.674±0.048	1.775±0.114	1.228±0.030
8	6.161	2	1.693	4	1.088	3	4.896±0.081	155	4.054±0.088	166	1.858±0.064	113	1.499±0.057	1.680±0.062	1.021±0.045
9	6.012	8			1.027	7	4.380±0.035	877			2.310±0.056	280	1.534±0.025		1.404±0.040
10	6 613	6	1.583	6	0.363	5	4.845±0.069	220	4.206±0.062	252	2.385±0.053	270	1.496±0.048	1.455±0.044	1.314±0.038
11					1.006	8					2.408±0.054	174			1.065±0.038
12	5.500	1			1.024	3	5.680±0.130	25			2.983±0.111	62	0.968±0.092		1.301±0.078
13	5.416	2			1.838	8	3.600±0.110	60			2.346±0.048	234	1.268±0.078		1.092±0.034
14	3.449	8	1.000	2	1.232	8	4.381±0.035	988	3.920±0.109	137	3.024±0.031	906	1.639±0.025	1.899±0.077	1.404±0.022
15	6.168	6	1.167	3	0.879	5	4.234±0.048	576	4.349±0.069	192	2.616±0.042	368	1.711±0.034	1.417±0.049	1.212±0.030
16	7.110	5	1.817	5	1.780	16	4.977±0.055	350	4.202±0.046	584	2.501±0.017	1652	1.534±0.039	1.660±0.033	1.054±0.012
17	6.656	19	1.722	7	1.299	12	5.885±0.036	1328	5.104±0.074	421	3.295±0.025	977	1.943±0.025	2.262±0.052	1.190±0.018
18	8.250	10	1.276	4	1.775	11	5.909±0.056	464	3.487±0.111	78	3.000±0.029	765	1.779±0.039	1.457±0.079	1.216±0.020
19	5.908	23	1.911	5	1.612	12	4.615±0.040	706	4.616±0.127	73	2.799±0.048	443	1.570±0.028	1.619±0.090	1.487±0.034

20	6.462	14			1.754	11	4.621±0.044	578			2.508±0.054	279	1.565±0.031		1.349±0.038
21	6.532	10			1.982	12	4.187±0.053	348			1.955±0.038	333	1.469±0.037		1.043±0.027
22	6.430	27			1.929	15	4.929±0.029	1004			2.416±0.035	519	1.389±0.021		1.201±0.025
23	6.245	14			2.000	8	5.269±0.040	490			2.932±0.037	396	1.314±0.023		1.104±0.026
24	6.568	15			2.000	7	5.174±0.042	483			2.466±0.046	210	1.387±0.030		0.986±0.032
25	6.587	12			1.967	7	4.895±0.070	200			2.483±0.046	215	1.468±0.049		0.997±0.032
26	6.453	9			2.000	11	5.591±0.051	264			3.427±0.040	297	1.237±0.036		1.030±0.028
27	6.829	7			2.000	5	4.386±0.072	290			2.280±0.057	143	1.813±0.051		1.013±0.040
28	6.082	21			1.757	11	5.148±0.041	765			2.796±0.044	330	1.677±0.029		1.177±0.031
29	6.723	11			1.788	8	4.890±0.053	383			2.778±0.050	203	1.534±0.038		1.053±0.035
30	6.479	11			1.771	12	6.156±0.066	192			3.013±0.058	216	1.353±0.046		1.271±0.041
31	6.599	9			1.653	6	4.945±0.054	328			2.543±0.086	127	1.464±0.026		1.429±0.060

ence may be understood as the result of the unconscious selection of the small and weakened flies on account of their fewer extra bristles.

As was the case in the high selected race, the means of the parents and their offspring show great independence in the return selected lines. This is the more noticeable when one compares the close parallelism between the curves of the high and return races which have totally different parentage.

Standard deviation

It has already been demonstrated that the means and the standard deviations in the high race form closely parallel curves. It may be supposed from this that finding close agreement in the standard deviations of the two races would argue for the close agreement of their distributions. The standard deviations of the various groups of the return selected race (dotted line) are compared with the standard deviations of the corresponding groups of the high selected race (broken line) in figure 8. It is evident that the variability in the two races is similar. The fluctuations in the return race are more striking, as should be expected with fewer individuals, but the general agreement as to the amounts and directions of the changes is unmistakable.

Conclusions

The data from the return selected race give ample evidence that the advance found in the early generations of the high selection has not been reversed. In the beginning, each high selection immediately raised the means higher; in this return selection during 8, and again during 6 generations, no general reduction of the means is discernible. Furthermore, it even appears that the offspring from the low parents are the same as those from the high selected race, with the exception of a slight lowering that seems to be due to the weakened condition of the return race. This similarity of offspring from high and low parents is of course, not incontestably proved by the facts of this race; were this true, it would be unnecessary to go fur-

ther. But no single proof of such a conclusion seems even theoretically obtainable. However, in the accumulation of evidence from different directions, the basis for a conclusion may be found.

The conclusion that high and low grade flies seem to have similar germ plasm sounds quite the reverse of the concluding statement of the preceding section, namely, that there are different sorts of germ plasm that differently influence the numbers of

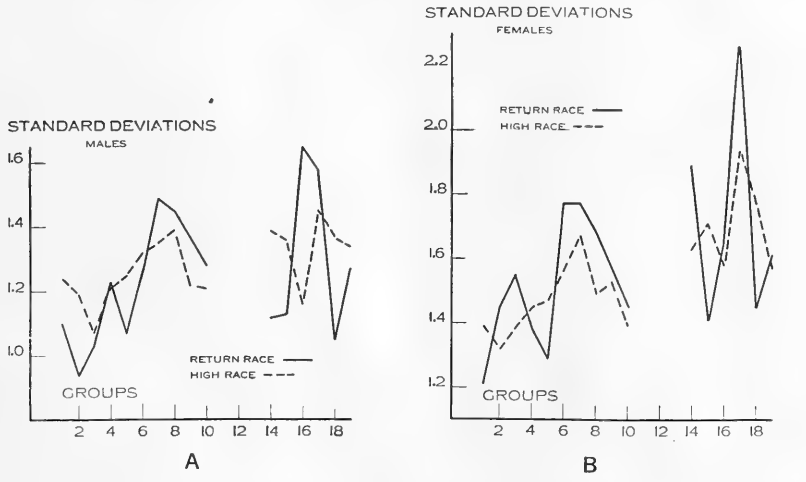


Fig. 8 Standard deviations in the return race compared with the standard deviations in the high race. The close agreement between the standard deviations in these two races supports the conclusion that the races are not differentiated.

extra bristles. The explanation of these rather conflicting statements will be found in the first section, where the course of the high selection was described showing a strong rise in the early generations. The conclusion that different grades do not have uniform germ plasm applied to the flies after the advance had occurred; the conclusion that different grades do have different germ plasm, applied to the flies before the advance had occurred. In view of the conclusions reached in the second section, the failure of the return selections to establish a low grade race can not be claimed to depend on the concealing influence of

environment (which was just as potent in the early generations) nor to depend upon a supposed lack of any genetic control at all of the numbers of extra bristles; it does depend upon a unification of the germ plasm brought about by the high selection.

EXTRACTED LOW SELECTED RACE

After a race of high grade germ plasm has been isolated by selection, the return selections are unsuccessful. This favors the hypothesis previously raised, that the germinal differences involved do not necessarily occur during the process of selection, nor does selection hasten or retard the occurrence of such differences. Selection has accomplished a sorting out of differences already present. Were it possible to regain the differences lost by the selection process, it would be possible to re-establish a low grade race. According to the theory formerly explained, such a regaining of germinal differences could be accomplished by crosses with normal flies. The modification of the distributions of extra bristled flies resulting from such crosses has been considered evidence of the regaining of germinal differences. With this fact in mind, the attempt was made to start a low grade race from the high selected race by first making a cross between the high selected and normal flies, and then selecting low grades from the extra bristled flies appearing as extracted extras in the F_2.

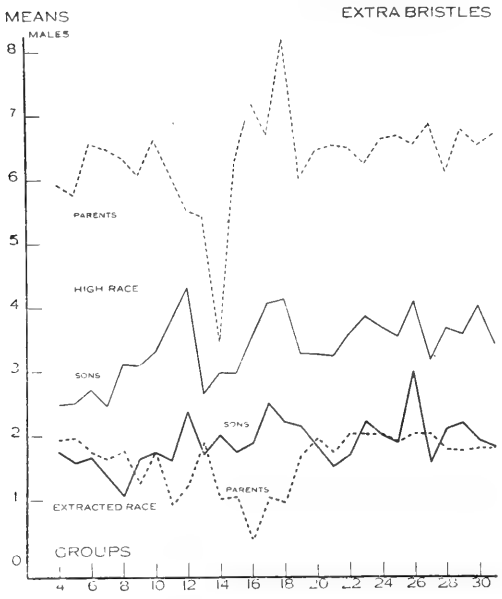
Three flies of the high selected race, generation 16, all double first cousins, were mated with normal wild flies from a stock that had been bred in the laboratory for two years or more. This stock had been inbred for many generations, and for a few generations single brother by sister matings were made by pairs. Four matings were made between the extra flies (grade 2) that appeared in the F_2 of this cross. The race started in this way was continued by using the lowest grade flies available as parents. Following the fourth generation of the race all the flies came from a single one of the four matings made in the first generation of this race. The data are to be found in tables 2, A and B, and plotted in figures 9, *A* and *B*.

The families in the 31 generations of this low selection of extracted extras are grouped by half month periods—the same method as used for the return selections. The last nine groups include total generations; all the matings for a generation in both the extracted and the high race in the same period were made on the same day. The means of the extracted low race are compared in figure 9 with the means of the high selected families grouped in the corresponding half month periods. The dotted lines give the means of the flies selected as parents in both races, these have been weighted according to the numbers of sons 'A' and daughters 'B' from each grade of parents.

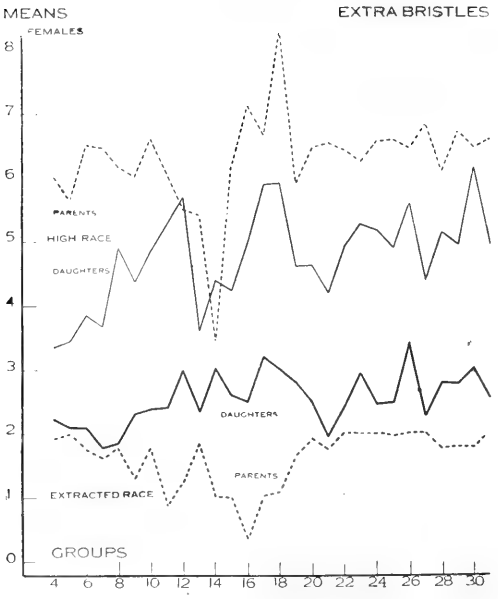
Means

The first fact shown by the curves of the means is, that the extracted race is unquestionably lower than the high selected race. The difference is less marked in the males than in the females, but the range of fluctuations is considerably less for the males in both curves. It may be suggested that the result of selecting low grade flies has been to weaken this race, as was supposed to be the case in the return race. But in this race there were so many low grade flies that were not small, that the same sort of selecting did not have the same tendency to weaken, and thus lower, the race.

The first selection of low grade extracted extras reduced the means at once. There was no series of preliminary selections that made little or no progress. However, after the initial differentiation, the means were lowered still further, for a few generations. Beyond this, selection seems to have had no power. During these early generations the curve of the parents is closely parallel with those of their sons and daughters, but this close agreement is not found in any other part of the whole series. Although the means of the parents and their offspring do not form parallel curves, one finds that the means of the extracted low race and the means of the high race do form parallel curves with coincident high and low points.


MEANS
EXTRA BRISTLES
MALES
8
7
6
5
4
3
2
1
0
PARENTS
HIGH RACE
SONS
SONS
PARENTS
EXTRACTED RACE
GROUPS
4 6 8 10 12 14 16 18 20 22 24 26 28 30

A


MEANS
EXTRA BRISTLES
FEMALES
8
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3
2
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PARENTS
HIGH RACE
DAUGHTERS
DAUGHTERS
PARENTS
EXTRACTED RACE
GROUPS
4 6 8 10 12 14 16 18 20 22 24 26 28 30

B

Standard deviations

In the return race the similarity of the standard deviations with those of the high race was cited as evidence of the similarity of the two lines. In this extracted race the standard deviations argue in like manner that the high race is different (fig. 10 *A* and *B*). The standard deviations have been omitted for groups 11, 12 and 13 since there were such a few individuals included in these groups. The standard deviations of the extracted race are lower than those of the high race. There seems to be something that hinders the appearance of the high bristle grades. It is not environment. One can see from the means that favorable conditions affect the two races in the same way, since both races rise at the same time; yet the rise in the extracted race is never as great. The standard deviations support the conclusion that the extracted race does not vary so much as the high race, that there is a genetic difference between these two races.

Conclusions

The establishment of a low race from the high selected race by means of a cross, stands in strong contrast with the unsuccessful attempt to produce a low race by selecting low grade parents directly from the high race. As in the high race, the first few generations of selecting were successful, and the curves of the offspring and parents are parallel; the lower parents pro-

Fig. 9 Means of the extracted race which was started from the extra flies extracted from a cross between the high race and normal wild. Parents and offspring are compared with parents and offspring in the uncrossed high race. The data are grouped according to the half month in which the matings were made. The last nine averages shown in each curve include all the flies from one generation; at this time all the matings for one generation in each race were made on the same day, so the similarity of the food in these last nine generations results in a greater similarity in the environment of the contrasted races, and there appears a closer parallelism in the means. Parents weighted as in preceeding curves; 'A' based on the sons, 'B,' on the daughters. The first selection immediately separated a race that was distinct from the high race. After the first few generations, fluctuations in the parental means do not occasion corresponding changes in the means of the offspring.

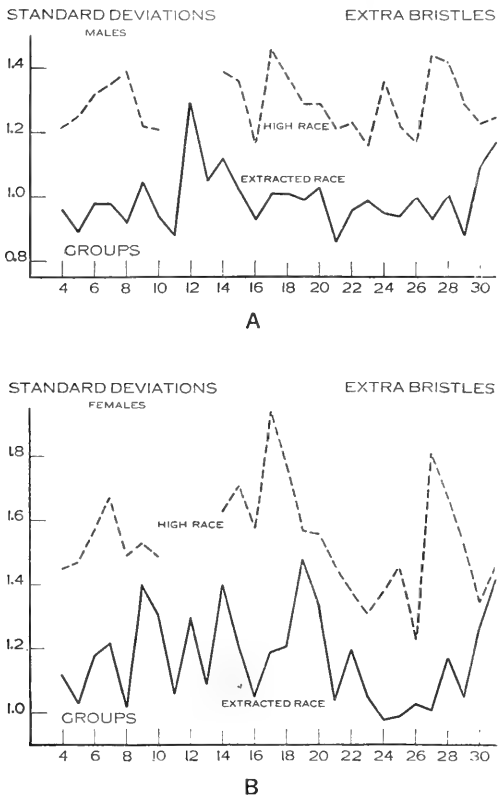

Fig. 10 Standard deviations of the extracted race compared with the standard deviations of the high race. 'A' based on sons, 'B,' on daughters. As the similarity of the standard deviations of the return and high races indicates similarity of the races, so the differences in the variability of the extracted and the high races indicate dissimilarity in these two races.

duced lower children. But after this, the means of the offspring were completely independent of the means of their parents. Although this extracted race responded to the same environment in the same general way as the high race, there was some sort of a restriction that inhibited the production of the higher bristle grades.

DISCUSSION

The rôle of environment

When families raised at the same time are compared, the means of the return, extracted and the main high race show a parallelism. Of these three races there is one with high parents and high means, one with low parents and high means, and one with low parent and low means, yet the means of all the offspring rise and fall with striking similarity. Differences in parentage, similarity in environment, similarity in variation; it is an evident conclusion that these variations are due to something other than differences in the germ plasm. There seems to be considerable justification for the belief that the greater part of the variations found in the curves is due to differences in the environments of the developing flies. An inverse relationship has been found to hold, in general, between the age of a culture and the number of extra bristles on the flies hatching; the older the bottle the smaller the flies are apt to be. The number of extra bristles appearing can be controlled to a certain extent. At any time small flies can be obtained by allowing the larvae only a small amount of food; this may be done either by allowing the bottle to dry, to mould, or to have too small an amount of banana. Optimum conditions for the production of small flies are the optimum conditions for the production of low bristle grades. When a unique set of atmospheric conditions are encountered, as in the constant temperature room, there appears a unique modification of the means (generations 29 to 31, high race) It seems hardly possible to escape the conclusion that the controlling factor in producing the variations in bristle numbers is environment and not heredity. This may incline some readers to immediately claim that, since the influence of environment is so strong, it would be impossible to discover any changes in the germ plasm, and forthwith condemn any conclusions that may be drawn from these data. At this point it will be well to consider the basis for our suppositions that we can know the character of the germ plasm by the condition of its somatic bearer.

It would be difficult to conceive of an organism that could mature free from all forces except those conditioned by the germ plasm. Every stage of an individual's growth is shaped by the reaction of the germinal to the extra-germinal, the hereditary to the environmental. In the case of the flies, the rôle of the environment in controlling the bristle numbers is obvious; we see changes in one, we see changes in the other. In some other cases of variation it is not possible to see, in this clear and simple way, changes in the environment associated with changes in somatic structures. Failing to find such a relationship, it is tempting to conclude that the variations in the soma do not indicate environmental changes but rather germinal variations. Can it rightly be supposed that in the absence of correlation between obvious fluctuations in temperature and food and such like, that there are no other controlling influences than those originating in the germ plasm? The anticipated criticism of this work is that the environmental influences are too potent for the germinal changes to appear. But suppose the power to influence bristle numbers, that seems as a matter of fact to lie in the amount of food eaten, etc., rested in some unknowable condition outside the germ plasm, such as the age of the sperm before fertilization, or the temperature of the mother's body during the maturation of the egg, then this objection would probable not be considered. The individual may be considered to be a reaction between the germ plasm and the environment but does it not sometimes seem that students of selection are so intent upon their consideration of the germ plasm that there is a tendency to ignore the scope of the influence of environment? In this way one of the most essential tools in attacking the problem is neglected. The point to be emphasized is that there is very little support for the supposition that the soma mirrors the germ plasm in all cases except when obvious environmental relations are found. It seems hardly possible that one can look forward to ever establishing firmly an exact relationship between soma and germ plasm, at least in bi-sexual multicellular animals. In the present case the difficulties are all to clear too permit the statement that the germ plasm did not

change in any way during the many generations of unsuccessful selection. However, in spite of the strong influence of environment, some conclusions that do bear on the problem of the germ plasm are to be drawn.

It may be concluded that environment was not responsible for the initial rise in the high race. Environment was as potent at this time as at any other, yet only at this time were the curves of the parents and offspring parallel. At the beginning low selection was successful, showing that genetic differences between low and high grade flies did exist. After this rise, the race as a whole was changed. This is shown by the failure of the return selection; at first, selecting these same low grades of parents established a low race. Crosses result in more modification after this rise than before. The analysis of individual families shows that before this rise the parents with higher grades produced children with higher mean grades, while after this rise, similar analysis shows that the highest grade parents were no more likely to produce offspring with especially high than with especially low mean grades.

It is certain that there were differences already present in the germ plasma of the flies first bred; that these same sorts of differences were not found among the flies whose ancestors had been selected for several generations; in other words, in regard to those germinal differences that account for the parallelism between parents and offspring in the early generations, that determine the difference between the successful low selections at the beginning and the unsuccessful return selections later on, that make the difference between an uncrossed selected race and a selected race that has been recovered from the dominance of a cross by normal, in regard to all such demonstrable differences, one finds that selection has sorted out a uniform race. Such a conclusion will stand however completely the environment may be proved to control the fluctuations in all but the early generations of the high race.

Accessory factors vs. one variable factor

Although the evidence was against it, the possibility was admitted[4] of applying to the first eleven generations of selection an alternative hypothesis involving one ever-varying factor, similar to the hypothesis adopted for the selection experiments of Castle.[5] The hypothesis of accessory factors has formerly been discussed in full; its application to all the data will now be made, and then the impossibility of so applying the alternative hypothesis, without numerous subsidiary hypotheses, will be shown.

The germ plasm of the original pair of flies, besides having a factor that permitted more than the four normal bristles to appear, had accessory factors that influenced the numbers of extra bristles that developed. Some of these accessory factors were in a heterozygous condition so that, due to their segregation, the germ cells of these flies were of different kinds. The effect of these germinal differences was strong enough to make the somas of the offspring fairly good relative indices of their germinal constitutions, even though the vitiating influences of environment were active. Selection of extreme variates moved the means in the direction of the selecting, raising them or keeping them low. Generation by generation the selecting reduced the heterozygosity of the accessory factors, so that the segregation was reduced and the germ cells that were produced were less varied. Shortly the differences between germ cells in an individual disappeared or at least became so small that their influence was overpowered by the influence of environment. Variations in bristle number then had no relation to the germinal condition. As long as brother by sister matings were made, the high and low grade flies produced similar offspring; no longer was it possible to move the means either up or down by selection. But as soon as these flies were crossed with normals, the distribution of the extra bristles was lowered; the

[4] MacDowell: loc. cit., p. 91.

[5] Castle, W. E.: Experiments in mass selection. Amer. Nat., vol. 49, no. 588, pp. 722–723.

heterozygosity of these accessory factors was regained in F_1, and in F_2 the segregation of these factors resulted in the lowered bristle averages, and again offered a chance for selection to modify the means. The differences between germ cells had become great enough to influence the somas in spite of the uncontrollable variations in the environment.

If a single Mendelian factor that varied in potency be assumed, the production of a high and a low race would be the expected result of selection. Furthermore, it would be expected that the transformation of the high race into a low race would be just as easy. Since this was not the fact, it becomes necessary to assume further that, because the factor had varied a certain amount in one direction, it was hindered from varying in the opposite direction—a supposition for which one would have difficulty in devising a mechanism. If one varying factor be made the basis of the explanation, a special hypothesis must be made to explain why the advance shown in the early generations of selecting was not continued. The physiological limit in bristle numbers had evidently not been reached; some limitation on the higher variations of this factor must be assumed. The last two hypotheses leave a variable factor that, after a series of selections is limited in its further variations in both directions. It becomes a matter of the number of generations before the variability of this factor no longer exists, before the end of the *cul de sac* is reached. Further subsidiary hypotheses would have to be added to account for the results of crosses with normals. It would be necessary to assume that the variations found in the germ cells of an individual heterozygous for this factor, are different from those in the germ cells of a homozygous individual. The 'Contamination theory' affords an explanation for this. The cross brought this factor and its allelomorphic mate together in the same nucleus. In order to explain any difference due to this heterozygosity, it must be assumed that there is an intimate fusion between these two members of the pair, and then that this fusion weakens the power of that factor for forming extra bristles. When the high race was selected longer, the modification of the means and modes found in F_2 was greater,

yet the extremes were as high as in the uncrossed race. To explain this one may assume that when the extra factor is more potent, it is more easily weakened by its contact with the normal factor, but that in some cases it is not weakened at all. If there be free variations in the factor it is difficult to understand the rigidity with which the normal four bristles are held as the lower limit of variation. There may be flies with only the normal four bristles in almost any generation of selection, yet, no matter how small a fly may be, the four normal bristles are inevitably found.

There can be no question as to which of the two main hypotheses is more fully supported by the interpretations that have been made of the facts. The supporting hypotheses required to make the hypothesis of a single varying factor fit the case are so numerous and, in some cases, so unthinkable as to render the main hypothesis of very slight value.

It may be claimed that, in time, in a hundred instead of fifty generations, selection could have accumulated enough germinal variations to bring the germ plasm again in contro of the numbers of extra bristles. But if there are differences in the germ plasm already present or continuously occurring, a long series of generations is not required to prove their existence. It would seem rather a forced conclusion to claim that the changes that might finally appear in a line that had been selected without success for a long time, were due to the long selection. It would not be hard to believe that if one waited long enough, without any selection at all, mutations could be found in almost any material. The point at issue is whether the changes in the germ plasm are continuous or spasmodic, whether they are like the continuous breakers on an ocean beach or the storm-caused splashings of an inland pond. It does not take long to find the breakers at the sea shore; one may spend weeks beside a little lake without seeing a single wave dash against the bank. In these experiments germinal differences were found to exist; their presence was found almost immediately. But after selection had separated the different kinds, no further differences were found.

As stated, this paper has not attempted to deal with the problem of absolute stability of germ plasm; it has questioned the theoretical possibility of a solution for that problem; but it has attempted to show that none of the various phenomena herein described require the assumption of any internal, or spontaneous, change taking place during the course of these experiments.

SUMMARY

I. A race of flies with extra bristles has been selected for 49 generations for the production of high numbers of extra bristles. From the study of this race the following points have been determined:

1. In any generation after the early ones the distribution of a single family is similar to that of the distribution of al the families taken together in that generation.

2. For about 8 generations the means rose; following this were two periods not comparable with each other, within neither of which was any evidence of further advance to be found.

3. Continued selection did not produce any high extremes that were not obtainable near the beginning of the experiment. The range of variation changed only very slightly; the low limits being most frequently at 0 or 1, the high limit at 9 for the females, at 7 for the males.

4. The standard deviations rose and fell together with the means; as the means of the females are higher than those of the males, so the standard deviations of the females are higher than those of the males. These relationships do not hold true when the complete yields of the bottles are not included (generations 33–49).

5. Changes in the means of the parents are not accompanied by changes in the means of their offspring, except at the beginning of the experiment.

II. By selecting low grade parents from the second generation of the extra bristled race a race of flies was established which had markedly lower means than the high selected race.

III. By selecting low grade flies from the 15th generation of the high race and continuing to select for low grades, it was

impossible in 8 generations to establish a race that was distinguishable from the high race. This attempt was repeated, starting from the 26th generation of the high race, and continued for 6 generations with similar results. Return selection does not reverse the progress made by the advance selection. Flies with high and low bristle grades appear to have very similar offspring.

IV. By selecting low grade parents from the F_2 of a cross between normals and flies from the 16th generation of the high race, a low race was established (extracted low) The following points were derived from the study of this race:

1. One selection was sufficient to establish this race as distinct from the high race.

2. For 4 generations the curves of the parents and offspring are parallel. After this, the two curves are completely independent.

3. For 4 generations the low selection continued to lower the means.

4. Except in the first few generations, the curves of the progeny rise and fall in harmony with the curves of the high race, when families raised at similar times are compared.

5. Besides being lower than the high race the variability of this race is less than that of the high race; in response to the same improvement in conditions this extracted race does not advance as far.

V. Comparing the different races it is found that no matter what the parentage, they all exhibit high points and low points at the same times. Environment is accountable for the variations in most of the generations. The initial rise in the high race however, was not due to environment, as this rise resulted in a genetic change in the race.

VI. The supposition of a single varying factor to explain the above results can not be justified, as it would require numerous further assumptions. All these results are simply explained on the assumption that there were genetic differences present among the original flies with extra bristles, that these genetic differences, or genes, are entirely independent of the main factor that occasions the monohybrid ratio in crosses with normal flies.

THE ACTION OF VARIOUS PHARMACOLOGICAL AND OTHER CHEMICAL AGENTS ON THE CHROMATOPHORES OF THE BROOK TROUT SALVELINUS FONTINALIS MITCHILL

JOHN N. LOWE

From the Department of Zoölogy, University of Wisconsin

THREE TEXT FIGURES AND ONE PLATE

CONTENTS

The reactions of melanophores (pigment cells) to pharmacologically active agents have been but little investigated. In the

majority of the physiological researches upon the melanophores, the experiments have only included the study of such physical agents as light, heat, etc. The problem here undertaken was to determine the reactions of the melanophores of young trout embryos in response to changes in their chemical environment. The trout embryos that were used in these experiments were too young to react to a change in the light conditions, and throughout the work gave no evidence of any psychic influence of the pigment cells.

MATERIAL AND METHODS

Young brook trout embryos, from two days to two weeks after hatching, were used. The melanophore of such young individuals are dark, much branched cells with deep black or brown pigment granules. These are the only kind of pigment cells present at this time. The xanthophores, the yellow or reddish pigment cells, appear after or a little before the yolk is absorbed. All the experiments were performed before the xanthophores appeared. After the yolk is absorbed the fish begin to react to the back ground. When placed in a dark dish, they become dark; when placed in a white dish, light in color. Microscopical examination shows that the pigment cells (melanophores) are expanded in the dark colored individuals and contracted in the light ones. The very young, two-day or two-week old embryos do not respond to changes of the back ground.

This constant condition is taken as a known factor. The contraction of the pigment cells was used as the criterion for determining stimulation, and their expansion (relaxation) as a mark of depression. The expansion of the pigment cells is characterized by the peripheral migration of the pigment granules within the processes of cell, and in contraction the movement is centripetal. My reason for considering contraction as stimulation and expansion as a depression is that certain reagents, alkaloids for example, if used in high concentrations produce no observable change in the pigment cells which under normal conditions are expanded. Small or 'therapeutic' doses produced a

contraction. Large doses produced an expansion of all the cells which had contracted in the weak solution. Inasmuch as it has been shown by various investigators that large doses of pharmacologically active agents produce a depression, and small doses incite a stimulation in other tissues, it is inferred that the condition is essentially the same with the melanophores.

All the chemicals used in these experiments were of Merck's, Kahlbaum's and Baker's manufacture. The solutions were made up with oxygenated distilled water. Chemically pure oxygen was bubbled through the water before it was used. This precaution was taken because the distilled water was very low in oxygen content and in it the pigment cells contracted. When oxygen was added no such contraction occurred. The details of the way in which the solutions were prepared are given under the respective heads.

The experiments with the salts and the alkaloids were carried on in Syracuse watch glasses, which were kept covered to prevent excessive evaporation. They were uncovered only when actual observations were made. The amount of the solution used was about 10 cc. Experiments were performed in stender dishes of 50 cc. capacity as a check on the Syracuse watch glasses. There was no difference in the results. The experiments with volatile substances were carried on in wide-mouthed, glass stoppered bottles, with a capacity of 50 cc. All precautions were taken to prevent evaporation.

Most of the experiments were carried on at room temperatures which varied between 69° and 72° F., although some were performed at the fish hatchery where the temperatures were from 46° to 50° F.

The experiments with the alkaloids and alcohols were started in solutions of 0.0001 per cent. The concentrations were increased in multiples of ten.

The experiments were repeated ten to fifteen times for each solution tested. In many cases the experiments were repeated double the number, in order to eliminate all possible individual variation and errors.

I wish, here, to express my chief indebtedness to Prof. M. F. Guyer, for his kindly criticism and suggestions during the progress of the work. To Prof. A. S. Loevenhart, I wish to acknowledge my appreciation of many courtesies extended. For the privilege and use of the fish hatchery and trout embryos, I desire to express my appreciation of the favor to Dean E. A. Birge and Superintendent James Nevin of the Wisconsin Fish Commission.

Reactions to gases

1. Oxygen. The oxygen used in these experiments was chemically pure. The pigment cells remained expanded in an atmosphere of oxygen, and the fish lived indefinitely.

The hydrogen used in these experiments was obtained by the action of chemically pure hydrochloric acid on Merck's highest purity zinc. The gas was passed through two towers of KOH and then through two towers of distilled water, of which one had red litmus, and the other blue litmus. The trout were in the fifth tower.

The pigment cells contracted completely in four to six minutes when the embryos were exposed to hydrogen. If oxygen was substituted before the fish died the pigment cells expanded. If the oxygen was again replaced by hydrogen the pigment cells contracted. The results of these experiments show (1) that the absence of oxygen caused a contraction of the melanophores; (2) that the oxygen is necessary for the maintenance of the expanded pigment cells.

2. Carbon dioxide. The carbon dioxide was generated through the interaction of chemically pure hydrochloric acid on marble. The gas was purified by being passed through a tower of sodium bicarbonate and then through a tower of acidified lead acetate, and lastly through two towers of distilled water.

The fish were exposed to water through which the carbon dioxide was bubbling in a steady slow stream. The carbon dioxide produced a complete contraction of the pigment in two and one-half minutes. The time of contraction was the same for all the

experiments performed. If an intense stream of oxygen was bubbled at the same time with the carbon dioxide, the pigment cells remained expanded. The proportion of the two gases which maintained the expansion of the melanophores was not determined. Briefly summarized the results prove that carbon dioxide produces a contraction of the pigment cells of trout embryos. The presence of oxygen antagonized the action of the carbon dioxide.

Effects of distilled water

The first experiments that were performed were to determine the effect of distilled water on the pigment cells of trout embryos. The normally expanded pigment cells contracted in ten to twelve minutes and the fish died usually in about twenty minutes—differing somewhat with the individual lots of fish. After an interval of ten to thirty minutes, following the initial contraction, the pigment cells began to expand. This secondary expansion of the melanophores in no way equaled the normal expanded condition. The processes of the cells were short and blunt. This expanded condition lasted for a short period; then the walls of the melanophores began to break down and the cell contents, viz., the pigment granules migrated into the interspaces of the epidermal layer. Often the pigment cells disintegrated without a previous expansion. Spaeth ('13) obtained essentially the same results with isolated scales of Fundulus in which the chromatophores (1) expanded, (2) contracted, (3) expanded a second time with a final degeneration. He did not try oxygenated distilled water. If 2 cc. of boiled tap water were added to 8 cc. of distilled water the results were the same. Then boiled tap water was tried and the pigment cells contracted in fourteen and twenty-two minutes. In distilled and boiled tap water through which oxygen had been bubbled the melanophores remained expanded and the fish lived indefinitely. The conclusion was obvious. It was oxygen want and not the absence of salts in the distilled water that caused the contraction of the pigment cells and the death of the fish.

Reactions to salts

The problem of salt action is one of the most interesting within the scope of physiology and has wide applications. The relation of various salts to heart beat is a long debated question.

Howell ('98), p. 49, is of the opinion "that the inorganic salts of the blood and liquids of the heart tissues especially of the calcium compounds, stand in a peculiar and fundamental relation to the initiation of the inner stimulus of the heart contractions." Loeb ('00 a, '00 b) believes that the sodium cations acting on the striped muscle to be the stimulating agents being counteracted by the ions of potassium and calcium. The position of Loeb is supported by Lingle ('00). Benedict ('05 and '08) is of the opinion that the anion probably plays an important rôle in the action of salt solutions upon heart beat.

Mathews ('04 a, '04 b, '05, '06) maintains that in the action of salt solutions on motor nerves, colloids, and sea urchin eggs, the ionic potential of the salt, which is the reciprocal of the solution tension, is an important factor in ionic action. R. S. Lillie ('11, '12 a, '12 b) working with the larvae of Arenicola and eggs of starfish, and McClendon ('10) on sea urchin eggs put forth the hypothesis that ionic action is due to the modification of the permeability of the plasma membrane. Loeb ('00 b) holds that ionic action is due to the formation of ion protein compounds, that is that the ions of the salt combine directly in some way with the protein molecules of the living protoplasm. True and Kahlenberg ('96) working with plants (Lupinus albus) believe that the anion is unimportant in the toxic action of the salt.

Spaeth ('13) working on the chromatophores in isolated scales of Fundulus heteroclitus concludes that the anion in potassium salts is of no importance in causing the initial contraction of the chromatophores, but that in the secondary expansion of the chromatophores the action of potassium is modified by the anions. On the other hand, the duration of the sodium expansion varies with the nature of the anion.

The above opinions tend to show that the part played by ions in stimulation is by no means a settled question. In an attempt to gain further insight into the subject brook trout embryos were subjected to solutions of pure potassium and sodium salts. The results have been so promising that the work is being extended to numerous other salts.

The salts used were of the purest of Merck's, Kahlbaum's and Baker's manufacture. The solutions were made up in a 0.2 molecular concentration with oxygenated distilled water. The solutions of the iodides which readily undergo decomposition were never older than thirty-six hours when used.

The experiments were carried on in Syracuse watch glasses in about 10 cc. of the solution. At times small dishes of 25 to 50 cc. capacity were used.

1. Effects of potassium salts. When the trout embryos are immersed in a 0.2 M. KI solution a rapid contraction of the normally expanded chromatophores results within two or three minutes. They then appear as minute dots with no peripheral processes. In placing a similar lot into a 0.2 M K_2SO_4 equivalent solution the change does not occur as rapidly, being completed in fifteen to twenty minutes. This at once suggested that there is a specific difference in the rate of contraction for potassium, varying with the anion. The experiments were extended to include the following neutral salts of potassium, viz., K_2SO_4, KCl, KBr, KNO_3 and KI. Practically the first experiment showed that there was a distinct difference in the rate of contraction varying with the anion. The rate and intensity of the contraction was most rapid in the order given (figs. 1, 2, 3, 4 and 5).

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4 >$$

In KI the contraction was complete before it had even begun in KCl or K_2SO_4. The experiments were repeated many times and as a check several of my colleagues were asked to come in and arrange the sets showing the greatest change. In all cases their arrangement was in the above order. This clearly indicates that if contraction in the melanophore is specifically induced by the

cation of potassium, it is unqualifyingly modified by its anion or the residual part of the undissociated molecules.

Another interesting feature observed was that after a longer or a shorter interval after the first contraction there followed a peripheral expansion of the pigment cells (figs. 6, 7, 8, 9 and 10), that is, the pigment cells put out processes which became longer and longer as time went on but which never reached the original size they had before treatment with the potassium salt solutions. This expansion set in earlier in KI where the contraction took place first, evidently the secondary expansion or paralysis is reciprocal of the first contraction. The expansion is in the order of the first contraction (figs. 6, 7, 8, 9 and 10).

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4$$

This peripheral migration of the pigment is in the nature of a paralysis. The paralytic state (depression) is soon followed by death of the pigment cell. The walls of the pigment cell disintegrate and the pigment granules flow into the interspaces of the body tissues. Death of the cells takes place often before the expansion is complete, and then premature disintegration of the pigment cells occurs. The condition or extent of the degeneration is dependent upon the 'physiological state' of the melanophores and the individual fish.

The maintenance of the irritability of the melanophores followed the same order, correlated with this was the longevity of the fish. The fish lived the longest in K_2SO_4 and KCl. They died very rapidly in KI.

The reactions varied with the concentration of the solutions, for in solutions of 0.1 M or less the changes were slightly slower. Molecular solutions gave no results but killed the fish immediately.

2. Effects of sodium salts. Here as in the potassium salts the embryos used had their melanophores expanded. It was observed that the neutral salts of sodium produced a contraction of the melanophores very slowly. In some instances the contraction did not take place in 92 to 116 hours, especially in the solutions of Na_2SO_4 and NaCl. The contraction in NaI was complete

in five to forty-five minutes. It was confirmed by repeated observation, that these contractions, slow as they may be for certain solutions (Na_2SO_4 and NaCl), were in the following order:

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4$$

A number of experiments were tried to determine if the sodium salts produced an expansion of the melanophores after the potassium salt contraction. The embryos were exposed to KCl from fifteen to twenty minutes when they were removed and rinsed in water to free them of the excess of KCl. They were now placed into the five neutral salts of sodium. The rate and degree of expansion was in the following order:

$$SO_4 > \quad Cl > \quad Br > \quad NO_3 > \quad I$$

The expansion was most rapid and complete in Na_2SO_4 and NaCl. In NaI there was no expansion.

The experiments were repeated with embryos that were not rinsed with water. The result was the same as in those that were washed in water. If the melanophores are contracted with KI instead of KCl the results are the same.

$$SO_4 > \quad Cl > \quad Br > \quad NO_3 > \quad I$$

It is interesting to note here that no expansion of the melanophores occurred in the NaI solution. Is this because the sodium cations are inhibited in permeating the cell membrane due to the presence of the dissociated iodine anions or some other factor? Are the cells permeable only to the iodine anions and not to the cations of sodium? Hamburger and von Lier ('02) claim that the blood corpuscles are permeable only for anions and are not permeable to the cations. If the expansion of the melanophore is specific for the sodium cation, it is overcome by the antagonistic action of the iodine anion, which produces a contraction. Nevertheless we must consider another factor, that is, the action exerted by the residual undissociated molecule which is present at all times in the solution. The expansion in-

duced by the sodium salts after a potassium salt contraction is followed by a contraction of the melanophores in the usual order. The position or order of the contraction was the same as for the expansion of the melanophores; but with one exception where the $NaNO_3$ changed places with the NaBr.

$$SO_4 > \quad Cl > \quad NO_3 > \quad B_r > \quad I$$

The extent to which the life of the fish and the irritability of the melanophores are preserved is possibly the function of the cation which is modified by the anion or the residual undissociated molecule.

3. Discussion. All these results seem to lend themselves to the interpretation that salt solution having a common cation are modified by their anions or the residual undissociated molecule. This is clearly shown by the rate and degree of the contraction of the melanophores by the potassium salts, where the contraction may be specific for the cation of potassium. Speath ('13) p. 547 says in speaking of the action of potassium salts: "The time of this contraction (K) is the same for the five salts within the limits of the variation of the individual scales. Since there is this common cation K^+ in all five salts it seems probable that the initial effect (contraction) is specific for the K^+ ions." My own results in the case of pigment cells of trout embryos are contrary to this conclusion. If contraction is specific for the positive cation of potassium (K^+), it should be the same in rate and degree in all the salts of potassium. Since the rate and degree of the contraction are not the same for the five potassium salts (figs. 1, 2, 3, 4, and 5) it must depend on some other or some modifying factor which is responsible for this difference.

A dissolved electrolyte conducts a current in proportion to the extent that it is dissociated or ionized. Its maximum conduction will be at complete ionization which occurs at infinite dilution. Therefore the degree of the dissociation or the coefficient of dissociations can be obtained from the conductivity of solution. The conductivity of an electrolyte divided by its num-

ber of gram equivalents in cms. is the molecular conductivity of the substance written as Λ. However, the conductivity is at its maximum at infinitely dilute solutions, therefore the value $\Lambda\infty$ is taken as a measure of the total number of ions that are produced by the dissociation of one gram equivalent of the substance. Therefore d the degree of dissociation is directly proportional to the conductivity; thus we have the simple formula $\alpha = \frac{\Lambda}{\Lambda\ \infty}$. The equivalent conductivity at infinite dilution for KCl is calculated to be 130.10. The equivalent conductivity of a two-tenth molecular KCl is 107.96 $\Lambda_{0.2\ M}$. The degree of dissociation at 18°C. is the ratio $\frac{\Lambda\ 0.2\ M}{\Lambda\quad\infty}$, or $\frac{107.96}{130.10}$ or 82.98 per cent. The values obtained in this way may be regarded only as approximate. The values are given in the following table.

TABLE 1

SALT	½ K_2SO_4	KCl	KBr	KNO_3	KI
Equivalent conductivity at infinite dilution $\Lambda\ \infty$	132.8	130.10	132.30	126.50	131.10
Equivalent conductivity at 0.2 M dilution Λ 0.2 M	87.76	107.96	110.40	98.74	111.2
Per cent or degree of dissociation $\alpha = \frac{\Lambda\ 0.2\ M}{\Lambda\ \infty}$	66.03	82.98	83.44	78.05	84.82

A study of the table leads one to believe that the rate and the degree of the contraction are in some way correlated with the degree of dissociation of the salts. The lowest rate and degree of contraction was found in K_2SO_4, where the degree of dissociation is 66.03 per cent. The most rapid and complete contraction occurred in KI where the dissociation is 84.82 per cent.

Potassium nitrate is out of place. It has a greater stimulating action than its degree of dissociation would indicate. It should fall between potassium sulphate and potassium chloride. The possible explanation for this break in the series may be that the

TABLE 2

SALT	½ Na_2SO_4	NaCl	NaBr	$NaNO_3$	NaI
Equivalent conductivity at infinite dilution $\Lambda \infty$	111.5	108.99	112.0	105.99	109.9
Equivalent conductivity at 0.2 M dilution Λ 0.2 M	71.4	87.73	91.2	82.28	90.2
Per cent or degree of dissociation $\alpha = \frac{\Lambda\ 0.2\ M}{\Lambda\ \infty}$	64.03	80.49	81.43	78.11	82.08

nitrate anion exerts an independent action or it may form nitrites which are more active.

In table 2 are shown the equivalent conductivities and degree of dissociation of the sodium salts.

The values were calculated in the same manner as those for the potassium salts. Here, as in the potassium salts, the reaction of the melanophores was correlated with the degree of dissociation.

There are two reactions of the melanophores which are characteristic of the potassium salts: (1) a primary contraction, (2) an expansion which is the sign of death or degeneration of the cell. The cell wall breaks down and the pigment granules escape into the surrounding tissues. The degree of the cytolysis is directly proportional to the degree of dissociation of the salt. In sodium salts we have two specific reactions: (1) the expansion and maintenance of the expansion for a certain period of time, (2) a slow contraction. The two reactions of sodium salts occur in an inverse order to those of the potassium salts, where contraction is followed by a cytolytic expansion. The contraction in sodium salts is not followed by a cytolytic expansion, but the disintegration takes place directly from the contracted pigment cell. This contraction in sodium salts is directly comparable to the cytolytic expansion observed in potassium salts, for both of these stages indicates the death of the pigment cell.

A. P. Mathews ('06) suggested that it is the ionic potential of the ions, and not the difference of voltage between the plate of

a metal and any solution of its salts, but rather the difference in pressure between a single ion and a single atom of the metal that determines the chemical action of the ions. Since solution tension is a measure of the difference in potential between the solution which contains a known amount of the ions of the metal and the metal itself, it is also the difference between the tendency of an atom of the plate to become an ion. When applied to living protoplasm the metal plate is replaced by the protoplasm. The value varies with the amount of electrolytic dissociation and the kind of plate present.

The solution tensions in volts of elements in normal ionic solutions.

K	2.92	Cl	1.694
Na	2.54	Br	1.270
		I	0.797
		NO_3	2.229

The ionic potential is the reciprocal of the solution tension. Ionic potential is the tendency of any ion in any concentration of solution to change into an atom of its metal.

The ionic potentials of the ions of metals in volts are:

K	2.92 (?)	Cl	1.694 (?)
Na	2.54 (?)	Br	1.270 (?)
		I	0.797 (?)
		NO_3	2.229 (?)

Mathews ('06) shows that the dissolving power of the salts of sodium and potassiun for edestine, a globulin of the hemp seed is in some way correlated with the ionic potential.

SALT	IONIC POTENTIAL	NUMBER OF CUBIC CENTIMETERS REQUIRED TO DISSOLVE ONE GRAM OF EDESTIN
KI	−2.123	5.7
KBr	−1.65	10.0
KCl	−1.226	15.1
NaI	−1.743	5.7
NaBr	−1.270	9.3
NaCl	−0.846	12.8

The more negative the value for the ionic potential the greater the solvent power of the salt for edestin. The negative value in potassium is much greater than that in the sodium. In the table we observe that it takes like amounts of the iodides and less of the other sodium solutions to dissolve the edestin. However, we should expect it to take less of the potassium salts than it does of the sodium. I find this to be true for the pigment cells of trout, where the potassium salts cause the contraction of the pigment cells more rapidly than do the salts of sodium. Unfortunately the solutions tensions for sodium and potassium are more or less indefinite which makes the results obtained for the salts of these metals incomparable. The ionic potential is not determined directly, but calculated only, thus making the explanation more difficult.

The results obtained in experiments on the action of salts on the pigment cells of trout are explicable on three assumptions; (1) that it is the antagonistic action between anion and cation, (2) that it is the independent action of the cation, (3) that the reaction is modified by the residual undissociated molecule.

The antagonistic action between anions and cations has been postulated by Mathews ('06), Benedict ('05, '08), and W. Koch ('09). The increased action of different salts having the same cation have been observed in different tissues. Loeb ('99) produced a better rhythmical contraction in striped muscle with NaI than he did with NaCl. Zoethout ('04) confirmed this observation, and extended it to KI which increased the muscle tone more than KCl. Benedict ('08) concluded that "the direct production of rhythmic activity by means of a salt's action upon heart muscle is due to the anion of the salt, while the chief function of the cation is apparently to maintain such a tone of the heart muscle that it will respond to the stimulus furnished by the anion." Mathews ('02) has shown that the presence of iodine, bromine anions stimulated the motor nerve more powerfully than the chlorine anion. Speath ('13) observed that the cytolytic expansion of the melanophores in potassium solutions, varied with the anions, but he did not note a difference in the rate of the primary contraction of the melanophores in the

different potassium salts. In sodium salts the expansion of contracted melanophores varied with the anion, and the contraction following this expansion was correlated with the anions.

In neutral salts of potassium there are two constant results produced on the pigment cells of trout; (1) a contraction of the pigment cells, (2) a cytolytic expansion. The times for each varied with the anion. If the antagonism existed between the potassium cations K +, and the negative anions SO_4-, Br−, Cl−, No_3-, I−, it was the least effective in KI and most potent in K_2SO_4. The order of contraction and expansion was

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4$$

In sodium salts there were two characteristic reactions, (1) an expansion, (2) a contraction. The rate and degree of the expansion of the melanophores was greatest in Na_2SO_4 and least in NaI. The rate of contraction was rapid in NaI and least in Na_2SO_4. The order of the expansion was

$$SO_4 > \quad Cl > \quad Br > \quad NO_3 > \quad I$$

The contraction rate of the pigment cells was inverse to the above.

$$I > \quad Br > \quad NO_3 > \quad Cl > \quad SO$$

The cationic action was modified by the nature of the anion. This anionic order was observed by Paul and Kronig ('96) on the disinfecting power of mercuric salts of chloride bromide and cyanide. Mathews ('06) has shown for the eggs of Fundulus heteroclitus that the fatal dose varied with the anion. Loeb and Cattell ('15) have shown that the hearts of Fundulus embryos, previously poisoned by KCl, and recovered by sodium salts was an anion effect inasmuch as it increased with the anion, apparently in agreement with Hardy's rule (ion effect = exponential function of the valency) for the acetate was much more efficient than the chloride.

2. That it is the cation of potassium or of sodium that causes the reaction of the pigment cells of trout embryos.

Loeb ('10, '12) and Loeb and Wasteneys ('11 a and '11 b) maintain that there is an antagonism between the sodium cation

Na + and the potassium cation K + and not between the potassium cation and the chlorine anion K + Cl −. This is supported in part by the foregoing experiments on the pigment cells of trout embryos. The pigment cells are expanded in sodium salts after a potassium salt contraction. But this is not true of all the salts of sodium. If the pigment cells are contracted in KCl or KI and are now placed in NaI there is no expansion. Apparently there is an antagonism between the dissociated anions of (Cl − and I −) and the sodium cation (Na +) for from the conditions of the experiment we should get an expansion. It is probable that Loeb underestimated the antagonism between the positive ions of K + and Na + and their negative ions Cl −. The longevity of the fish is better protected in sodium salts than in potassium salts. But again some of the sodium salts are more protective (Na_2SO_4 or NaCl) than others (NaI). That the potassium and sodium cations do exert some such modifying action is undeniable, but to say that it is independent of its anion is not warranted by the facts at our command.

3) That it is the residual undissociated molecules in the solution that modify the action of the salt.

In 0.2 M, KI the degree of dissociation is much greater than in an equivalent 0.2 M, solution of K_2SO_4. Correspondingly KI initiates more intense responsiveness of the pigment cells than does K_2SO_4. The rate and degree of the reactions of the pigment cells decline as the number of the undissociated molecules increases. In the potassium salts the primary contraction and the expansion vary with undissociated molecule, thus,

$$SO_4 > \quad Cl > \quad Br > \quad NO_3 \quad I$$

The degree of dissociation for 0.2 M solutions are

66.03 82.98 83.44 78.05 84.82

The fact that the nitrate is out of place was mentioned before. As already stated, this may be due to the independent activity of the nitrate, which may break down to form a nitrite.

In sodium salts the dissociation percentages are slightly less than in potassium salts. The rate of contraction is much slower.

The fish live longer in sodium solutions, and the pigment cells retained their irritability longer. The pigment cells expand if transferred after they are contracted in potassium salts. Is this reaction specific for the sodium ion or is it due to the increased number of undissociated molecules in sodium solution? It cannot be positively concluded whether this difference in residual molecules is enough to account for the difference in the rate of contraction of the melanophores in sodium and potassium salt solutions. The ascribing of the principles of salt action to the anion or cation without the consideration of the residual undissociated molecule is just as out of proportion in the field of physiology as to say that the undissociated alkaloids and other substances have no action.

Reactions to alcohols

Whether or not alcohols have a stimulating action is a much debated question. The Schmiedeberg school of pharmacologists maintains that alcohols produce no primary stimulation of the central nervous system. According to this view the giving alcohol to a mammal, if followed by an increased muscular activity, is said to be due to the depression of the cerebral centers, thus removing the restraint from the motor areas. Binz and his followers hold to the view that alcohol first stimulates and then depresses the nerve cells.

The literature on the pharmacological action of alcohol on the heart and other tissues is very extensive, but to my knowledge there are no records of any attempts to determine its action on the melanophores. Whatever action the alcohols exert on the melanophores will not settle the question whether alcohols stimulate or depress the nervous system, as the melanophores in the very nature of their origin and structure must be looked upon as specialized mesenchymal cells. While it is not at all improbable that the general facts observed with melanophores may be

[1] After these experiments were completed Spaeth 1916 published results, where he subjected isolated scales of Fundulus to vapors of alcohol, ether and chloroform and always obtained a contraction of the melanophores, and larger amounts of these vapors inhibited the contraction.

true also of other tissues, I refrain from applying the results to such an interpretation. The literature is used in a comparative way, but not in the sense that the results obtained with melanophores are directly comparable.

Ten per cent stock solutions of methyl (Sp. G. O. 796), ethyl (Sp. G. O. 796–800) propyl (Sp. G. O. 8066) alcohols of Merck's manufacture were made up with oxygenated distilled water. The dilutions were made from these stock solutions with oxygenated distilled water. The experiments were carried on in glass stoppered bottles of 75 cc. capacity. All work was done at room temperature of 18° to 20°C.

1. Methyl alcohol. Overton ('01) showed that methyl alcohol has a less powerful narcotic action on tadpoles than ethyl alcohol. Vernon ('11) confirmed that the same was true in the depressing action of methyl alcohol on the heart muscle of a turtle's heart.

Young brook trout embryos of the same age and condition were subjected to the action of the respective alcohols of the various concentrations. The contraction of the pigment cells was taken as the criterion of stimulation, the relaxation (expansion) as that of a depression.

Ethyl alcohol in solutions of 1.6 to 2.5 per cent produced a complete contraction of the pigment cells. Methyl alcohol of an equal concentration did not cause a contraction. In a 3.5 per cent solution there was a slight retraction of the pigment cells, but the contraction was not complete. A 4.5 per cent solution produced a complete contraction of the melanophores. Solutions of 5 per cent to 5.5 per cent produced a slight contraction of pigment cells. This partial contraction was followed by an immediate expansion. If embryos in which the melanophores were just contracted in a 0.005 per cent strychnine solution were subjected to 5 per cent to 5.5 per cent methyl alcohol the pigment cells expanded. In 7 per cent to 10 per cent solutions of methyl alcohol there was no visible change in the expanded melanophores.

Thus it may be concluded that (1) methyl alcohol in high concentration acts as a depressing agent, (2) in medium concentra-

tion it has a stimulating action, and (3) in very weak solution it has no effect on the melanophores of trout embryos. Methyl alcohol has a less pronounced stimulation action than ethyl alcohol on pigment cells of trout. It was necessary to double to concentration so as to bring about reactions in any way comparable to those produced by ethyl alcohol. The action of methyl alcohol was less striking and the stages of stimulation and relaxation were slower in appearing than in ethyl alcohol.

2. Ethyl alcohol.—When trout embryos were exposed to weak solutions (0.01 per cent to 0.8 per cent) of ethyl alcohol, no change took place in the pigment cells. The embryos did not show any signs of depression and appeared perfectly normal. In solutions of 1 per cent to 1.5 per cent the embryos became more restless and the pigment cells exhibited a partial contraction. In concentrations of 1.6 per cent to 2.5 per cent of ethyl alcohol the fish became more active, the pigment cells showed a complete contraction; while in solutions of 3.0 per cent to 4.5 per cent they showed a transitory contraction, followed by an expansion. This result could be very easily overlooked. In 6 per cent to 10 per cent solutions the trout embryos died rapidly in from fifteen to twenty-five minutes, and there was no contraction of the pigment cells. If embryos that had their pigment cells contracted in the 2 per cent solution were transferred to a 7 per cent the pigment cells expanded rapidly.

If the embryos in which the pigment cells were contracted were transferred to a 4.5 per cent to 6 per cent solution an immediate expansion resulted. This expansion was due to the depression caused by the high concentration of the alcohol, which was far beyond the maximum threshold of stimulation. When the fish which had their melanophores contracted in a 2 per cent solution were placed in a 0.5 per cent solution they expanded. Here the dilution of the alcohol was below the threshold stimulus. If embryos that were exposed to $7\frac{1}{2}$ per cent solution for an interval of four to six minutes, were placed in water or very weak alcohol, there was observed a contraction of the pigment cells which was of a very short duration. This result was no doubt due to the washing out or the dilution of the alcohol within

the tissues, to the threshold stimulus and as the process of dilution continued the point was reached where the concentration fell below the threshold and a relaxation (expansion) of the melanophores occurred. After a complete recovery of the embryos from the effects of the alcohol the pigment cells reacted normally to other stimuli.

These results show clearly that very weak solutions of ethyl alcohol do not have any effect on the pigment cells of the trout embryos. This is in harmony with the work of Kobert ('82) on the frog's muscle, Lee and Salant ('02) on the gastrocnemius muscle of the frog, and Carlson ('06) for the heart muscle and heart ganglion of Limulus, all of whom observed that weak or very weak solutions of ethyl alcohol had no stimulatory action. Ethyl alcohol in contractions of 1.3 per cent to 2.5 per cent water shows a decided stimulatory action on the pigment cells of brook trout embryos. This is in accord with results of others on the primary stimulation of ethyl alcohol. Pickering ('95) has shown that alcohol excites the embryonic heart muscle of the chick. Scheffer ('00) has observed that in the frog's gastrocnemius when it was treated with alcohol the capacity for work was increased. If the muscle was curanized the stimulating effect of alcohol was nil. O. Loeb ('05) has noted that in solutions of 0.13 to 0.3 per cent that the action of the isolated mammalian (cat) heart was augmented. Wood and Hoyt ('05) have shown that small amounts of ethyl alcohol increased the force of the heart beat in the frog, snake, tortoise, and turtle. Lee and Salant ('02) have demonstrated that in medium concentrations of ethyl alcohol there was an increased rate of contraction and relaxation in frog's muscle (gastrocnemius). Carlson ('06) has observed that for the heart muscle and heart ganglion of Limulus, alcohol stimulated. Vernon ('10) has shown that alcohol has an excitatory effect on the isolated heart of the turtle (Emys). The ('02) observed a marked increase in the number of contractions of the bell of the Medusa Gonionema in ethyl alcohol of 0.5 to 0.25 per cent.

In a strong concentration of 4.5 per cent there was a marked depression or an expansion of the pigment cells. In this con-

centration there was no primary stimulating period observed. If it is to be found, it may be so short as to be very easily overlooked. Alcohol in large amounts decreased the rate of contraction in the gastrocnemius frog's muscle, Lee and Salant ('02). Romanes ('77) found that strong solutions of ethyl alcohol produced increased and spasmodic contraction of the medusa bells of Sarsia (sp.) and Tiaropsis (sp.). These were followed by a depression.

Lee ('02) observed that in solutions of ethyl alcohol of a greater concentration than 2 per cent the contractions of the bell of the medusa, Gonionema, were much reduced in volume and in number. Dogiel ('77) has shown a depression in the heart rhythm of Corethra plumicornis. Vernon ('10) observed that large doses of ethyl alcohol depressed the rate and volume of the contraction of a turtle's heart (Emys).

3. Propyl alcohol. Weak solutions of propyl alcohol 0.01 per cent to 0.04 per cent did not effect the melanophores. In a 0.06 per cent there was a noticeable contraction of the pigment cells. Solutions of 0.08 per cent to 0.125 per cent produced a rapid and complete contraction. In 0.7 per cent to 1.3 per cent the contraction was only temporary, and was followed by an immediate relaxation of the pigment cells. A 1.5 per cent to 2 per cent produced no visible change in the expanded melanophores, and when embryos with contracted melanophores were exposed to the solution the melanophores expanded. In these concentrations there was observed a marked disintegration (cytolysis) of the cells. Higher concentrations (2.5 per cent to 4 per cent) killed the embryos without inducing any change in the expanded pigment cells. Contracted cells exposed to these solutions expanded instantaneously and after this response gave no reactions to other stimuli.

It is obvious from these results that the stimulation of the pigment cells by propyl alcohol begins in solutions of lower concentrations than it does in ethyl and methyl alcohol. It will be seen that my results for methyl, ethyl, and propyl alcohols are in perfect agreement with the results on the toxicity of the above alcohols of other investigators.

Joffroy and Serveaux ('95) studied the toxicity of alcohols on mammals by intravenous injections. Bear ('98) introduced the alcohol directly into the stomach of the mammals. Picaud ('97) placed fish and amphibians in the solutions of the alcohols and in this way determined the toxicity of the alcohols. Bradbury ('99) and Cololian ('01) used fish, Overton ('01) on tadpoles employed the same method in their investigations. Wirgin ('04) determined the concentrations at which the various alcohols inhibited the growth of Micrococcus pyogenes aureus. He also investigated the laking power of the alcohols on the red corpuscles of the rabbit. Vernon ('11) studied the depression of an isolated tortoise heart by the alcohols. In table 3 the toxicity of ethyl alcohol is taken as unity and the values given are the comparative toxicities of the other alcohols. The values are only approximate.

TABLE 3

ALCOHOL	JOFFROY, MAMMALS	BAER, MAMMALS	PICAUD, FISH	BRADBURY, FISH	COLLOIAN, FISH	OVERTON, TADPOLES	WIRGIN, BACTERIA	WIRGIN, RED CORPUSCLES	VERNON, TORTOISE HEART	STIMULATION OF THE PIGMENT CELLS OF TROUT EMBRYOS	DEPRESSION OF THE PIGMENT CELLS OF TROUT EMBRYOS
Methyl.....	0.46	0.8	0.67	1.0	1.1	0.73	0.73	0.84	0.72	0.45	0.55
Ethyl.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Propyl.....	3.5	2.0	2.0	1.0	3.6	2.0	1.5	2.1	2.1	2.0	3.0

The stimulation level is lowest in methyl alcohol (4.5 per cent); next is ethyl alcohol (1.6 per cent to 2.5 per cent); and lastly propyl alcohol (0.08 per cent to 0.125 per cent). This is in harmony with the results of other investigators on the toxicity of alcohols where it was found that methyl was less potent in bringing about narcosis, and the potency increased for the other alcohols directly with the molecular weight. It was shown by Baer ('98) that the toxicity of the alcohols varied directly as their boiling points. Meyer ('99) and Overton ('01) discovered that the narcotic action of the alcohols varied with their solvent power for fats or lipoids. It may be suggested that in addition to the above physical factors involved in the action of the alcohols, that the dielectric constant of the alcohols probably plays

an important part in their action. It was observed that the greater the dielectric constant of the alcohols used the lower the stimulating or depressing power, and conversely the lower the dielectric constant the more striking were the reactions. Whatever may be the relation of these physical factors of the alcohols in stimulation or depression, their chemical structure must not be overlooked; for as the length and complexity of the chain in monohydric alcohols increases so does the strength of their action.

ALCOHOLS	MOLECULAR WEIGHT	DIELECTRIC CONSTANT AT 20°C	BOILING POINT, °C.	STIMULATING POWER—ETHYL ALCOHOL TAKEN AS 1
Methyl.....	32.03	31.2	65.7	0.45
Ethyl.....	46.05	25.8	78.4	1.0
Propyl.....	60.06	22.0	97.4	2.0

Reactions to alkaloids

The study of the action of drugs on the pigment cells of trout was undertaken with a threefold purpose, viz., to compare the action of drugs on the pigment cells with that of other tissues; second, to determine if possible the controlling mechanism of the pigment cells, and third, to see if the drugs had a specific action on the pigment cells.

The literature on the pharmacology of the pigment cells of fish is not very extensive. The earliest historical record of experiments on the action of drugs on the pigment cells is that of Redi (1664), who observed that eels which died in a tobacco decoction were light in color. Pouchet ('76) observed that Gobius niger changed in color when placed in strychnine. Morphine, quinine, and santonin had no effect. Lode ('90) concluded that curare destroyed the nerve endings of the pigments cells of trout (Salmo fario). von Frisch ('11) found that chloral hydrate contracted the pigment cells of the minnow and crucion. He also concluded that the action of cocaine was through the central nervous system.

The pigment cell may be stimulated or depressed by the drug acting: 1) on the pigment cell in such a way as to increase or

decrease its irritability; 2) on the nerve endings leading from the ganglia controlling the pigment cells; 3) on the central nervous system. I have no direct evidence to offer which will enable us to determine which of these or combination of these three factors are operative in the action of the drugs on the pigment cell, for I was unable to separate the nervous and pigment cell tissues for experimental purposes. It is obvious that large doses have no selective action. At certain optimal concentrations all the drugs show a selective action on the pigment cells or their controlling mechanism. This selective action of drugs on the mechanisms of the pigment cells will further our knowledge as to their function.

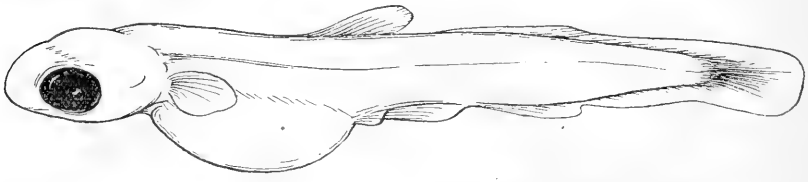

Fig. 1 A normal brook trout embryo showing the general alignment of the body.

In interpreting my results I have given special emphasis to their relation in a comparative way to the observations of other observers on various vertebrate and invertebrate tissues. This comparative method makes the results easier of interpretation and is less liable to lead to an erroneous conclusion.

The drugs used were all of Merck's manufacture. They were dissolved in oxygenated distilled water. The stock solutions were made up from 0.25 per cent to 0.5 per cent. Dilutions were made from these solutions. The experiments were carried on in Syracuse watch glasses in 10 cc. of the solution. These results were checked by experiments in small stender dishes of 50 cc. capacity. The conclusions are based on experiments repeated ten times in 1913 and again in 1914 another series of ten was tried. Five to ten animals were used at one time in each dilution. The trout embryos used were from four days to two weeks after hatching. In no case were the individuals of the different ages mixed.

[2] Figures 1, 2, and 3 were drawn by Miss H. J. Wakeman.

Other experiments are in progress to determine the action of drugs on pigment cells isolated from the nervous system.

1. Strychnine. In 0.5 per cent oxygenated solution death resulted without primary stimulation of the pigment cells. In 0.05 per cent strychnine solution the results were the same. Solutions of 0.005 per cent strychnine caused a contraction of the pigment cells rapidly, the contraction was complete in five minutes. There was a remarkable thing observed in this concentration of strychnine. The irritability of the fish was increased to a high degree. The fish went into typical strychnine spasms. The head was thrown backward and the tail curved upward and forward, describing a half circle (as shown in text fig. 2). A

Fig. 2 Showing a brook trout embryo in a typical opisthotonos response in 0.005 per cent strychnine.

passing shadow over the disk brought on a new spasm. Shadows in rapid succession increased the concavity backward. If the dish was tapped very lightly the same responses occurred. This period of heightened excitability lasted from eight to twelve minutes. During this interval the pigment cells remained contracted (fig. 13). As this convulsive period disappeared, the pigment cells expanded (fig. 14). This expansion showed that the depression and paralysis of the pigment cell controlling mechanism had occurred. In 0.0005 per cent the pigment cells were contracted in ten minutes. No convulsions were observed in this concentration. In weak solutions of 0.00005 per cent to 0.000025 per cent no contractions of the melanophores was produced.

Pouchet ('76) observed that the pigment cells of Gobius niger contracted in strychnine solutions. Romanes ('77) noted that in the medusa Sarsia (sp.) the swimming motions were much accelerated by strychnine, also that convulsions occurred in this and three other forms—Cyanaea capillata, Tiaropsis indicans, and Tiaropsis diademta. Hedborn ('99) has shown that strong doses of strychnine augment the beat of the isolated mammalian heart (cat). Dogiel ('77) demonstrated a slight increase in the rate of the heart beat of Corethra larvae. Pickering ('93) observed that weak solutions of strychnine had a primary stimulating action on the heart muscle of an embryonic chick. Carlson ('06) has found that strychnine in very weak concentrations had a distinct stimulatory action on the heart ganglion of the Limulus heart. Stronger solutions produced augmentation followed by paralysis. He was unable to note any primary stimulation on heart muscle. Laurens ('15) observed that if a drop or two of a 1 per cent solution of strychnine was injected into the body cavity of Amblystoma larvae the pigment cells contracted.

All the above experiments on other tissues show that strychnine has a primary stimulating action and especially on the motor ganglia. From the evidence of Ballowitz ('93) who demonstrated that the pigment cells of fish have a connection with the nervous system, and from the fact that strychnine stimulates the nervous system, we are warranted in concluding that strychnine acts directly on the nervous mechanism controlling the melanophores of trout embryos, rather than on the melanophores themselves. The seat of strychnine poisoning is in the spinal cord, therefore, the melanophores of trout embryos are in all probability controlled in part by the spinal nervous system.

2. Picrotoxin. Picrotoxin is used as a fish poison. It produces a medullary stimulation and ultimately results in death. When trout embryos are exposed to a 0.25 per cent solution of picrotoxin the pigment cells contract rapidly. The contraction is complete in two to five minutes. The contraction remains for forty-eight to sixty-four hours, if the fish are kept in this solution. The fish live in 0.25 per cent solution for one hundred

and twenty-six hours. There is no convulsive period. In a weak solution of 0.025 per cent of pictoroxin the contraction is slightly less rapid, and lasts indefinitely (fig. 11).

When the tail is cut away the pigment cells in the tail portion expand (fig. 12). They remain expanded for six hours and then degeneration sets in. The melanophores in the anterior or head end remain contracted. The contraction continues for eight to twelve hours and then disintegration of the pigment cells occurs. This justifies the conclusion that the reactions of the pigment cells of trout embryos are in some way controlled by the higher nerve centers.

If the pigment cells that are contracted in picrotoxin are expanded in 0.2 M. NaCl and are now placed in picrotoxin the contraction is much slower than the first time. The sodium chloride seems to counteract the action of the picrotoxin.

3. Morphine. In embryos exposed to 0.5 per cent solution of morphine hydrochloride the pigment cells remain expanded. In a 0.12 per cent most of the pigment cells were expanded but there were a few isolated areas that showed a contraction. After an exposure of three hours these isolated areas of contracted pigment cells had increased. In a 0.06 per cent solution of morphine the result was the same. In a 0.012 per cent solution no change occurred, all the pigment cells remained expanded. There was no contraction of the pigment cells in a 0.005 per cent solution. Pigment cells contracted by picrotoxin, potassium iodide or strychnine were expanded by morphine. According to Pouchet ('76), morphine did not cause any change in the pigment cells of Gobius niger. Romanes ('77) has found that in Aurelia aurita morphine had a highly depressing action. Pickering ('93) found that morphine acetate depressed the action of the heart muscle of embryo chicks. Cushny ('10) says that the action of morphine on the central nervous system is a mixture of stimulation and depression which are not equally marked throughout the system; also, "there is a selective action on the medulla oblongata in which certain centers are entirely paralyzed before neighboring ones undergo any distinct modification." Waller ('96) found that morphine applied directly to the nerve had but little effect on its irritability.

The explanation for the localized areas of contracted pigment cells may depend upon the selective action of morphine upon the nervous system.

The foregoing experiments support the conclusion that the pigment cells are controlled by the medulla or the spinal cord. It is probable that the localized areas of expanded and contracted pigment cells are in direct response to the mixture of stimulations and depressions caused by the action of morphine on the medulla. Or if the pigment cells are controlled by the reflex irritability of the spinal cord which may be depressed for a period and then may be followed by an increased irritability. On the latter hypothesis all the pigment cells should contract during the heightened irritability or expand during the diminished irritability; but since this is not the case it is probable that all the regions of the spinal cord are not involved at the same time.

4. Caffeine. In embryos exposed to a 0.2 per cent to 0.25 per cent solution of caffeine citrate no change occurred in the pigment cells. The animals died in a much distorted condition. The pigment cells disintegrated in two hours. A 0.05 per cent solution of caffeine citrate caused the pigment cells to contract in 4.25 minutes. There was a peculiar twitching of the muscles which lasted twelve minutes. A depression occurred in fourteen minutes. The pigment cells expanded very rapidly. In 0.025 per cent caffeine citrate solution contraction of the pigment cells took place in 5.25 minutes. The depression or paralysis was elicited in thirty minutes in some, while in others it took forty-five to sixty minutes. A solution of 0.005 per cent caffeine citrate caused no contraction of the pigment cells in two and one-half hours.

The convulsions observed were quite similar to those that occurred in strychnine. In caffeine the responses to shadows were absent. If the dish was jarred the reactions were weaker and lasted a short interval. These reactions occurred in solutions ten times as strong as in strychnine. The response was not opisthotonus, but the head was drawn toward one side and the tail toward the other. There was no difference in the sides to which the curvature occurred (as shown in text fig. 3). The

animal was in the form of the letter S. The convulsive period lasted a short time and gave from one to six spasmodic reactions. The pigment cells remained contracted during this period. As the convulsive tremors gave way to a complete paralysis the pigment cells expanded. The convulsive period and the contraction of the pigment were simultaneous. Weak solutions of 0.0005 per cent had no effect on the trout embryos or their pigment cells.

If the embryos are removed from a 0.05 per cent caffeine citrate solution during the period of convulsions, and if the poison is washed out rapidly there is a complete recovery. The pigment cells expand normally. If removed during paralysis after con-

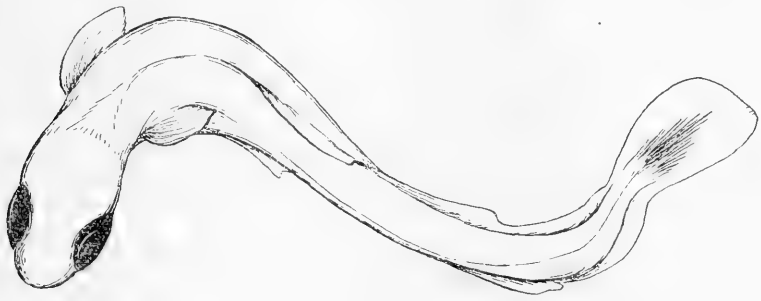

Fig. 3 Showing a brook trout embryo in a typical caffeine convulsion

vulsions the fish may recover very slowly or not at all. In weaker solutions of 0.01 per cent to 0.025 per cent there are no convulsions, but only a contraction of the pigment cells; there is a complete recovery when they are placed in water.

Carlson ('06) has shown that caffeine caused a primary augmentation in the heart muscle and primary stimulation of the heart ganglion of Limulus. Hedborn ('99) observed that caffeine stimulated the isolated mammalian heart (cat). Pickering ('93) observed an increase in the number of heart beats in the embryo chick's heart, and concluded from his work that it is not necessary to introduce a nervous hypothesis to explain the action of caffeine. Romanes ('77) has found in Sarsia (sp.) exposed to a sea water saturated with caffeine there was a great increase of the contraction and at the same time a diminution

of the potency of the beat. Soon the pulsations became of a fluttering nature and spontaneous movements ceased.

In the pigment cells of trout there is a stimulation which is at its height during the convulsive period. This is soon followed by a paralysis and an expansion of the pigment cells results. There is a direct resemblance in the results obtained with caffeine and strychnine, in that the reflex irritability is remarkably increased. The pigment cells contract in both instances during the convulsive period. There is a similarity in the results on the pigment cells of trout and the work of other investigators on other tissues. Caffeine may act directly on the pigment cells as it does on muscle (Pickering, '93, Carlson, '06), or it may stimulate the reflex centers in the medulla and spinal cord, which give off the fibers which control the pigment cells.

5. Curara. In very strong solutions of curara of 2 per cent to 1 per cent, a few pigment cells contracted. When trout embryos were exposed to a 0.5 per cent solution they moved about rapidly for eight to ten minutes. In one case one showed a complete contraction of the melanophores in three minutes while the other nine fish in the same lot showed no change. The one that showed this contraction had its pigment cells completely expanded in thirteen minutes. In 0.25 per cent solution there occurred a partial contraction of the pigment cells. The pigment cells along the lateral line were not contracted. The fish died in an hour and were covered with a colorless jelly-like slime. In the following dilutions of curara, viz., 0.05 per cent, 0.025 per cent and 0.001 per cent a partial contraction of the pigment cells occurred in two minutes and thirty seconds. In a 0.0025 per cent curara solution the change took place in fourteen to forty minutes. In all the experiments the contraction of the pigment cells was not evenly distributed but occurred in spots (fig. 15). The tail portion showed many contracted pigment cells, but in the head region there were the largest number of contracted melanophores. Along the lateral line the pigment cells remained expanded. After fourteen or fifteen minutes all the contracted pigment cells were expanded. This mixture of responses was constant for all the experiments.

Pouchet ('76) observed that curara did not modify the reaction of the pigment cells of turbot, viz., the pigment cells remained in an expanded condition. Lode ('90) has found that subcutaneous injection of a mixture of curara and glycerine caused a dark coloration in the adult trout (Salmo fario). He ligated the aorta and found that in the anterior end with the intact circulation expansion of the pigment cells occurred, while in the posterior end with the interrupted circulation, the pigment cells remained contracted. These experiments are not conclusive because the removal of the circulation interfered with normal metabolism of the cells. Moreover, the pigment cells are very sensitive to the changes in their oxygen supply. He observed that if the spinal cord of a curarized trout was stimulated, no contraction of the pigment cells occurred. If the pigment cells were stimulated directly, the pigment cells contracted. He concluded that the curara destroyed the nerve endings but did not affect the pigment cells. Laurens ('15) found that if Amblystoma larvae were placed in a 0.2 per cent solution of curara their movements were abolished and the pigment cells remained expanded under all conditions. He concluded that this failure on the part of the pigment cells to react was probably due to the direct effect of the solution on the animal; or asphyxiation of the larvae by the curara, and the consequent increased amount of CO_2 in the blood may have caused the melanophores to remain expanded. If a small amount of 1 per cent solution of curara was injected into the body cavity the larvae were rendered unmotile but the melanophores reacted to light (expanded) and to darkness (contracted) as usual. He concluded here that this experiment did not prove that curara had no effect on the melanophores, for it has been shown that melanophores will contract and expand after all nervous connections have been destroyed.

Carlson ('06) has shown that in weak solutions of curara there was a primary stimulation of the heart ganglion of Limulus. It had a little effect on the heart muscle. Young ('81) observed that in Mya (sp.) and Solen (sp.) there was a distinct acceleration in the number of heart beats, and sometimes a diminution,

and even a complete arrest. Plateau ('80) found that curara did not modify the frequency of the amplitude of the Decapod heart. Larger doses diminished the amplitude. Dogiel ('77) showed there was a primary stimulation by curara of the heart of Corethra plumicornis. Boehm and Tillie ('04) have observed a primary stimulation of the isolated mammalian heart (dog).

Since Curara stimulates the central nervous system Cushny ('10) and other ganglia Carlson ('06), it is possible that it acts as a stimulant on the medulla and spinal cord which transmit the impulse to the chromatophores, and a contraction results. Later as the curara destroys the nerve end plates the stimulus does not reach the pigment cell from the center. The pigment cells retain their independent irritability for a long time. The mixture of contracted and expanded melanophores is probably due to the unequal action of the curara on the peripheral nervous mechanism of the melanophores.

6. Nicotine. When trout embryos were exposed to 0.5 per cent nicotine solution, their muscles twitched for a moment and then all activity ceased. The heart beat continued for twenty-eight minutes. There was no change in the pigment cells. The pigment cells disintegrated soon after death. There was a very marked maceration of all the tissues. The whole fish was covered by a colorless slime. In a 0.125 per cent nicotine solution there was a slight primary contraction of the melanophores which was followed almost simultaneously by an expansion. The eyes bulged out of the head which caused the fish to appear grotesque. A 0.005 per cent solution of nicotine caused a complete contraction of the pigment cells in two and one-half minutes. The paralytic expansion occurred eight minutes after the contraction. In 0.0025 per cent nicotine the contraction time was the same as in the preceding experiment. The period of paralysis was delayed which occurred in eleven minutes. A nicotine solution of 0.0005 per cent produced a complete contraction in eleven minutes. The paralysis or depression of the pigment cells appeared in thirty-five minutes. In a 0.0001 per cent nicotine solution there occurred only a very slight change in the form of the pigment cells. In diluted 0.00005 per cent

no change was elicited. In all the cases where paralytic expansion occurred, it appeared first on the ventral side. The reason for this is not understood.

Redi (1634) according to van Rynberk ('05) observed that eels which died in a tobacco solution became lighter in color. Cushny ('10) says, that in nicotine the spinal cord is thrown into a condition of exaggerated irritability and that the medulla seems to be involved to a greater degree than the spinal cord. The stimulation does not involve the higher brain centers. Carlson ('06) observed that nicotine in weak solutions stimulated the heart ganglion of the Limulus heart. This primary stimulation was followed by a depression. There was no primary stimulation of the heart muscle. Gee ('13) has found that in a solution of 0.00066 per cent of nicotine leeches were vigorously stimulated, which was followed by a depression of movements. Romanes ('77) found that violent spasms were incited in the medusae Sarsia (sp.) and Tiaropsis when exposed to nicotine. He also observed various distortions. Langley and Dickson ('90) concluded that nicotine acts directly on the nerve cells and not on the muscle.

It is known that nicotine first stimulates and later paralyzes the ganglionic cells of the sympathetic system, whether applied directly to them or injected into the circulation. It is quite probable that nicotine affects the sympathetic system of the pigment cells, for there is first a contraction of the cells which is later followed by an expansion.

7. *Atropine.* Strong solutions (0.5 per cent) produced no change in the pigment cells. The fish lived four hours in this concentration. In 0.025 per cent solution of atropine sulphate, there was no change in the pigment cells. All possible concentrations were tried, but none of them produced a contraction of the pigment cells.

Pigment cells were contracted in 0.005 per cent strychnine solution and then were transferred to solutions of 0.05 per cent to 0.0025 per cent of atropine, where all the pigment cells expanded rapidly. The expansion was complete from two to four minutes. Pigment cells contracted in potassium salts were expanded just as those that were contracted by strychnine.

All the experiments show conclusively that atropine does not have any direct stimulating action on the pigment cells of trout embryos. Cushny ('10) says, that atropine acts on the higher centers of the brain and less on the lower divisions, viz., the medulla and the spinal cord, which is just the reverse of strychnine. This acts on the lower centers and not on the central system. The results obtained justify the conclusion that the pigment cells are controlled by the lower reflex centers.

Romanes ('77) in his experiments on the medusae Sarsia (sp.) and Tiaropsis found that atropine caused convulsive swimming movements by a marked depression. Pickering ('93) showed that 0.012 gm. of atropine to 1 cc. of normal saline solution reduced the normal heart beat of the embryonic heart of the chick. Carlson ('06) found that atropine stimulated the heart ganglion and not the muscle of the Limulus heart. Cushny ('10) says most secretions are depressed by the administration of atropine. This is not due to the inactivation of the secretory cells, but to the failure of the nervous impulses. The action of atropine on other tissues, from all evidence, shows us that it does not act directly on the vascular and secretory elements, but on their nerve terminations. It is therefore possible that atropine acts on the pigment cells through their nerve fibers, paralyzing them, but does not act directly on the pigment cells.

8. Cocaine. One-half per cent solutions killed the trout embryos rapidly. There was a momentary stimulation of the pigment cells which was followed almost simultaneously by an expansion of the pigment cells. In a 0.125 per cent solution of cocaine the behavior of the pigment cells was the same as in the 0.5 per cent solution. In 0.025 per cent to 0.05 per cent cocaine solution the pigment cells were contracted in four minutes. The contraction was followed by an expansion. Solutions of 0.005 per cent of cocaine produced a complete contraction in five minutes. The expansion followed in twelve minutes. Very weak, 0.00033 per cent solutions, had no effect on the melanophores.

These results show that cocaine has a primary stimulating action on the pigment cells of trout embryos. This primary

stimulation is followed by an expansion of the pigment cells. The action of cocaine on the nervous system is in a series, namely, the cerebrum is first affected, then the cerebellum and medulla, and lastly the spinal cord. It also acts on the sensory fibers and their terminations.

Von Frisch ('11) observed that the local application of cocaine caused the contraction of the melanophore in the minnow and Carssius (sp.). An injection of a 5 per cent solution into the body cavity caused a contraction of the pigment cells after the sympathetic nerves were severed. He concluded that the action of cocaine was through the central nervous system. Carlson ('06) showed that weak solutions of cocaine had a primary stimulating action on the heart ganglion of Limulus, but had no effect on the heart muscle. Hedborn ('99) observed a slight primary stimulating action on the isolated heart of the cat.

It is probable that cocaine acts on the reflex center which controls the pigment cells. It may act on the nerve endings of the pigment cells. It is obvious that it will require a great deal more of work to determine the relation of cocaine to the pigment cells before any generalization can be made.

9. Veratrine. Solutions of veratrine of 0.5 per cent concentration caused a rapid contraction of the pigment cells. The contraction was complete in two minutes. Paralysis set in at six minutes, and pigment cells were completely expanded in two more minutes. In a 0.25 per cent solution the stages were the same. In a 0.005 per cent veratrine solution the pigment cells were completely contracted in nine minutes. The first signs of paralysis appeared in eighteen minutes. Veratrine was a very active agent in causing the contraction of the pigment cells. Dilutions of 0.0005 per cent to 0.00005 per cent caused a contraction of the pigment cells in eighteen to twenty minutes. The paralytic expansion occurred in thirty minutes. A solution of 0.00001 per cent veratrine caused no change in the pigment cells.

Veratrine acts on the medullary center and the spinal cord, where a marked increase of irritability is elicited. After large doses there is a paralysis of the centers. It acts on the periph-

eral ganglia and nerve endings. It is highly probable that the action of veratrine on the pigment cells is through the lower centers of the nervous system rather than local.

Carlson ('06) found that weak solutions of veratrine had a primary stimulating action on the heart ganglion of Limulus. In strong solutions the period of stimulation was followed by a depression in two minutes. The ganglion free heart did not respond to the poison. Plateau ('78) observed a primary stimulation in the heart of Carcinus moenas and Homarus (sp.) which was followed by a depression. Romanes ('77) found that in the medusa Sarsia (sp.) the first effect of veratrine was an increase in the number and potency of the contractions. This period of increased responsiveness was followed by a gradual depression into complete quiescence.

Summarizing the action of veratrine on the pigment cells, it may be stated, that it first stimulates the contraction of the pigment cells. This period of stimulation is followed later by a paralysis of the mechanism controlling the pigment cells. The pigment cells are expanded during this period of depression. This is in harmony with the observations of other workers on various tissues, where there is observed a primary stimulation followed by a depression.

10. Quinine. Quinine in a 0.5 per cent maintained for a long time the pigment cells in an expanded condition. Dilutions were made from 0.25 per cent to 0.0005 per cent of quinine hydrochloride solution, and in all of these dilutions no change occurred in the pigment cells. Solutions of 0.000033 per cent to 0.0000165 per cent gave the same result.

The pigment cells were first contracted in picrotoxin and were then placed in the quinine solutions of 0.025 per cent to 0.005 per cent and in every case a rapid expansion of the pigment cells occurred. The rapidity of the expansion was greater in the quinine than it was in the ordinary process of washing out of the picrotoxin.

Quinine differs from most drugs in that its action is very widespread, and it is often called a general protoplasmic poison. Binz ('68) observed that quinine inhibited the beat of the cilia

in protozoans. Also, that it stopped the movements of the leucocytes. Santesson ('93) found that quinine depressed the rhythm of an isolated frog's heart. Hedborn ('99) observed that quinine depressed an isolated mammalian heart (cat). O. and R. Hertwig ('87) observed that sperm treated with quinine had their movement paralyzed. Eggs when treated with quinine after the sperm entered, the conjugation of the pronuclei was delayed. Carlson ('06) has found that quinine did not stimulate the ganglion or the muscle of the Limulus heart.

It is obvious from the experiments that quinine exhibits no primary stimulating action on the pigment cells of trout embryos. Any accurate interpretation of the depressing action of quinine is not possible, since the drug acts in the same way on the nervous tissues and the pigment cells as well.

SUMMARY

1. The experiments were performed on the melanophores (pigment cells) of the brook trout embryos, Salvelinus fontinalis Mitchill. Such young trout have only one kind of pigment cells, the melanophores. The young two-day or two-week old trout do not yet react to back ground. The first sign of reaction to back ground appears only after the yolk is absorbed.

2. In the presence of oxygen the pigment cells remain expanded and the fish live indefinitely. When hydrogen (oxygen want) is substituted for the oxygen the pigment cells contract and the embryos die. Oxygen is necessary for the maintenance of the expansion of the melanophores and life of the trout embryos.

3. Carbon dioxide excess caused a contraction of the melanophores. If oxygen was bubbled with the carbon dioxide, the presence of the oxygen had an antagonistic action.

4. Distilled water caused a rapid contraction. A mixture of distilled and boiled tap water gave the same result. In boiled tap water the pigment cells contracted. Oxygenated distilled water and boiled tap water maintained the pigment cells in a normal expanded condition. It was the absence of oxygen and not of the salts that caused the contraction.

5. In the potassium salts, K_2SO_4, KCl, KBr, KNO_3, and KI there occurred a rapid contraction of the expanded melanophores. The rate and degree of the contraction was the order given

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4 .$$

This primary contraction was followed by a cytolytic degeneration (expansion). The time required for the appearance of this degeneration was greatest in

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4$$

If the contraction or degeneration of the melanophores is specific for the potassium cation, it is unqualifiedly modified by its anion, or the residual part of the undissociated molecules.

6. The neutral salts of sodium, Na_2SO_4, NaCl, NaBr, $NaNO_3$, and NaI, caused a slow contraction of the melanophores. The contraction was most rapid in NaI and slowest in Na_2SO_4 and other salts were intermediate as

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4$$

Degeneration appeared first in NaI and last in Na_2SO_4 and varied in this order

$$I > \quad NO_3 > \quad Br > \quad Cl > \quad SO_4$$

The irritability of the chromatophores and life of the fish was maintained longest in Na_2SO_4 and NaCl from (118 to 132 hours) in NaI from one to two and one-half hours.

7. The pigment cells that were contracted in potassium salts, when placed in sodium salt they expanded. The order of expansion was

$$SO_4 > \quad Cl > \quad Br > \quad NO_3 > \quad I$$

There was no expansion in NaI.

8. The results obtained in the experiments on the action of the salts on the pigment cells of trout are probable to be explained on one or more of three assumptions: (1) That it is due to the antagonistic action between anion and cation; (2) that it is the independent action of the cation; (3) that reaction of the melanophores is likely modified by the undissociated molecule.

9. The narcosis or depression of the pigment cells of trout by the homologous alcohols corresponds very closely to their narcotic action as determined by Overton and numerous other investigators.

10. Very dilute solutions of methyl, ethyl, and propyl alcohols exert no action on the pigment cells of trout.

11. The pigment cells of trout embryos respond to alcoholic stimuli. Their mode of reaction is comparable to the reaction of other tissues to alcohols inasmuch as they are stimulated by small doses and depressed by large doses.

12. Strychnine in moderate doses causes a primary contraction of the expanded melanophores. Large doses cause a depression without a primary stimulation (contraction). The action of the strychnine is on the nervous system rather than on the pigment cells directly.

13. The action of picrotoxin causes a rapid contraction of the pigment cells. The mechanism controlling the pigment cells is in the higher centers, because if the spinal cord is severed the pigment cells expanded.

14. Morphine induces a contraction of the melanophores in isolated areas. This is probably due to the selective action of morphine upon the nervous system. Large doses produce no change in the expanded melanophores. Morphine expands the pigment cells that were contracted in picrotoxin, KCl, and strychnine.

15. Curara causes a mixture of responses, that is, there are areas of expanded and contracted melanophores. This is likely due to the unequal action of the curara on the peripheral nervous mechanism of the melanophores.

16. Medium solutions of nicotine cause a contraction of the pigment cells. Strong nicotine solutions have no effect on the pigment cells. The action of nicotine is directly on the nervous controlling mechanism of the pigment cells.

17. Atropine in all concentrations has no stimulating action on the pigment cells of trout. Atropine paralyzes the fine nerve connections of the pigment cells.

18. Cocaine has a primary stimulating action on the pigment cells of trout. This action is probably on the nerve endings of the pigment cells that connect them with the reflex center.

19. Veratrine causes a primary contraction of the pigment cells which is followed by a rapid depression (expansion). The action of veratrine is through the reflex center of the spinal cord and medulla rather than local.

20. Quinine exhibits no primary stimulating action on the pigment cells. The drug has no selective action on tissues, therefore it is a general 'protoplasmic poison.'

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PLATE 1

EXPLANATION OF FIGURES

In figures 1 to 10 are shown five sets of brook trout embryos which were exposed to the action to 0.2 M solutions of Kl, KNO_3, KBr, KCl, and K_2SO_4, 1 to 5 for an interval of fifteen minutes, and figures 6 to 10 for a period of three hours.

1 The melanophores were completely contracted in Kl.

2 The contraction was not as pronounced in KNO_3.

3 In KBr the melanophores had longer processes than in the two preceding solutions.

4 In KCl the processes were more distinct and showed the finer arborizations.

5 An exposure of fifteen minutes to K_2SO_4 produced no observable change in the melanophores.

6. After an exposure of three hours to Kl the melanophores showed a distinct secondary expansion.

7 In KNO_3 an exposure of three hours produced a less extensive secondary expansion than Kl.

8 In KBr the processes were very much shorter than in Kl and KNO_3.

9 After three hours in KCl the melanophores were still spherical, but there was a suggestion toward a peripheral migration of the pigment as indicated by the swollen condition of the cells.

10 After three hours in K_2SO_4 there was no expansion of the melanophores.

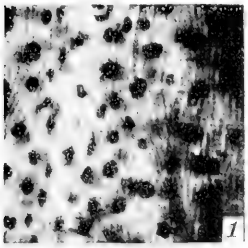
11 All melanophores contracted, photograph taken after twenty-four hours of exposure to Picrotoxin.

12 The melanophores expanded after severing the tail in an individual which was exposed to Picrotoxin for twenty-four hours. Photograph taken five minutes after cutting.

13 All melanophores contracted during the period of Strychnine convulsions.

14 Showing the expansion of the melanophores after the strychnine convulsion had subsided.

15 The contracted and expanded melanophores as they occurred in 0.0025 per cent curara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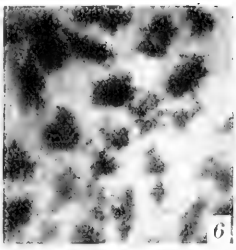
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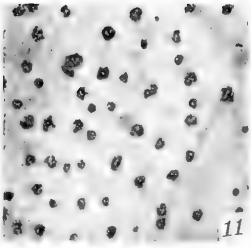
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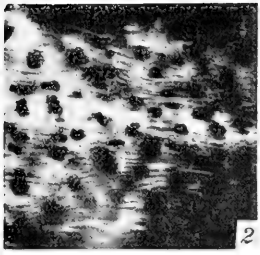
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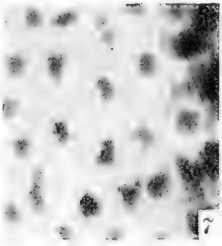
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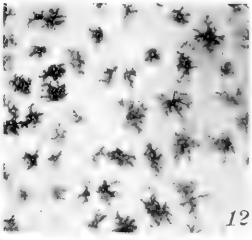
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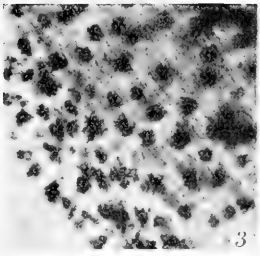
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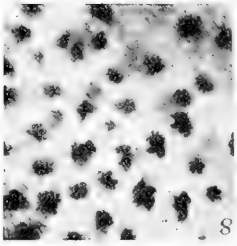
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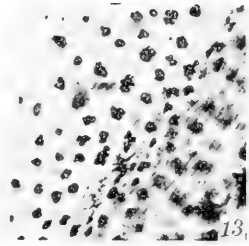
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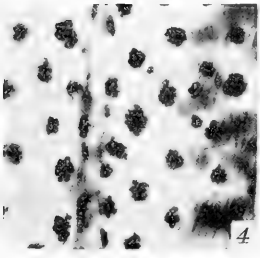
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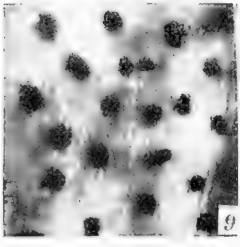
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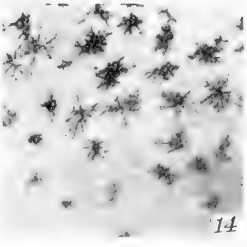
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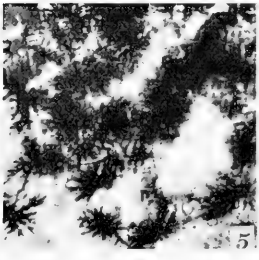
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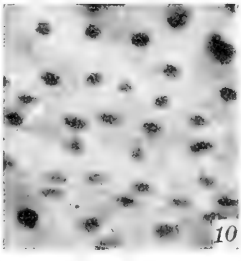
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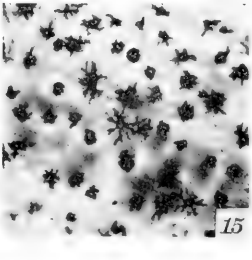
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THE REACTIONS OF THE MELANOPHORES OF AMBLYSTOMA TIGRINUM LARVAE TO LIGHT AND DARKNESS

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SIX FIGURES

INTRODUCTION

In two earlier papers (Laurens '15 and '16) the reactions to light and darkness of the melanophores of normal and eyeless larvae of A. punctatum and of A. opacum were described. These differed in some details from the corresponding reactions described by Babak ('10) for normal and blinded Axolotl larvae. The present paper is concerned with the description of similar reactions of the melanophores of larvae of Amblystoma tigrinum, the tiger salamander, the so-called Axolotl. Owing to the close similarity of these results with those obtained with A. punctatum and A. opacum they can be briefly dealt with. A few observations were also made of the melanophores of larvae of A. microstomum.[1] These showed responses slightly different from the others, but owing to the fact that only a few larvae were available at the time the observations were made, not very much can be said about them beyond the statement that the melanophores apparently do not show the secondary reactions which have been described for punctatum and opacum.

Babak ('10), it will be remembered (see Laurens '15, p. 620 and '16, p. 237), found that there was a difference between the reactions of the melanophores of normal and blinded Axolotl larvae to light and darkness. In bright diffuse light the melan-

[1] The eggs of A. tigrinum were sent to me by Prof. C. Judson Herrick, of the University of Chicago, those of A. microstomum by Prof. G. E. Coghill, of the University of Kansas. It is a pleasure to thank both of these gentlemen for their kindness.

ophores of the normal larvae contract, those of the blinded larvae expand. In darkness the melanophores of normal larvae expand, while those of blinded larvae contract. This opposite reaction of the melanophores of normal and blinded larvae he found not to occur until the larvae were 17 mm. long, and he supposed that before this the retina had not acquired the pigment motor function which it later has, and the melanophores respond, therefore, merely to direct stimulation, the reaction to which is the same in normal and blinded individuals. After this time, by means of the control which the eyes gain through the central nervous system, the sense of the reactions of the melanophores is reversed, the effect of indirect stimulation through the eyes being opposite to that of direct. Babak explains this difference between direct and indirect stimulation by assuming that both phases of the movement of the chromatophores of normal Axolotl larvae are governed by the central nervous system, and that this double innervation is conditioned upon the retinae which have opposite influences upon the nervous system according as to whether they are illuminated or darkened. The darkened retinae are believed to exert a positive influence on the chromatophores through the nervous system just as the illuminated retinae do, but in the reverse direction. The destruction of the retinae has an entirely different result from that obtained by darkening them. In other words, the retinae in complete darkness are active and exert a positive influence which is directly opposite to that caused by illumination.

Neither of these two opposite effects of the retinae upon the chromatophores are, according to Babak, inhibitory, but they are both tonic influences. The impulses bringing about the expansion of the chromatophores originate in the darkened retinae, and are so strong that they overcome the tendency of the darkened chromatophores to contract and cause their expansion. On the other hand, the impulses for the contraction of the chromatophores originate in the illuminated retinae and are in turn so strong that they overcome the tendency of the illuminated chromatophores to expand and bring about their contraction.

Pernitzsch ('13) also found that when Axolotl larvae were kept in darkness they became dark, due to the expansion of the melanophores.

The results which were obtained from the study of the reactions of the melanophores of A. punctatum and A. opacum larvae (Laurens '15) were such that Babak's explanation could not be applied to them. They threw no doubt, however, on the assumption that both phases of the movement of the melanophores are normally, by means of the eyes, under the control of the nervous system, although it seemed necessary to regard one of the influences of the retinae as inhibitory and opposite in effect to that which causes the pigment cells to contract.

It was found that the melanophores of normal and eyeless larvae of A. punctatum and of A. opacum react in identically the same way, expanding in light and contracting in darkness, the only difference being that the reactions came about more quickly in the normal than in the eyeless larvae. Later the melanophores of normal larvae were found in the opposite conditions, in light and in darkness, to what they were in before, for after having been kept for from three to five days in light the melanophores are contracted, and after having been for five days or more in darkness, the melanophores are expanded. The melanophores of eyeless larvae did not show these secondary reactions.

EXPERIMENTAL

The same methods employed in my former work on the pigmentation of Amblystoma larvae were carried out here. The optic vesicles of the eyeless individuals were removed at the tail bud stage (Laurens '14, p. 197), and the eyeless and normal larvae were kept in individual dishes. The melanophores were observed under the binocular microscope, attention having been already called to the inaccuracy of the method of simply observing the general coloration of the animals as an index of the contracted or expanded condition of the pigment cells.

As in the case of A. punctatum only the subepidermal or corial melanophores are referred to in the following description. The

epidermal pigment cells do not show sufficient regularity of response to light and darkness to fall in line with those of the corial melanophores. The xanthopores seem always to remain expanded.

RESULTS

The state of the melanophores of normal and eyeless larvae of A. tigrinum under different conditions are in complete agreement with those of A. punctatum and A. opacum. If normal seeing larvae are placed in bright diffuse light over an indifferent background and in darkness, and kept there, it will be seen after several days, that the melanophores of the animals kept in the light are partially contracted ($\frac{1}{8}$ to $\frac{1}{4}$ expansion) (fig. 5), while those of the animals kept in darkness are partially expanded ($\frac{3}{4}$ to $\frac{7}{8}$ expansion) (fig. 6). Eyeless larvae show the reversed condition, the melanophores of those in the light being expanded, of those in darkness, contracted. Table 1, which is reproduced from my earlier paper ('15), summarizes these observations, the conditions of the melanophores over a white and a black background being also included.

TABLE 1

State of the melanophores after long continued illumination and darkness

BACKGROUND	LIGHT			DARKNESS
	White	Black	Indifferent	
Normal	Contracted	Expanded	Contracted ($\frac{1}{8}$ to $\frac{1}{4}$ exp.)	Expanded ($\frac{3}{4}$ to $\frac{7}{8}$)
Eyeless	Expanded	Expanded	Expanded	Contracted

But the reactions, or primary responses, to light and darkness of the melanophores of the normal larvae are different (table 2), and do not agree with the conditions of the melanophores, which have just been described.

When the melanophores of larvae, normal and eyeless, that have been in darkness for only a few hours, are examined, it is seen that they are completely contracted, appearing as fine black dots. If these larvae, over an indifferent bottom, are now exposed to bright diffuse light the melanophores soon (five min-

TABLE 2

The reactions of the melanophores of normal and eyeless larvae to light and darkness

	LIGHT INDIFFERENT BACKGROUND	DARKNESS
Normal		
I. reaction	Expansion ($\frac{7}{8}$) (1 to 2 hours)	Contraction (2 to 3 hours)
II. reaction	Contraction ($\frac{1}{8}$ to $\frac{1}{4}$ exp.) (4 days or more)	Expansion ($\frac{3}{4}$ to $\frac{7}{8}$) (5 days or more)
Eyeless		
I. reaction	Expansion (2 to 3 hours)	Contraction (4 to 5 hours)

utes) begin to expand, though it is sometime before expansion is completed, the time varying between one and two hours for the normal larvae, and between two and three hours for the eyeless (figs. 1 and 2). If, after the melanophores have expanded, they are transferred back to darkness, the melanophores contract (figs. 3 and 4). This reaction does not begin so quickly as that to light, and it takes longer to be completed, between two and three hours for the normal larvae, and between four and five hours for the eyeless.

It is clear then that, as in A. punctatum, there is no difference between the responses of the melanophores of normal seeing and eyeless larvae of A. tigrinum. In light they expand (figs. 1 and 2), in darkness they contract (figs. 3 and 4). This reaction always take place, from the time the melanophores are first responsive to light and as long as they have been examined, the reactions of the melanophores having been observed in larvae ranging in length from 20 to 70 mm. It may be mentioned in passing that, as the larvae grow older and the number of melanophores increases, the reactions take longer and are less complete.

Due to the fact that, although the reactions of the melanophores of A. punctatum and A. opacum was to expand in light and contract in darkness, the state of the melanophores of seeing larvae, when these were kept for some time in light or darkness respectively, was different, a distinction was made be-

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Photographs at about $1\frac{1}{5}$ magnification of living animals, not anaesthetized, taken by Prof. Alexander Petrunkevitch, to whom it is a pleasure to express here my thanks. Figures 3 and 4 are unfortunately slightly out of focus. Larvae that have been in darkness for several hours when brought into the light are rather restive, and it is some little time before they can be quieted sufficiently

tween these conditions, the first being known as the primary reaction of the melanophores, the changed condition as the secondary reaction.

It has been found that, as indicated above, such reactions take place also in the melanophores of the larvae of A. tigrinum. When these are placed in darkness the melanophores at first contract, but remain so for only a limited time, for after five or more days of darkness,—interrupted by illumination with very weak red light only long enough to clean out the dishes and to add food,—the larvae are dark in appearance, the melanophores having expanded (fig. 6). This is a so-called $\frac{3}{4}$ to $\frac{7}{8}$ expansion. Eyeless larvae never show this secondary reaction of the melanophores, for these remain contracted, no matter how long the larvae are kept in darkness.

Very long tenure in darkness naturally affects the melanophores, both of seeing larvae, where they are secondarily expanded, and of eyeless larvae, where they are contracted. The number of melanophores does not increase so rapidly as normally occurs with the growth of the larvae, and the amount of pigment decreases, so that the animals eventually assume a golden, and then a transparent silvery appearance, due to the lack of pigment. When such individuals are brought into the light, the melanophores increase in number and eventually the larvae cannot be distinguished from those that have been always in the light. Light then seems to have more influence on the number of pigment cells than does the expanded condition

to snap them. The melanophores therefore have usually expanded slightly before the pictures can be taken.

Fig. 1 Normal seeing larva showing expanded melanophores after having been exposed to bright diffuse daylight for four hours.

Fig. 2 The same for an eyeless larva.

Fig. 3 Normal seeing larva showing contracted melanophores after having been in darkness for five and one-half hours (slightly out of focus).

Fig. 4 The same for an eyeless larva (slightly out of focus).

Fig. 5 Normal seeing larva showing the melanophores in their secondarily contracted condition ($\frac{1}{8}$ to $\frac{1}{4}$ expansion) after having been six days in bright diffuse light over an indifferent bottom.

Fig. 6 Normal seeing larva showing the melanophores in their secondarily expanded condition ($\frac{3}{4}$ to $\frac{7}{8}$) after having been six days in darkness.

of the melanophores (see von Frisch, '11, and Babak '13). This is supported by observations on the number of melanophores in seeing larvae that have been kept permanently in light over white bottoms where the pigment cells are always contracted. That the expanded condition is ineffective in this regard is not claimed, for comparisons between larvae kept in the light over white bottoms and those kept over black bottoms show that the number in the latter is greater.

In seeing larvae that have been kept in the light over indifferently colored bottoms for some time the melanophores also secondarily react. Continual or constant illumination does not seem to be necessary for this secondary reaction. Comparative observations have been made on larvae placed over an indifferent bottom in bright diffuse daylight, on those that have been continuously illuminated in a dark room by an electric lamp placed above the dish, the light being passed through ground glass and thus diffused, and on those continuously illuminated by light from a nitrogen-filled Mazda lamp passed through 'daylite' glass. All show after a few days the melanophores, which expand at first when they are illuminated, to be contracted (fig. 5). This, as was before found for A. punctatum, is not a complete contraction, but what has been called a $\frac{1}{8}$ to $\frac{1}{4}$ expansion. It takes, on the average, four days to come about. In a few individuals it sometimes takes a shorter time, in some it takes much longer, and in a few it never takes place. Table 2 summarizes all of these reactions of the melanophores.

Normal seeing larvae of Amblystoma tigrinum kept in an aquarium with Elodea and other plants are when young a pale, dirty, greenish gray which as the larvae grow older becomes a rich brown color. Eyeless larvae, on the other hand, are much paler in general appearance, having been described as 'anemic looking' by some who have observed my animals. In such an environment as has been described the general background is a black or dark one, and it is to this that the rich dark brown appearance of the normal larvae is due. It is dependent upon the eyes as is shown by the pale appearance of the eyeless individuals.

Seeing larvae when reared over a white bottom are very pale in appearance, due not only to the contracted condition of the melanophores, but also to the fact that the number of the melanophores is not so great. Eyeless larvae reared over no matter what bottom are darker than these, the melanophores being always expanded in the light. But, although the number of the melanophores is greater than the number in seeing larvae on white backgrounds, it is smaller than the number in seeing larvae under the general environmental conditions of the average aquarium dark bottom described above, and over a black one.

That the reactions of the melanophores are adaptive is shown by the above observations and also by the following. Seeing and eyeless larvae were placed when young (25–30 mm. long), in a large aquarium containing Elodea and other water plants. Coarse, brownish sand composed the bottom. As time went on, with the sinking to the bottom of decayed leaves and stems and the collected faeces of the animals, the bottom became a very dark brown, almost black, over which the seeing larvae were relatively inconspicuous, while the eyeless larvae were distinctly to be seen. Periodically, the aquarium was divided into two parts by a plate of glass placed diagonally across it and the nature of the bottom in one half changed by sprinkling white sand over it. The seeing larvae promptly changed over the 'white' bottom, becoming markedly paler, due to the contraction of the melanophores. The melanophores of the eyeless larvae did not respond. When the dividing plate of glass was removed the difference between the seeing larvae that have been over the 'white' bottom and those that have been over the dark one became very apparent, while the eyeless were alike over both bottoms, and different from each of the kinds of seeing larvae, being darker than the seeing larvae that had been over the 'white' bottom, and paler than those that had been over the dark bottom.

No further evidence regarding the cause of the secondary responses of the melanophores of seeing larvae to light and darkness is advanced beyond that which has been earlier expressed.

In light it is thought that the constant illumination or stimulation of the retinae starts impulses by certain photochemical changes in the retinae, the end effects of which are opposite to those of direct stimulation, and in this way bring about the secondary reaction of contraction. In darkness the same kind of thing is supposed to take place, long continued absence of light producing impulses by chemical changes, the end effects of which result in the expansion of the melanophores. That the secondary responses do not take place in the eyeless larvae shows that the seat of the causes of them must be sought in the retinae. The eyes through the nervous system cause the melanophores to secondarily contract in light and to expand in darkness. The results obtained with the larvae of Amblystoma tigrinum are thus in complete agreement with those earlier obtained with the larvae of A. punctatum and A. opacum, and do not agree with those of Babak.

SUMMARY

1. The reactions of the melanophores of the larvae of Amblystoma tigrinum are exactly like those earlier obtained with the larvae of A. punctatum and A. opacum. In light the melanophores expand (figs. 1 and 2) and in darkness they contract (figs. 3 and 4), in both seeing and eyeless larvae. The melanophores of seeing larvae that have been kept for some time (four days or more) in bright diffuse light over an indifferent bottom are, however, partly contracted ($\frac{1}{8}$ to $\frac{1}{4}$ expansion) (fig. 5) while the melanophores of seeing larvae that have been kept for some time in darkness (more than five days) are expanded ($\frac{3}{4}$ to $\frac{7}{8}$ expansion) (fig. 6), thus showing, under long continued illumination and darkness, what has been called a secondary reaction.

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EVIDENCES ASSOCIATING PINEAL GLAND FUNCTION WITH ALTERATIONS IN PIGMENTATION

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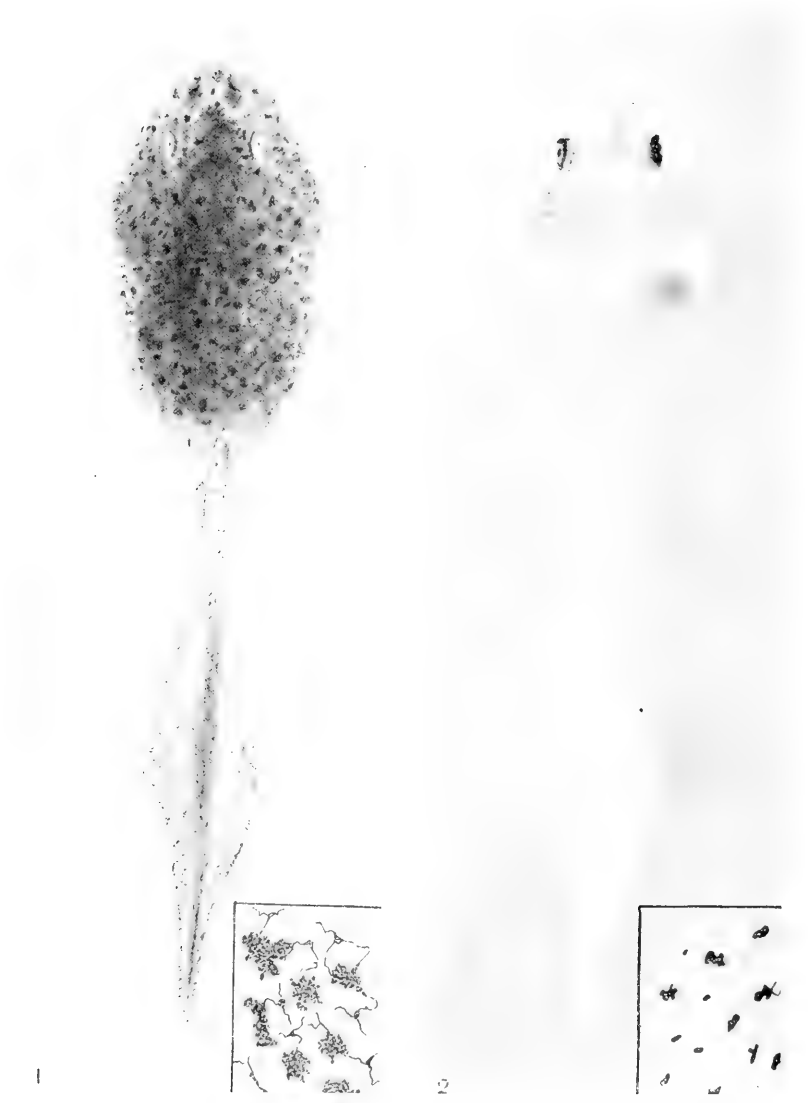
SEVEN FIGURES

INTRODUCTION

In the spring of 1915 we undertook to establish the influence of pineal gland substances upon growth and differentiation processes in tadpoles. The tadpoles employed were hatched in the laboratory and immediately placed upon a diet of pineal gland. On the tenth day of larval life it was readily observable that in the pineal fed groups the coloration was uniformly lighter than in the control, muscle-fed groups. This alteration was at first attributed to some unknown difference in environmental conditions—light, background, etc., as the color of these organisms was known to vary considerably in response to such stimuli. These changes when first noticed were trivial in degree, but as development progressed the alterations in pigmentation became correspondingly greater. Thirty minutes after feeding pineal tissue, the tadpoles which prior to the feeding had been uniformly dark, became so translucent that all the larger viscera were plainly visible through the dorsal body wall (figs. 1 and 2). This translucency appeared with such regularity and so punctually after pineal feedings and was so markedly absent in the control groups that the phenomenon was made the subject of special study. Out from this work have come many acceptable evidences of a pineal gland influence upon pigmentation, upon the phases of colloidal state, and upon the vegetative nervous system.

The following report is the record of the unfolding of this further work.



Figs. 1 and 2 Drawing of the same tadpole just prior to (fig. 1) and forty-five minutes after (fig. 2) pineal feeding.

NATURE OF PIGMENTATION

The color phenomena observable in many animal life forms are due to the absorption or reflection of light rays by chemical substances in the integument of the animal. These materials are usually found as granules of pigment lying in specialized cells, the chromatophores. Chromatophores are divisible into several types, but of these only two are found in frog skin. First, the melanophores, lying in the skin, peritoneum, etc., contain granules of dark-brown or black melanin and are often contractile. Second, the xanthophores, which are found only in the adult skin, contain granules of light yellow xanthin and are never contractile.

The relationships between pigmentation and environment are perfectly obvious but little understood. The color changes of the common tree toad (Hyla arborea) have been accounted for in various ways. One writer holds that the pale condition is the result of the stimulative effect of light upon the chromatophores and that the dark phase is due to the absence of that stimulant (1). Another observer (2) claims that light alone has very little effect, but that changes of temperature control the coloration. The problem is complicated by the fact that the same individual may not react in the same way under exactly similar conditions. Certain it is, however, that animals do respond to environmental changes by alterations in their coloration. It has been shown by numerous experimenters that these changes are to a great extent, under the control of the eyes. A change of environmental light is caught by the eye and the resulting stimulus transmitted to the chromatophores by the central nervous system. That this is the usual method of procedure is indicated by the atypical reactions of blinded individuals.

The mechanism of these changes is entirely dependent upon the contraction or expansion of the melanophores. Both xanthophores and melanophores enter into the color effects but the former play a passive role and always present the same appearance. The melanophores of frog tadpoles are of two distinct

types. The simpler and less conspicuous form is limited to the epidermis. It consists of a cell-body with two or more simple processes (*Ep. M.*, figs. 5 and 6). They may lie singly in the epidermis or, in such abundance as to form a definite reticulum. These melanophores are not contractile (3).

The second type of melanophore is found in greatest abundance in the sub-epidermal connective tissue. During late metamorphosis these cells migrate to the corium. In the expanded condition the sub-epidermal melanophores present a very typical 'mossy' appearance. The cells lie so closely approximated that they form a nearly continuous sheet (*Sub. M.*, fig. 6). There is a lighter, central space in each cell probably representing the nucleus. In the contracted condition (*Sub. M.*, fig. 7) the melanophores appear as irregular dots in which no structure is visible.

It has been fairly well established that the contractile melanophores are innervated by motor fibres proceeding along both sympathetic and spinal nerve paths (4). Hooker has shown physiologically that the reactions of the melanophores of the frog are synchronized by the action of the central nervous system. By histological methods, Ballowitz (5) has demonstrated motor nerve endings in melanophores of bony fishes. Laurens (6) working with Amblystoma larvae has shown that the melanophores may contract as the result of direct stimulation. Often, however, this primary reaction is overcome by an opposite, secondary reaction initiated by the central nervous system.

There are three principal theories to account for the mechanics of melanophore contraction. Ballowitz (1) claims that the contraction is an intra-cellular migration of the pigment granules within fixed cells. The protoplasm of a chromatophore is filled with numerous, extremely fine, radially arranged, anastomosing canals within which the pigment is forced back and forth by the alternate contraction and relaxation of the protoplasmic canal-walls.

Spaeth (7) believes that the chromatophores of fishes are fixed stellate cells, within which the pigment granules, carried in a rather fluid cytoplasm, stream into and out of the processes

during expansion and contraction. He explains this migration as a strictly colloidal phenomenon, the contracted and expanded conditions representing respectively the aggregate and disperse phases of a colloidal suspension. This is perhaps the most widely accepted and tenable view.

Hooker (8) has advanced a third explanation. He believes that the melanophores of frog larvae lie in preformed spaces and that the cells expand and contract as a whole within the spaces which enclose them. The acts of expansion and contraction, according to this theory, are brought about by pseudopodia, the pigment granules being carried in the cell cytoplasm. On this premise the pigment cells are to be considered amoeboid.

These expansions and contractions are commonly brought about by changes in the intensity of light or heat, but many other agencies will cause a specific reaction. Spaeth (7) has made a detailed study of the reactions produced by a great variety of stimuli upon the melanophores of Fundulus. He notes that the reactions are in every way comparable to those obtained by the same agents upon smooth muscle. He raises the very pertinent question as to whether melanophores may not be considered as modified smooth muscle cells.

In 1910 Babak (9) noted the reversal of the normal reaction to light when Axolotl larvae were blinded. In diffuse light the melanophores of normal seeing larvae contract. After painting the eyes with an opaque substance the melanophores expand in light. In the same way the melanophores of normal larvae expand in darkness, while those of blinded larvae contract. Fuchs (10) explained the phenomenon as due to the intervention of the parietal organ (the pineal gland of higher organisms). He reached this conclusion from a consideration of the phylogeny of this organ. The embryology of the parietal organ in some of the lower reptiles indicates very clearly that this body is a remnant of a third eye. Fuchs assumed that it had retained some of its controlling power over the melanophores. In the normal larvae its influence is completely over-shadowed by the superior power of the functioning eyes. In the blinded

larvae the parietal organ again assumes control. Laurens has completely disproven this hypothesis (11).

It is noteworthy from the present study that although the pineal gland does not exert a controlling influence upon pigmentation comparable to that arising from environmental stimulation of the retina, nevertheless it contains an active substance capable of directly inducing pigmentation changes irrespective of environmental conditions.

MATERIAL AND METHODS

Eggs of the species Rana pipiens, Rana cantabrigiensis and Bufo Americana were collected in the vicinity of Detroit, and hatched in the laboratory. Immediately after hatching and before the oral orifice had opened they were grouped in trays into colonies of 200 and food placed in the trays. The food was weighed, each colony receiving the same amount triweekly. All foods were taken voraciously by the tadpoles. By means of a water dropping and disposal system the tadpoles were at all times in fresh, aerated tap water. Moreover the trays were frequently shifted to average environmental conditions of light and temperature. In the observations on pigmentation we used some 12,000 tadpoles.

The food consisted of desiccated glandular material and fresh plant food. Of the glands we tested the effect of pineal (adult and preadult) thyroid, parathyroid, and suprarenal. Brain tissue and beef muscle were used as controls. Different lots of tadpoles were fed upon Spirogyra, bread crumbs and hemp seed as a further check. A single lot of tadpoles was fed on desiccated retinae from beef eyes as a particular experiment. Of these tissues the pineal gland alone produced the phenomenon we have called the pineal-pigment cycle.

EXPERIMENTAL EVIDENCE

Effect of whole pineal tissue on pigmentation

Certain endocrinous tissues are known to alter pigmentation in tadpoles. This was noted by Gudernatsch (12), in his feed-

ing experiments on these animals. Concerning the pigment altering tissues he states:

> After five weeks feeding, those (Tadpoles) fed on adrenal cortex became much lighter than those fed on adrenal medulla or any other food. This difference in color became more evident as the experiment proceeded, until the cortex-fed tadpoles had an extremely light, greenish yellow tint. The spleen and thymus fed tadpoles became extremely dark during the course of the feedings. Those fed on liver developed a dark greenish color, those on ovary a yellowish color.

The type of pigment variation observed by Gudernatsch is obviously distinct from that observed by us. In his animals the pigment alteration was slow in appearing and persistent. In ours the change appeared early in life, occurred sharply in relation to feeding, was cyclic and transient.

After the tenth day of larval life pigment changes were always evident after every feeding of the pineal tissues and the animals continued to react until their forelegs protruded. Sufficient blanching of the bodies occurred within thirty minutes after pineal feedings to differentiate these colonies from their controls. A maximum condition of translucency was attained in about forty-five minutes, and three to six hours later restoration to the original color was complete. The difference was first noticeable in the region about the eyes due to the absence of larger viscera. It can be demonstrated, however, that the reaction occurs simultaneously over the whole body. At the height of the reaction the integument was so transparent that the brain, the olfactory tracts, the kidneys, the beating heart and the intestines were all clearly visible through the dorsal body wall.

Figures 1 and 2 are drawings of a single tadpole just prior to and forty-five minutes after feeding pineal material. The darker portions in 2 are due to the denser viscera, the pigment conditions being the same over the entire animal.

Photography fails to give a true picture of this phenomenon but since actual photographs are more valuable as exact evidence than drawings figures 3 and 4 are here included. These are respectively photographs of the same group of tadpoles

just prior to and thirty minutes after feeding 5 mgm. fresh pineal gland. A true evaluation of the relative pigmentation may be had by comparison of the tadpoles of figure 3 with the periphery of those in figure 4. The dark color in the center of the bodies in figure 4 is due to the opacity of the denser viscera and intestinal contents and not to a difference in the pigmentation of the skin.

Figs. 3 and 4 Photographs made by reflected light.

Fig. 3 Normal tadpoles—just prior to pineal feeding.

Fig. 4 Same tadpoles 30 minutes after feeding acetone extract of pineal gland. The darker portions of these tadpoles are due to denser viscera—heart, gills, intestinal contents, etc. The degree of translucency is identical in all parts of the skin.

If a portion of the skin from a light and from a dark tadpole be dissected loose and examined under a microscope the reason for the difference in shade will be readily apparent, (figs. 6 and 7). The two types of melanophores are present. In the normal (dark) piece of skin the sub-epidermal melanophores (fig. 6, *Sub. M.*) are expanded to such an extent that they form an opaque sheet in which there are left a few scattered openings. In the

light piece of skin these melanophores are contracted to rough spheres of pigment (fig. 7, *Sub. M.*). The epidermal melanophores exhibit an unchanged appearance in both drawings. A sagittal section of normal skin (fig. 5) shows the relation between the two types of melanophores and the various layers of the integument.

These described alterations in pigmentation are invariably induced in tadpoles upon the administration of pineal materials, be they the fresh minced glands, simple desiccation preparations, or simple aqueous extracts. In an effort to associate these changes with certain constituents of the pineal gland, various fractions of the pineal were prepared and employed and are now about to be described.

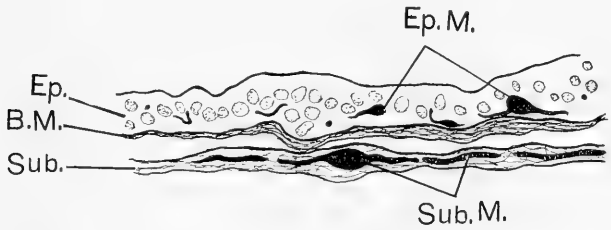


Fig. 5 Sagittal section of integument taken from the eye region of a normal tadpole. Section illustrates the two types of melanophores and their position relative to the tissue layers.

ABBREVIATIONS

Ep., epidermis
Int., integument, including epidermis and sub-epidermal tissue
Ep.M., epidermal melanophores
Sub.M., sub-epidermal melanophores
B.M., basement membrane
Sub., sub-epidermal connective tissue

Effect of pineal fractions on pigmentation

In the preparation of these split materials the fresh glands were either ground up and immediately extracted or desiccated and subsequently extracted. From the results of a wide variation in fractionation methods, chief interest centers around the acetone and alcohol extractives and their residues. In the case of acetone the process was carried out in a Soxhlet apparatus. On freeing the extractives from acetone there resulted

a brownish-black fatty mass with an odor suggestive of crude fish oil. Portions of these extractives and the residue were preserved intact for experimentation. Other portions of both were reextracted with alcohol. Likewise fresh pineal material

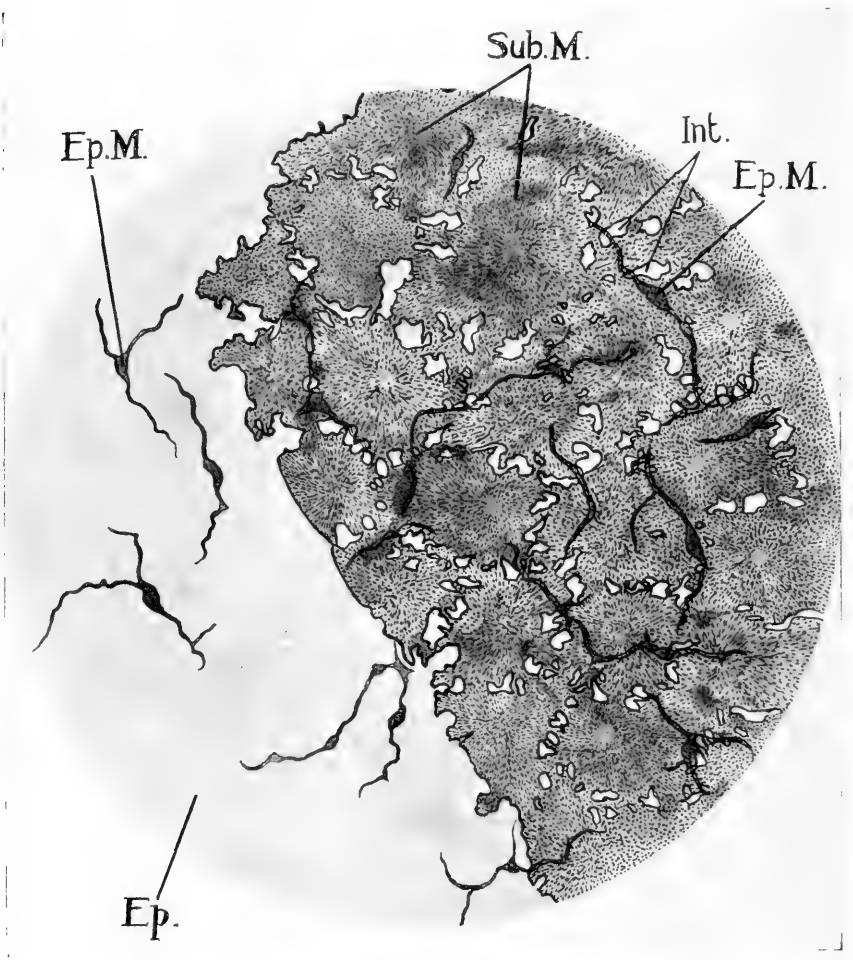


Fig. 6 Surface view of integument from normal tadpole. On the left side of the drawing the sub-epidermal connective tissue has been torn away, leaving the epidermis alone. Thus on the right side both types of melanophores are visible, on the left the epidermal type alone. The pigment granules in the sub-epidermal melanophores are evenly distributed throughout the cytoplasm, illustrating the disperse phase.

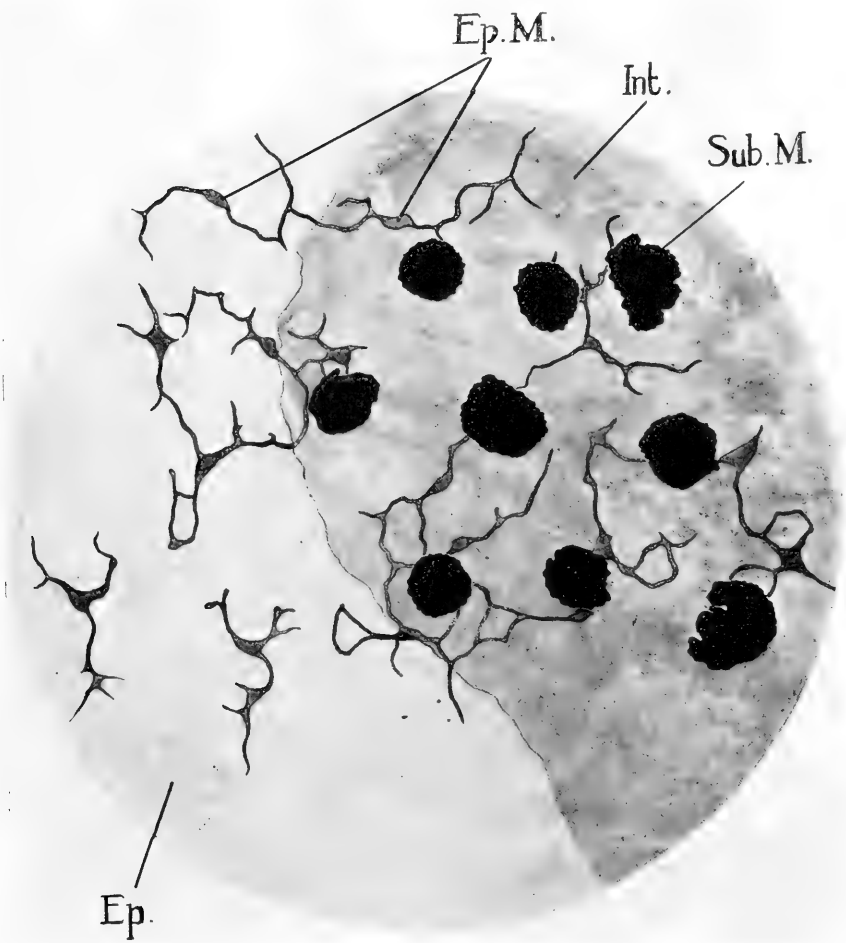


Fig. 7 Similar view of integument from tadpole fixed 30 minutes after feeding acetone extract of pineal gland. In this case the pigment granules in the subepidermal melanophores are collected into a mass in the centre of the cell—the aggregate phase.

The material was fixed in Bouin's fluid, dehydrated, cleared in xylol and mounted without staining in balsam. The drawings were made with the aid of an Abbe camera lucida, with Leitz objective 6 and ocular 4, giving an approximate magnification of 590 diameters, but have been reduced $\frac{1}{3}$ for publication. All material was taken from the region between the eyes.

was extracted with alcohol and the residue and extractives respectively extracted subsequently with acetone. These several preparations were tested as to their influence upon pigmentation on several hundred tadpoles from the same hatchings. At once it was apparent that the pigment altering principle was completely dissolved in acetone. The typical pigment cycle was induced by this extract while the residue and all acetone extracts of muscle tissues induced no pigment changes. The residue from acetone extraction was, however, capable of inducing the growth stimulating action that McCord has described for the pineal gland, while the acetone extracts which were exquisitely active in inducing pigment alterations were only slightly active in stimulating rapid growth. The inference is that at least two separate distinct principles exist in the pineal, the one producing the pigment phenomena, the other stimulating rapid growth. In the case of the alcoholic extraction the active substances were not readily soluble, for the alcoholic extractives, the alcoholic residue, and the acetone reextractives, all yielded positive pigment results. The acetone extractives yielded quite readily to aqueous emulsifying and this form proved to be the most convenient mode of employing this material.

Quantitative relations in time and amount of contraction

In the feeding of pineal materials to the tadpoles the time interval necessary to establish maximum contraction of the pigment cells increased as the concentration decreased. Tadpoles placed in a 1:500 pineal emulsion were noticeably lighter in five minutes and required but thirty minutes to arrive at maximum translucency. In higher dilutions the maximum translucency was attained only after a longer interval and in very high dilutions producing only qualitative changes, the maximum was not attained. The dilution of 1:100,000 was the highest that produced a macroscopically discernible qualitative action. These reasonably constant quantitative relations have afforded us a means for the evaluing of the strength of our several preparations and may on extended study prove to be

a trustworthy method for the standardization of pineal products for at the present time no method exists for testing the strength of such preparations.

The quantitative relations between concentration and time will be evident from the following table.

DILUTION OF ACETONE EXTRACT	MAXIMUM REACTION ATTAINED IN
	minutes
1:500	30
1:1000	45
1:2000	60
1:5000	105
1:10,000	Qualitative change but maximum not attained.
1:100,000	Maximum not attained.

The maximum reaction was determined by comparison with a standard consisting of several tadpoles which had been placed in a 1:500 pineal emulsion thirty minutes prior to the beginning of the experiment. The translucency thus obtained was found to be the greatest possible. It served as the criterion for comparison as to the degree of depigmentation induced by pineal preparations of unknown activity. In the practical standardization of pineal preparations, the end reaction of greatest feasibility was the comparative time intervals necessary to attain to maximum translucency. Such a method is obviously open to the criticism that frog larvae are only obtainable for a short period in the year. With Bufo Americana pigment changes were found to be too trivial to be of value in standardization. Rana pipiens were exquisitely responsive and amirably suited for standardization purposes except in that only during the spring months are they obtainable. We are now experimenting with certain amphibian larval forms that may be obtained throughout the year.

We have determined that the growth stimulating principle in the pineal is distinct from the principle concerned in pigment changes and this on further investigation may militate against this proposed means of standardization.

Effect of pineal gland upon melanophores of other forms[1]

Pineal gland extracts have no demonstrable effect on either type of chromatophore of Loligo (squid) or upon the melanophores of adult Fundulus. The extracts however determine distinct contraction of the melanophores of young Fundulus as may be noted in accompanying table.

No. 1 ONE WEEK-OLD FUNDULI IN BOILED SEA-WATER		No. 2 ONE-WEEK-OLD FUNDULI IN EMULSION OF ACETONE EXTRACT OF BEEF MUSCLE IN SEA-WATER		No. 3 ONE-WEEK-OLD FUNDULI IN EMULSION OF ACETONE EXTRACT OF PINEAL GLANDS IN SEA-WATER	
Time		*Time*		*Time*	
9.15	Complete expansion of melanophores	9.15	Complete expansion	9.15	Complete expansion
9.38	Complete expansion of melanophores	9.40	Complete expansion	9.37	Beginning contraction
9.46	Complete expansion of melanophores	9.47	Complete expansion	9.45	Contraction increasing
9.59	Complete expansion of melanophores	10.00	Complete expansion	9.58	Contraction almost complete
10.31	Complete expansion of melanophores	10.32	Complete expansion	10.30	Complete contraction
11.51	Complete expansion of melanophores	11.52	Complete expansion	11.50	Complete contraction
11.55	All transferred to fresh sea water				
1.55	p.m. Complete expansion	1.56	Complete expansion	1.54	Partial expansion
3.01	p.m. Complete expansion	3.02	Complete expansion	3.00	Complete expansion of some of the melanophores, especially in tail; partial expansion of others

Mode of absorption of the pigmentation altering principle

Several experiments were tried with the object of showing whether the pineal tissue must be ingested or not in order to produce the reaction.

[1] Experiment conducted by A. Noble at Woods Hole, Mass.

1. Pineal emulsion on anesthetized tadpoles. Several tadpoles of equal depth of pigmentation were completely anesthetized with of ether after which half were placed in an emulsion of acetone extract of beef pineal, the others in a like emulsion of muscle tissue. Five minutes later those in the pineal emulsion were preceptibly lighter, later acquiring a marked translucency. The latter remained unchanged. The tadpoles recovered from the anesthetic and gradually regained their original appearance.

2. Hypodermatic injection of pineal emulsion. Several tadpoles of equal depth of pigmentation were divided into two groups. One received 0.01 cc. of an emulsion (1–500) of acetone extract of pineal gland, injected hypodermatically into the peritoneal cavity; the other received the same amount of normal saline solution injected in a similar way. Shortly after injection the pineal treated animals became lighter, eventually reacting to the maximum degree of translucency. The other tadpoles remained practically unchanged in appearance. As a further control a third group was immersed in the pineal emulsion for the length of time consumed in making the injection and the tadpoles were then washed in tap water. They remained unchanged. This control showed that the effect produced was due to the injected pineal material and not to any accidental absorption through the skin.

3. Effect of pineal emulsion on eviscerated tadpoles. A number of tadpoles were completely eviscerated. These tadpoles live and swim about as freely as their fellows. Following this procedure part of the animals were removed to an emulsion of the acetone extractive of beef pineal gland and shortly passed through the same pigment changes as normal tadpoles placed in this emulsion at the same time. The eviscerated and normal tadpoles remaining in tap water did not change in color.

These observations prove conclusively that the principle involved is directly absorbable through the gills or skin. The effect is produced without the intervention of the processes of digestion. There is no indication, however, as to whether the principle acts directly upon the melanophores, or indirectly through the medium of the central nervous system.

Effect of pineal extract upon unstriated muscle

Aqueous extracts of fresh pineal glands were tested according to the method of Dale and Laidlaw (13) upon isolated strips of guinea-pig uterus. The extract produced a typical though feeble contraction of the uterine muscle. Three cc. of a 20 per cent pineal extract was roughly equivalent in activity to 0.004 cc. of a 20 per cent pituitary extract. Thus pineal extracts stimulate certain smooth muscle cells as well as pigment cells to contraction. This similarity of action goes far to confirm Spaeth's hypothesis that the melanophore is a type of smooth muscle cell.

COMMENT

Many acceptable evidences associate the pineal gland with an earlier optical function. In the reptilian stage of evolution this parietal eye probably attained to its highest development. In the embryos of certain lizards (Lacerta agilis) the typical eye structure is still evident, but in no form living at the present time does the pineal gland retain an ocular function, of high order (14).

The color changes in forms are obviously in adjustment to environmental conditions. The eye is the essential controlling factor in this adjustment. When in blinded animals certain definite changes in pigmentation still occur, on theoretical ground it is tenable to assume that the pineal body retains sufficient ocular mechanism to exert its influence upon the pigment cells. This is the hypothesis suggested by Fuchs (10). Laurens has established experimentally that such an activity on the part of the pineal is highly improbable (11). Accepting the contentions of Laurens, it is the gist of our work that while the pineal does not act in the rôle of its ancient ocular function, it contains within itself an active principle capable of inducing pigment changes independent of and wholly apart from environmental conditions. The pineal pigment changes dominate and appear despite environmental conditions tending toward the

opposite phase. The salient observations that we have recorded in detail on the foregoing pages are:

1. Up to near tenth day of larval life in tadpoles, pigmentation is not influenced by pineal feeding. Evidences relative to this are not precise, but suggest that this is due to incomplete development of the nervous mechanism involved.

2. Beginning at this time and continuing until near the termination of metamorphosis, the addition of traces as small as 1 part acetone extract in 100,000 parts water determine distinct cyclic pigment changes peculiar to these preparations. Prior to feeding, both controls and experimental animals are uniformly dark colored. Shortly after feeding the pineal fed groups begin to lose color until within thirty minutes, all macroscopic pigment is lost so that all the larger viscera are clearly visible (figs. 1 and 2). The condition is transient and the cycle is complete with full restoration of color within from three to six hours, unless further pineal food is added to the trays. As metamorphosis is completed the pigment is no longer altered by pineal materials, due to rearrangements of chromatophore types and sites in the adult animal.

3. The response in pigment change is quantitative. A method is described for the standardization of pineal preparations.

4. The pineal substance responsible for the pigment changes is wholly extracted by acetone. The residue after acetone extraction is an inert substance as to pigment influence. However this residue has an influence on growth and differentiation. The inference is that the gland contains more than one active substance.

5. The reactions produced by pineal extracts add some evidence to Spaeth's contention that the melanophores are modified smooth muscle cells. The similarity of contraction of certain smooth muscle organs under the influence of pineal extracts and the contraction of melanophores is in keeping with Spaeth's hypothesis.

The very nature of this pineal-pigment cycle affords an excellent method of approach to the mechanics of melanophore function and from this the larger problems of colloidal state.

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STUDIES ON SEX IN THE HERMAPHRODITE MOLLUSK CREPIDULA PLANA

II. INFLUENCE OF ENVIRONMENT ON SEX

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CONTENTS

INTRODUCTION

In a former paper (Gould, '17) the writer has followed out the sexual cycle of Crepidula plana and has shown that the sexual life of the adult may be divided into (A) the male phase, (B) the transitional phase and (C) the female phase. During the transition period the testis degenerates and eventually all the primordial male cells in the gonad disappear; while the primordial female cells, present from the beginning, multiply, and form the ovary.

It was also pointed out that there was great irregularity in the development of the male phase. A number of specimens of the same size and apparently of the same age, taken at the same time of year, may show widely different sexual states. One may be a fully developed male; one may be a partially developed male; one may exhibit evidence of having been a male though the male characters are being lost; and one may furnish no suggestion that any male characters have ever developed. The analysis of this phenomenon was reserved for the present paper;

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and while the analysis is by no means complete, a number of facts have been disclosed which have to do with the influence of environment on sexual development.

Light was first thrown on the question by the observation that those animals which we may call 'sexually inactive' or 'neuter,' though of a size equal to the males, were most frequently found in the 'new' hermit crab shells (i.e., Gastropod shells only recently acquired by the hermit), in which there were only a few small Crepidulas, and no large females. Of 216 specimens taken from such situations, there were 11 males (5 per cent). The other 205 (95 per cent) were either immature males, degenerate males or sexually inactive. This could be determined in the live animals by the amount of development or the non-development, of the external sex organs, the penis and seminal groove. The majority of all Crepidulas found in the 'new' shells had the thin, round, smooth shell which is characteristically formed on an unobstructed surface.

In the large colonies, or in fact in any shell where one or more large female Crepidulas were present with the smaller ones, the proportions were strikingly different. Material of this kind was more abundant and 1066 small individuals were examined, of which 662 (62.1 per cent) were adult males, 404 (37.9 per cent) were either immature males, degenerate males or sexually inactive.

DEGENERATION OF THE TESTIS

An accident disclosed another interesting fact. About 50 males had been removed from the colonies in the hermit crab shells, and placed by themselves in a dish under running salt water. They were neglected for several weeks and when subsequently examined seemed to be perfectly normal except that the penis had in every case disappeared, leaving only a small brown hump behind the right tentacle. Sections of the gonad showed that it had shrunken to small strands which ran here and there in the visceral sac. This chance observation coupled itself with the fact that males are far more numerous in large Crepidula colonies than in 'new' hermit shells having only a few small

inhabitants. It seemed advisable to try a controlled experiment of removing the males from the colonies, and of examining their gonads after various periods of time.

Experiment 1 (tables 1 and 2). Carried on in salt water aquaria of Princeton University vivarium. The smaller Crepidulas were removed from shells inhabited by hermit crabs and also containing colonies of C. plana, and the males were selected from them. The only Crepidulas left in each hermit shell were the two or three large females. Some of the males were then placed on and around the females to serve as controls (table 2) while the rest were allowed to attach themselves in other shells from which all the Crepidulas had been removed

TABLE 1

Specimens removed from large colonies containing female, found to be males, and transferred, two or three together, to hermit shells in which were no other Crepidulas

SPECIMEN	LENGTH	DAYS	PENIS	GONAD	SEMINAL VESICLE
61	?	10	Long	Shrunken to small strands; spermatogonia; sperm	Full of sperm
62	?	10	Long	Shrunken to small strands; spermatids; sperm; distorted nuclei	Full of sperm
63	?	10	Short	Small; spermatogonia; spermatids; sperm	Full of sperm
67	15	13	Short	Small strands; inactive	Thick wall; empty
68	7	13	Short	Small strands; few spermatogonia and sperm	Thick wall; few sperm
69	7	13	Stump	Small; spermatogonia only	Not included in sections
73	12	18	Short	Inactive; type A and B cells in wall; few sperm	Small; full of sperm
74	12	18	Short	Has considerable lumen, empty except for a few spermatogonia and sperm	Full of sperm; unusually large number of apyrenes
75	12	18	Stump	Empty; inactive	Reduced to indifferent gonoduct
79	9	34	Stump	Small; inactive	Thick wall; empty
80	8	34	None	Small strands; inactive	Thick wall; empty
81	8	34	Stump	Small strands; inactive	Reduced in size; a few sperm
82	10	34	None	Preparation for oogonial division.	Not included in sections
83	$7\frac{1}{2}$	34	Stump	Small strands; inactive	Not included in sections

TABLE 2

Specimens removed from large colonies containing females, found to be males, and replaced in hermit shells in which were from one to four large female Crepidulas

SPECIMEN	LENGTH	DAYS	PENIS	GONAD	SEMINAL VESICLE
64	?	10	Long	Normal testis	Full of sperm.
65	?	10	Long	Normal testis	Full of sperm
66	?	10	Long	All stages of spermatogenesis except spermatocytes	Very large and full of sperm
70	13	13	Long	All stages of spermatogenesis but testis small	Full of sperm
71	11	13	Long	Good size; normal testis	Very large; full of sperm
72	9	13	Long	Good size; normal testis	Full of sperm
76	11	18	Long	Normal testis	Full of sperm
77	11	18	Long	Normal testis; very active	Very large; full of sperm
78	9	18	Long	All stages of spermatogenesis, but testis small	Full of sperm
84	16	34	Long	All stages of spermatogenesis, but testis small	Full of sperm
85	18	34	Long	All stages of spermatogenesis, but testis small	Full of sperm
86	15	34	Long	All stages of spermatogenesis, but testis small; minority of spermatocytes	Full of sperm
87	9	34	Long	Normal testis	Full of sperm
88	7	34	Long	Normal testis	Full of sperm
89	6	34	Long	Normal testis	Very large; full of sperm
90	5	34	Long	Normal testis	Very large; full of sperm

(females as well as males). Several males were put in each hermit shell as was the case with the controls. The two sets of hermits were then shut up in two crates and sunk in the aquarium.

After ten days three Crepidulas were taken from each lot and sectioned. Specimens 61, 62 and 63 of table 1 were taken from the hermit shells without female Crepidulas. In two of the three the testis was completely and in the third, partly degenerated. In the controls with females (specimens 64, 65 and 66 of table 2) two were normal males, the third showed no spermatocytes. After thirteen days three more were taken from each lot, and showed a still more marked difference (specimens 67, 68, 69; controls, 70, 71, 72). The external genitalia, as well as the testis, degenerates in the segregated males. Still other samples were taken after eighteen days, and at the end of thirty-four days all those left in each crate which had been males originally, were fixed and sectioned. As the tables show, the males which had been separated from the females suffered regressive changes in the testis

and the accessory male organs. Some of the controls showed partial regressive changes but the majority remained perfectly normal.

The significance of the words at head of columns in the tables is as follows: 'Specimen', identification number; 'length', length of shell of specimen in millimeters; 'days', number of days duration of experiment; 'Penis', 'Gonad', 'Seminal vesicle', condition of those organs in the prepared specimens.

Experiment 2 (Tables 3 and 4). Carried on in floating live-cars of the Marine Biological Laboratory, Woods Hole. This is similar to the preceding experiment except that it was performed under more nearly normal conditions. It shows the same degeneration of organs in the segregated males. Here and there among the controls (table 4) we find a specimen in which the activity of the testis is reduced, or there is slight degeneration; this is not unexpected, since a few degenerate males are found in normal colonies.

The results of experiments 1 and 2 strengthened the suspicion that the large female Crepidulas of the colony exercised some influence upon the small male members of the same colony. The preservation of the male character seems to depend on the presence of the larger animals in the same neighborhood with the males. Another experiment confirmed this still further.

Experiment 3. All the large females were removed from two colonies leaving the smaller animals which had been clustered about them untouched. It was assumed, though it could not be made certain, that the usual majority of males was present. The shells occupied by the colonies and inhabited by the hermit crabs were replaced, after removal of the females, in the aquarium and left for fifteen days. At the end of that time all the smaller Crepidulas were fixed and sectioned. Nine out of the eleven specimens showed that they had been functional males, but that the testes had degenerated to various degrees. The presence of the seminal vesicle with sperm in it proved that the testis had recently been active, even though the testes of some individuals were reduced to the neuter condition.

Two of the eleven specimens showed that they had not been males when the experiment was begun; for the seminal vesicle was not developed.

MALE DEVELOPMENT IN NEUTER INDIVIDUALS

It is possible to show, in Crepidula plana, not only that the preservation of the male phase depends upon the proximity of larger individuals, but that 'sexually inactive' or 'neuter' specimens will rapidly develop male characteristics when brought into

TABLE 3

Males treated as in table 1

SPECIMEN	LENGTH	DAYS	PENIS	GONAD	SEMINAL VESICLE
109	16½	14	Long	Small strands; distorted nuclei	Thick wall; full of sperm
110	12	14	Long	Small strands; spermatids; sperm	Full of sperm
111	11	14	Half usual length	Small strands; inactive	Full of sperm, mostly apyrenes
112	10½	14	Long	Normal testis	Full of sperm
113	10½	14	Half usual length	Few spermatogonia; spermatids; sperm	Full of sperm, largely apyrenes
114	10	14	Long	All stages spermatogenesis, but testis small; some follicles nearly empty	Not included in sections
115	9½	14	Very short	Very much reduced; some spermatids and sperm; a few *resting oocytes*	Full of sperm
116	9½	14	Long	All stages spermatogenesis but much reduced in size	Full of sperm
117	6	14	Long	Small strands; degenerating cells; some follicles empty	Full of sperm
118	6	14	Long	Small; interior nearly empty; some spermatogonia and sperm	Contains sperm and coagulated matter
129	14	19	Half usual length	Oocytes in early synapsis	Thick wall; few apyrene sperm
130	14	19	¾ usual length	Very small; inactive	Small; thick wall
131	12	19	Half usual length	Small; sperm and coagulated matter	Full of sperm
132	12	19	Short	Small strands; few sperm	Small; thick wall; few sperm
133	11	19	Long	All stages of spermatogenesis; reduced size	Full of sperm
134	10½	19	Long	All stages of spermatogenesis; only few spermatocytes; reduced size	Small; full of sperm
135	10	19	⅓ usual length	Spermatogonia, spermatids and sperm; reduced size	Full of sperm

TABLE 3—Continued

SPECIMEN	LENGTH	DAYS	PENIS	GONAD	SEMINAL VESICLE
136	9½	19	Short	Small; lumen empty except for few sperm	Small; full of sperm
137	9	19	Short	A few spermatogonia; spermatids; sperm	Full of sperm
138	8	19	Stump	Spermatogonia; spermatids; sperm	Small; few sperm
139	7	19	Short	Spermatogonia; spermatids; sperm	Small; few sperm
150	17	33	Short	Small; inactive	Not included in sections
151	14	33	Very short	Small; degenerating spermatogonia	Thick wall; some parts full of sperm, some empty
152	13	33	⅔ usual length	All stages spermatogenesis, but reduced size	Full of sperm
153	12	33	Stump	Spermatogonia; few sperm	Thick wall; few sperm
154	11	33	None	Small; empty; distorted nuclei	Shrunken to nearly straight tube; occasional sperm
155	11	33	Stump	Shrunken to solid strands; distorted nuclei	Shrunken to nearly straight tube; occasional sperm
156	11	33	Stump	Small; nearly empty; occasional sperm; distorted nuclei	Small; thick wall; empty

a colony containing individuals larger than themselves (e.g., the large females).

Experiment 4. Carried on in float-cars, Woods Hole, June to August, 1915. The writer collected as many hermit shells as possible where there were no female Crepidulas but only a few small specimens in each hermit shell, all about of the same size. The majority of these had thin, flat smooth shells. They were examined with a lens and the few which showed any development of accessory male organs were discarded. The rest were divided into two lots. Those of one lot (table 5) were allowed to attach themselves inside hermit shells where one or several large females were already attached. Those of the other lot were returned, several together, to hermit shells where there were no other Crepidulas whatever (table 6). It was subsequently found that the sexual condition of an individual is somewhat influenced by the presence of another of only slightly larger size; the experiment

TABLE 4

Males treated as in table 2

SPECIMEN	LENGTH	DAYS	PENIS	GONAD	SEMINAL VESICLE
119	15	14	Long	Normal testis	Very large but nearly empty; probably after copulation; this condition seldom seen
120	13	14	Long	Normal testis	Small; full of sperm
121	12	14	Long	Normal testis	Very large; full of sperm
122	12	14	Long	Normal testis	Very large; full of sperm
123	9½	14	Long	Normal testis. Very large	Small; many apyrenes
124	9	14	Long	Normal testis	Full of sperm
125	8½	14	Long	Normal testis	Full of sperm
126	8	14	Long	Normal testis	Full of sperm
127	6½	14	Long	Normal testis. Very large	Small; full of sperm
128	5	14	Long	Normal testis	Full of sperm
140	13	19	Long	Reduced in size; all stages of spermatogenesis	Full of sperm
141	13	19	Long	Normal testis	Full of sperm
142	12½	19	Short	Early stages of spermatogenesis but no late stages	Few sperm, largely apyrene
143	12	19	Short	Spermatogonia; sperm	Small; full of sperm
144	10	19	Long	Normal testis; very large	Large; full of sperm
145	10	19	Long	Normal testis	Full of sperm
146	10	19	Long	Normal testis	Full of sperm
147	9½	19	Long	Normal testis. Large	Large; full of sperm
148	9	19	Long	Normal testis	Full of sperm
149	8	19	Long	Normal testis. Very large	Full of sperm
157	17	33	Half usual length	Spermatogonia; sperm	Small; thick wall; some sperm
158	14	33	Long	Normal testis	Full of sperm
159	13	33	Long	All stages spermatogenesis, but reduced in size	Full of sperm
160	13	33	Long	Normal testis; small	Full of sperm
161	12½	33	Long	Normal testis	Very large; full of sperm
162	12	33	Long	Normal testis	Full of sperm
163	11	33	Long	Normal testis	Full of sperm

would have been more striking, therefore, if in the controls each specimen had been completely isolated. The difference of behavior of the two groups is plainly to be seen, however, in tables 5 and 6, made up of observations on the sectioned specimens at the end of the experiment, which lasted thirty-four days.

TABLE 5

Neuter Crepidulas transferred to neighborhood of large females. Sectioned after thirty-four days. Specimens from each hermit's shell listed together. Wherever sufficiently marked, increase in length of shell ('growth') and amount of development of penis, during course of experiment, have been recorded. Summary of results: with adult testis, 18; testis to spermatids, 6; testis to spermatocytes, 2; with spermatogonia only, 1; sexually inactive, none. Total, 27

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
			Hermit 1		
175	13		Stump	Young testis to spermatids; small	Not included in sections
176	13		Stump	Young testis to spermatids	Seminal vesicle in process of formation
177	12½	1	None	Few spermatogonia, spermatocytes and spermatids	Undifferentiated
			Hermit 2		
178	10	3½	Long	Spermatogonia; spermatocytes; spermatids; small	Not included in sections
179	9½		Long	Adult testis	Vesicle, full of sperm
180	9	1½	Short	Adult testis, large	Vesicle, full of sperm
181	9		Very short	Adult testis; very large	Vesicle, full of sperm
182	7	1½	Long	Adult testis; small	Small vesicle, full of sperm
			Hermit 3		
183	11	2½	None	Spermatogonia; spermatids	Not included in sections
			Hermit 4		
184	10	2	Long	Adult testis, large	Vesicle, full of sperm
185	9		Long	Adult testis	Vesicle, full of sperm
186	8		Long	Adult testis	Vesicle, full of sperm
187	8		Long	Adult testis	Vesicle, full of sperm
188	7½	2	Stump	Adult testis	Vesicle, full of sperm
189	7½		Stump	Spermatogonia and spermatocytes, very active	Empty tube, somewhat convoluted
190	7	1	Long	Adult testis	Vesicle, full of sperm

TABLE 5—Continued

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
191	7		None	Spermatogonia; spermatocytes	Small tube, somewhat convoluted
192	7	1	Long	Adult testis	Seminal vesicle, full of sperm
193	6	1½	Long	Adult testis, large	Seminal vesicle, full of sperm
				Hermit 5	
194	8	2	Stump	Adult testis, small	Small convoluted tube
195	7	1½	Long	Adult testis, large	Seminal vesicle, full of sperm
196	7		None	Spermatogonia	Small tube, straight
197	7		Long	Adult testis, large	Seminal vesicle, full of sperm
198	7		Half usual length	Young testis to spermatids	Small convoluted tube
199	6	1	Long	Adult testis, large	Seminal vesicle, full of sperm
200	6		Long	Adult testis, large	Seminal vesicle, full of sperm
201	6		Long	Adult testis, large	Seminal vesicle, full of sperm

The above tables show that the great majority of neuter animals become males when associated with large females; that in the absence of large females, only a very few neuter animals develop male characters, and these few are always found in proximity to somewhat larger individuals. It is to show this fact that the specimens from each hermit shell are listed together in table 6. In no case does the largest individual in a colony ever become a male.

The stimulus from the larger to the smaller individuals is quantitative. A number of the latter in table 6 have gone through the first stages of male development, but the change is not often complete. The number of sexual products formed is small, motosis rare; the specimens of table 5, on the other hand, nearly all show full male development, both in primary and sec-

TABLE 6

Neuter Crepidulas returned to hermit's shells similar to those which they formerly occupied, i.e., without large female individuals. Sectioned after thirty-four days. Specimens from each colony listed together. Summary of results: with adult testis, 3; testis to spermatids, 2; testis to spermatocytes, 1; with spermatogonia only, 11; sexually inactive, 5; with oogonia, 2. Total 24

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
			Hermit 1		
202	16	4	None	Many primordial egg cells	Undifferentiated
203	15	3	None	Many primordial egg cells	Undifferentiated
204	13	2	None	Small; few spermatogonia	Undifferentiated
205	13	2	None	Spermatogonia, degenerating	Undifferentiated
206	12	4	None	Spermatogonia, degenerating	Proximal part somewhat wider and slightly twisted
207	$12\frac{1}{2}$		None	Spermatogonia	Undifferentiated
208	$10\frac{1}{2}$		Short	Small adult testis	Small seminal vesicle with a few sperm
			Hermit 2		
209	15		None	Inactive	Undifferentiated
210	13		None	Inactive	Undifferentiated (transparent cells)
211	11		None	Few spermatogonia	Undifferentiated
212	11		None	Spermatogonia, degenerating	Not included in sections
213	$10\frac{1}{2}$		Stump	Spermatogonia	Proximal end somewhat widened and convoluted
214	9		Stump	Adult testis; small	Small seminal vesicle; sperm
215	8		Short	Adult testis	Seminal vesicle; small; sperm
			Hermit 3		
216	9	2	None	Inactive	Slightly widened proximally
217	9	2	None	Inactive	Undifferentiated

TABLE 6—Continued

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
				Hermit 4	
218	15	6	None	Few spermatogonia	Undifferentiated
219	13	4	None	Few spermatogonia	Undifferentiated
220	10	2	None	Young testis to spermatids	Not included in sections
221	10	2	None	Inactive	Undifferentiated
222	9½	2	None	Spermatogonia and spermatocytes	Undifferentiated
223	9½		None	Spermatogonia	Not included in sections
224	9		None	Spermatogonia and spermatids	Slightly widened and twisted proximally; empty
225	8	1½	None	Spermatogonia	Undifferentiated

ondary characters, great numbers of sperm and active spermatogenesis. The results of all experiments indicate that the neuter animal, living in isolation and having reached a considerable size, requires only a very slight stimulus to start the development of male characters, but a greater one to complete it.

It is a question whether the production of a limited number of spermatogonia is a sure indication of a stimulus from the outside; certain specimens which had each been kept isolated in a small vial produced a few spermatogonia; but development never went any farther.

Experiment 4 was repeated and samples were taken from the colonies at various times. Space is not available to submit the results in tabular form. In general, the neuters quickly took on male characters when placed in colonies with large females; while the neuters kept apart rarely did. Nine days after having been transferred to the neighborhood of large females, the formerly neuter specimens had testes developed as far as spermatids; adult males were found in sixteen days. The specimens had been marked by notches in the shells to obviate any possible mistake in identity.

When left for long periods, the small Crepidulas living near large females become more and more divergent from those living

in the absence of large females, both in size and in sexual condition. This is indicated briefly in tables 7, 8, 9, and 10.

The experiments on neuter Crepidulas indicate that the 'sexually inactive' animals are so because male development cannot take place in the absence of a certain stimulus which proceeds

TABLE 7

Neuter Crepidulas transferred to neighborhood of large females; sectioned after sixty-seven days

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
242	13½	None	Long	Adult testis	Seminal vesicle, full of sperm
243	10	None	Long	Adult testis	Seminal vesicle, full of sperm

TABLE 8

Neuter specimens returned to hermit shells free from other Crepidulas, sectioned after sixty-seven days

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
244	23	11	None	Early growth period of oocytes	Not included in sections
245	29	20	None	Ova with yolk	Not included in sections

TABLE 9

Neuter Crepidulas transferred to neighborhood of large females; sectioned after seventy-five days

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
246	15½	2½	Long	Adult testis	Seminal vesicle, full of sperm
247	14	2½	Long	Adult testis	Seminal vesicle, full of sperm
248	10½	2	Long	Adult testis	Seminal vesicle, full of sperm

TABLE 10

Neuter specimen returned to hermit shells free from other Crepidulas; sectioned after seventy-five days[1]

SPECIMEN	LENGTH	GROWTH	PENIS	GONAD	GONIDUCT
249	26	11	None	Ova with yolk; (larvae in mantle cavity)	Not included in sections
250	19	5	Long	Adult testis	Seminal vesicle, full of sperm
251	26	15	None	Early growth period of oocytes	Not included in sections
252	25	11	None	Synaptic stages of oocytes	Transitional

[1] Specimen 250 was found in the same hermit shell with 249 and attached directly behind the latter. The male development in 250 was undoubtedly due to the proximity of the larger animal; 251 and 252 were each alone in a hermit shell.

from larger to smaller individuals. The stimulus may act upon animals of quite advanced size. Its effect is seen in a very short time. If male development is to go on uninterruptedly the stimulus must be constantly supplied. Whenever it is removed male development ceases and the male organs degenerate.

Individuals which are free from the male-producing stimulus grow much more rapidly than those which remain in the male phase; and the attainment of the large size is correlated with female development (tables 8 and 10). (For other factors influencing growth, see former paper.)

INFLUENCE OF LARGER ON SMALLER MALES

In the experiments so far recorded, only large female Crepidulas were used for the purpose of causing the male condition to appear or preserving it, in the smaller animals. It is important to know whether the quality of inciting and sustaining male development is limited to animals in the female phase. The results of experiment 4, table 6, indicated that larger Crepidulas, though not females, might influence smaller ones. The following tests were made to determine whether the presence of very large males would sustain the male condition in smaller specimens.

Experiment 5. (Carried on in float cars, Woods Hole.) The largest males which could be found (18 to 22 mm.) were removed from their colonies and transferred to hermit shells in which there were no other Crepidulas. Much smaller males were transferred to the same hermit shells and allowed to attach themselves near the larger. It was of course realized that the male organs of the large specimens would soon degenerate after being transferred but in the meantime their effect on the smaller males could be observed, and their effect while they were in the transition period. Several of the small males were fixed and sectioned from time to time, and the observations on them may be briefly tabulated as follows:

A. Fourteen days after transferring; 6 normal males, 4 more or less degenerate.

B. Fifteen days after transferring; 2 normal males, 2 degenerate.

C. Twenty-two days after transferring; 1 normal male, 4 more or less degenerate.

D. Thirty-one days after transferring; 2 normal males, 2 more or less degenerate, 2 with evidence of regeneration after degeneration.

E. Thirty-six days after transferring; 1 normal male, 2 degenerate.

F. Thirty-nine days after transferring; 1 normal male, 2 with evidence of regeneration of testis. A fourth specimen in this lot, not one with which the experiment was started, had secured an attachment to the hermit shell probably after being dislodged from another; it shows evidence of *recent* male development, undoubtedly caused by its new association with the large (formerly male) specimen on which it was found mounted.

G. Forty-eight days after transferring; 1 normal male, 1 male with evidence of regeneration of testis.

H. Seventy-four days after transferring; 1 normal male, 1 male with evidence of regeneration of testis.

I. Seventy-eight days after transferring; 5 normal males, 1 immature male.

Examination of the material from experiment 5 brought out several interesting things:

Fewer small males degenerated than in experiments where they were completely removed from larger animals; but more than when they were left near large females.

At the end of thirty-one days a hitherto unobserved phenomenon is discovered. Two specimens from D show very clearly that after having undergone degeneration *the testis has developed anew*. The accessory male organs do not yet show regeneration; the penis has disappeared leaving only a small stump and the seminal vesicle has the thick-walled condition assumed when the sperm are being absorbed (see former paper). There

are adult sperm in the vesicle. The testes of these specimens however, plainly exhibit great activity, having all stages of spermatogenesis to spermatocytes in one case, spermatids in the other. Similar conditions are seen in two specimens of F. By this time the large animals about which the smaller are clustered are no longer males. Sections through the gonads of several showed that there was no longer a testis, but no sign of femaleness had yet appeared. Eventually, the large specimens gradually assumed the female condition, growing rapidly as they did so, and the smaller individuals clustered about and upon them were then nearly all found to be in the active male condition (H and I). All the small males which had gone through a period of degeneration subsequently experienced regeneration and finally show no sign of the former cessation of activity. It is possible that some males have not degenerated at all, since every lot taken showed at least one individual with normal testis.

Summing up the facts gained in this experiment: 1) Large males will have an effect on the smaller ones similar to the effect of large females on males, but not so marked. 2) A large animal with a degenerate testis will also give a stimulus to a smaller individual. 3) An immature female, which was formerly a male, will likewise give a stimulus. 4) After the degeneration of the testis, a specimen may subsequently *regenerate it again*, if it receives a stimulus from a larger individual. 5) The largest individual in such an artificial colony as has been described never shows regeneration of the testis following degeneration; there is no indication that a smaller animal can affect a larger.

Some attempt has been made to find whether a male freshly removed from a normal colony will cause male development in a smaller neuter; the evidence is incomplete, but suggests that it may do so.

REVERSIBILITY OF FEMALE DIFFERENTIATION

Although an adult or nearly adult female can apparently not again assume the male condition, it is clearly shown that the partial development of the female organs may pause in its early

stages and give place to the male phase. Occasional instances found in nature indicate this, and in a few cases the superposition of male on female development has been accomplished experimentally. Two such cases are described in table 11.

TABLE 11

Superposition of male on female development. Specimen 313: collected as neuter; placed close to two considerably larger immature females for eight days. Specimen 314: collected as neuter; placed between two large females in fingerbowl for nineteen days

SPECIMEN	LENGTH	POSITION WHEN FOUND	GONAD	GONIDUCT
313	12	3 mm. distant from a 20 mm. specimen. Other large specimens also near	Various synaptic and early growth stages of oocytes around periphery of gonad; also, *rapid spermatogonial multiplication*	Proximal part becoming convoluted
314	13	Close to and between two large females in fingerbowl	Various synaptic stages of oocytes; but frequent division of primordial male cells	Proximal part slightly enlarged and convoluted

The above accounts for the occasional presence of oocytes in the male gonad of C. plana. It is due to spermatogenesis interrupting the first stages of female differentiation. In such an event the oocytes do not persist very long, but if male development continues, degenerate. Undoubtedly in C. plana the separation of the two sexual phases is an adaptation so fastened upon the species that sperm and eggs cannot develop under the same conditions in the gonad.

The attempt was made several times to cause nearly adult females to return to the male state. All results go to show that after the oocytes are well on in the growth period no more male development is possible. Careful examination of ovaries was made after the effort to incite spermatogenesis, in order to find whether there were at least any mitoses of primordial male cells; and not only were such mitoses absent, but primordial

male cells were also lacking. If they completely disappear, as seems to be the case, the inability of females to re-assume the male phase is explained.

NATURE OF THE STIMULUS

As soon as it became evident that there was actually a stimulus passing from the larger to the smaller individuals of C. plana, affecting the sexual development of the latter, experiments were undertaken to discover the nature of that stimulus. Unfortunately up to this time all experiments have given negative results. Yet since they have served to limit the field of inquiry and furnish additional proof of the presence of the unique influence, it is thought worth while to include them in this report. Other more precise tests are planned for the future.

Copulation

When it was first found that males would degenerate on being removed from the vicinity of the females it was suggested that the sex gland atrophied through loss of functional activity; but the development of male organs in neuter animals which had not previously the power of copulation disposed of this possibility.

Motility

Males are more motile than females (Conklin, '98) or than sexually inactive individuals. Is it possible that frequent movement of small animals in large colonies has anything to do with development of male organs? To this it may be answered that motility is not necessarily a property of males; a considerable number of males have been found whose shells indicated by the conformation to the underlying surface that they had not moved at all during nearly the whole period of their lives; and one neuter animal remained constantly in the same spot for several weeks while undergoing male development under experimental conditions.

Food

In view of the work which has from time to time purported to show that nutrition has an influence on sex, an experiment was performed to show whether a difference in amount of food material might be responsible for the difference of sexual development of small Crepidulas. The experiment is based on the assumption that the Crepidulas take the same sort of food as the hermit crab, and that they do in fact live on the fragments thrown free in the water about the hermit's crab's head as he tears up food with his chelae and jaws.

Experiment 8. Carried on in the laboratory aquarium, Woods Hole. Table 12. Small neuter individuals were marked by a notch in the front edge of the shell and transferred to hermit shells from which all but the large females had been removed. All the hydroids were first scraped from the hermit shells in order to remove all possible sources of food about the Crepidulas. The new colonies were then placed in a glass aquarium about 1 foot in diameter and the same in depth, into which ran sea water which had first been passed through a Berkefeld filter. After periods specified in the table, the animals were fixed and sectioned. The position in the colony with reference to the females at the end of the experiment is noted in the table. The hermit crabs though unfed remained active and apparently unaffected.

Table 13. Other specimens from the same lot as those used above were notched and transferred to hermit shells from which all but the large female Crepidulas had been removed, as had been done with the others, and the new colonies were put in a similar glass aquarium. Sea water which had not been filtered was allowed to run slowly into the jar, and the hermit crabs were fed every day with crushed spider crabs, small fish, etc., large quantities of which they devoured. Specimens from the colonies were fixed and sectioned at the same times as from the starved colonies.

It will be observed that the individuals which show least amount of male development or none (specimens 362, 363, 368) had settled themselves after attachment, at some distance from the females.

The foregoing indicates that male development is not a matter of food or the lack of it; for development takes place equally well whether the neuters have plenty of food or none at all, provided only they are near the larger animals.

A similar experiment, not tabulated here, in which males were used instead of neuters, indicated clearly that starving or feed-

TABLE 12

Neuters transferred to neighborhood of large females; starved. Specimen 357 to 362 fixed after fourteen days; 363 and 364 after eighteen days

SPECIMEN	LENGTH	POSITION WHEN FOUND	PENIS	GONAD	SEMINAL VESICLE
357	9	Close behind female	Long	Adult testis; very active	Small, full of sperm; apyrenes in distal end
358	8	Mounted on female's shell	Long	Very active young testis to almost adult sperm	Small; very few sperm, not quite adult
359	7½	Mounted on female's shell	Long	Young adult testis, very active	Small; very few sperm
360	7	Close behind female	Long	Young testis to spermatids; small; very active	Small; very few sperm
361	7	Close behind female	Long	Young testis to spermatids; small; active	Small; empty
362	7	10 mm. distant from female	Stub	Inactive	Undifferentiated
363	11	10 mm. distant from female	None	Inactive	Very small; slightly convoluted
364	9	Mounted on female's shell	Long	Adult testis; very active	Very large; full of sperm

ing would not cause degeneration of male organs if large females were near them in the hermit shell.

Stimulating secretion?

Several experiments have been made to determine whether the females or any of the large Crepidulas are constantly giving off a substance into the sea water, which may be taken up by the smaller individuals and passing to the sex glands, affect their development. We may already discount the possibility of an ovarian secretion; for it has been seen that the stimulus to male

TABLE 13

Neuters transferred to neighborhood of females; fed. Specimen 365 to 370 fixed after fourteen days; 371 and 372 after eighteen days

SPECIMEN	LENGTH	POSITION WHEN FOUND	PENIS	GONAD	SEMINAL VESICLE
365	10	Mounted on female's shell	Long	Testis to spermatids; very active	Small; few sperm in distal part, majority apyrene
366	10	3 mm. to one side of female	Half usual length	Young testis to spermatids; very active	Small; empty
367	9	Mounted on female's shell	Short	Young testis to spermatocytes of the 2nd order; very active	Small; just beginning to twist
368	9	10 mm. behind female	Stump	Very small; spermatogonia	Nearly straight tube; small
369	7	Mounted on female's shell	Short	Young testis to spermatids; very active	Very small; empty
370	7	Mounted on female's shell	Short	Young testis to spermatids; very active	Small; few sperm in distal part, majority apyrene
371	11	4 mm. distant from female	Short	Adult testis; small; not very active	Very small; few sperm
372	7	Mounted on female's shell	Stump	Spermatogonial multiplication	Small, thick wall, occasional sperm

development may come from an animal which is not yet an adult female, but has its gonad in a very rudimentary or inactive condition.

So far all experiments to discover the presence of a stimulating secretion have given only negative results. They will be described here. The writer proposes to institute more critical tests along the same line.

Experiment 9. A number of females were taken from colonies and placed in a small fingerbowl of sea water. Three males freshly removed from colonies were placed in another fingerbowl and the water in which the females were was poured into the fingerbowl containing the males. Every day the water standing on the males was poured out and replaced by water in which the females had been; the latter were each time covered with fresh sea water. After sixteen days the specimens which had been males were fixed and sectioned; all showed very advanced degeneration of the male organs.

Experiment 10. This experiment was repeated in a slightly different way as follows: A sufficient number of large females were taken from colonies to cover the entire inner surface of an 8-inch evaporating dish. A very small stream of water was allowed to run slowly into this dish from a salt water tap. Small glass tubes then led the water from the evaporating dish full of females to two fingerbowls, one of which contained small neuter animals and the other males. Care was taken to keep the small individuals in each fingerbowl separated from each other by some distance. This was left running for a month, and at frequent intervals the small animals in each fingerbowl were examined. At no time did any of the neuter animals begin to develop a penis. The penes of the males gradually atrophied and disappeared, and did not reappear. The numbers were considerable (20–25 specimens in each fingerbowl), and the result was so uniform that it was not considered worth while to fix and section the specimens. Now if a secretion were being given off in the water, one might suppose that this experiment would prove its existence; for each of the small individuals would receive secretion from many more females than is the case in the usual colonies. There were 20–25 large females in the evaporating dish, whereas few hermit shells contain more than two or three. The dishes were shallow and whatever substance was present would not be very much diluted, although it might not be in quite so concentrated form as it would be close to a female Crepidula in the recesses of a hermit's crab's shell. In so far as it went, the experiment failed to establish the fact of a stimulating secretion. It might be yet conceived that a secretion is given off by the females which is effective only at the moment of liberation, or in the nascent condition. This remains to be investigated.

Experiment 11. The bodies of females were ground in a mortar and the extract was added to a fingerbowl of sea water containing neuters. Change of sea water in the fingerbowl was made twice a day, extract of freshly killed female being added at each change. No sign of male development appeared in the neuters. The experiment of course lacks conclusiveness in one respect; the extract was made from the whole body of the large animal, because it is not known what part of the body may give rise to the hypothetical secretion; and it has been shown that for many secretions formed by animals there are also formed antagonistic or inhibitory substances (e.g., anti-fertilizin of F. R. Lillie).

In view of the negative results of the above mentioned experiments, the writer does not wish to do more than suggest the possibility of a chemical stimulus, until the point has been further investigated.

GENERAL CONSIDERATIONS

It must be understood that the peculiar condition which has been found in Crepidula plana, the dependence of the male phase on conditions of the environment, has been developed within the species. So far as the writer is aware no similar phenomenon has been described in any other Mollusc. Small specimens of the nearly related Crepidula convexa and Crepidula fornicata have been kept in aquaria by the writer, in the absence of larger specimens of the same species, under the same conditions as the segregated individuals of C. plana; yet the treatment did not interefere with the development and maintenance of the male phase. Many specimens were fixed and sectioned which had been found living in nature where no larger ones were in the vicinity; those of the 'male sizes' had fully developed, active testes. Apparently, then, the peculiarity of C. plana is a special and not a generic one. It is true, however, that there is great variation in the activity of spermatogenesis in C. convexa and C. fornicata. Occasionally specimens are found (not necessarily segregated) where the gonad is very nearly inactive. It would be interesting to learn whether the European and South American species of Crepidula show any sexual behavior similar to that of C. plana.

Outside the Molluscs there is one striking instance of sexual behavior somewhat similar to that which the writer has found. Baltzer (10) in investigating the development of the marine worm Bonellia viridis, has found that if the free-swimming larva attaches itself to a female of the same species, and develops there in a parasitic manner, it becomes a male; but that if it develops solitarily, it becomes a female.

The case of Bonellia is clearer than that of Crepidula plana, in that there is an actual contact between the animal which gives the stimulus to male development and the animal which receives

it; and in that a substance can be demonstrated passing from one to the other. There is not the least suggestion of 'parasitism' in the case of Crepidula plana.

An instance of external conditions modifying a male gonad toward the female condition, though not at all as in Crepidula plana, is seen in certain Crustacea. Potts ('06) and G. Smith ('10) have found in the cases of the hermit crab (Eupagurus) and the spider crab (Inachus) respectively, that the presence of parasites attached to the body causes a degeneration of the testis in the male, and the subsequent appearance of what the authors believe to be ova, in the gonad. Parasitic infection in the female does not result in a modification toward the male condition. Smith believes that the parasitic castration brings out a 'latent hermaphroditism' in the male but not in the female. The male would then be the heterogametic sex, the female homogametic. The secondary sex characters are also modified from the male toward the female condition in all degrees. On account of the loose correlation between the changes in the secondary sex characters and those in the gonad, Potts believes that the former are not directly consequent upon the latter, but that "both are attributable to some change in the general metabolism."

SUMMARY

In the protandric hermaphrodite Crepidula plana the development of the male phase is dependent upon the presence of a larger individual, not necessarily a female, of the same species. It is evident that some stimulus passes from the larger to the smaller individual. The greater the difference in size between the animal giving the stimulus and the animal receiving it, the more certain and complete is the male development of the smaller. A small stimulus will initiate male development, but a greater one is necessary to complete and maintain it.

When a male becomes removed from the neighborhood of the larger animal, the male organs degenerate, a condition of sexual inactivity ensues, later replaced by female development.

If a larval C. plana settles and grows during the first part of its life where no larger individuals are present, the male phase

probably never occurs; but if at any time up to the female stage the small individual comes within the sphere of influence of a larger one, it will immediately develop male organs, attaining the male condition in about two weeks. Whether or not the male phase is realized, the female phase is eventually developed.

The degeneration of the male organs does not prevent a second or third male development if the small Crepidula comes within the sphere of influence of a larger one after the degeneration. Partial degeneration may be halted and male activity resumed.

During the male phase the growth of the body is retarded; after degeneration of the testis and during the sexually inactive condition, or in neuter animals which have never developed the male condition, growth is rapid.

The first steps of female development may be interrupted and replaced by male development, under experimental conditions. In this case the oocytes degenerate and the activity of the primordial female cells is suspended; while the primordial male cells multiply and undergo maturation. After the oocytes are advanced in the growth period, male development is no longer possible.

The nature of the stimulus to male development in C. plana is at the present time not certainly known. The following statements may be made in regard to it:

1. The stimulus depends upon the presence of the actual body of a large Crepidula plana; for if the large specimens be removed, leaving all the other conditions of the colony unchanged (even the shells of the large specimens still in their former positions) the stimulus is no longer given.

2. The movement of the smaller individuals, from whatever cause, does not furnish the stimulus; for some males are developed while in a fixed position.

3. Male development does not depend upon the amount of food received; for starved neuter specimens develop a testis as quickly as well-fed ones, when in the presence of large females; and they do not develop any *more* quickly.

4. No experiment has so far demonstrated the existence of a stimulating secretion; this possibility has not been thoroughly tested.

5. The stimulus does not depend upon the presence of the hermit crab with which the Crepidulas are associated; it will have its effect even if the home of the colony is a fingerbowl.

Whatever the manner of working of this peculiar adaptation, there is no doubt of its great advantage to the species; for it provides that the male members of every colony of Crepidula plana shall quickly develop into females as soon as there is no longer a larger female which requires fertilization; and also that there shall be adult males in the colony ready to function as soon as any individual has reached the adult female phase.

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REACTIONS OF THE WHIP-TAIL SCORPION TO LIGHT

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FOUR FIGURES

CONTENTS

STATEMENT OF THE PROBLEM

During the past summer I was fortunate in having an opportunity to work with a number of specimens of the whip-tail scorpion (Mastigoproctus giganteus, Lucas). The fact that this Thelyphonid has median eyes which are well separated anatomically from the lateral eyes, made it appear a promising subject for experiments to determine the relative effectiveness of the two types of eyes.

Since there have been no extensive observations published concerning the effects of light on any of the Thelyphonids, the present paper has been devoted to presenting data on the reactions of normal animals. No attempt was made to treat exhaustively all phases of their behavior under the influence of light. The object was rather to obtain such reaction measurements as would best serve as a basis of comparison for subsequent work directed toward determining the relative effectiveness of the various parts of their photoreceptive mechanism.

GENERAL CHARACTERISTICS AND BEHAVIOR

Fabre has said of the Languedocian Scorpion, that he is an "uncommunicative insect, occult in his manners and unpleasant to deal with, so much so that his history, apart from the findings of anatomy, is reduced to little or nothing." Of the whip-tail scorpions this is even more true. The general structure of the group is well covered in an extensive paper on the Pedipalpi by Börner ('04). There is, however, little detailed information concerning the structure of the eyes. Parker's paper ('87) on the eyes of scorpions deals with a species so far removed from Mastigoproctus giganteus, that it would be unsafe to assume the eye structure was similar in the two cases; the anatomical position of the eyes is certainly quite different. As far as I was able to ascertain, there are in the literature only a few casual and fragmentary references to the behavior of this or closely allied forms. The limited distribution and our consequent unfamiliarity with the Thelyphonids, together with the very meagre information available as to their habits, makes desirable a brief description of certain points in their anatomy and general behavior.

The sketch reproduced in figure 1 shows sufficiently the general appearance of Mastigoproctus giganteus. Of the four pairs of legs, only the posterior three pairs are used in walking. The anterior legs are modified and serve as antenna-like feelers. They are long, slender, and flexible. Whenever the animal is moving or is about to move, the anterior legs are constantly waving about feeling out the path ahead. The feelers, as I shall call the anterior pair of legs, were found to be sensitive to touch, to heat, to chemicals, and to moisture; there was no indication that they were photosensitive. The delicacy of their responsiveness may be well demonstrated by breathing on them when they are at rest. Even this slight stimulus will send the feelers into restless activity. The scorpion is, moreover, able to follow up or to avoid stimuli received by the 'feelers.' I have seen an animal which was aimlessly wandering about the table chance to get the tip of one of its feelers into a watch

glass of water; it immediately swung about, thrust its other feeler into the water, then climbed half into the dish and began greedily to scoop water into its mouth with its chelicerae. Substituting a dish with very dilute hydrochloric acid for the water, a clear-cut avoidance reaction was obtained the instant the feelers came in contact with the acid.

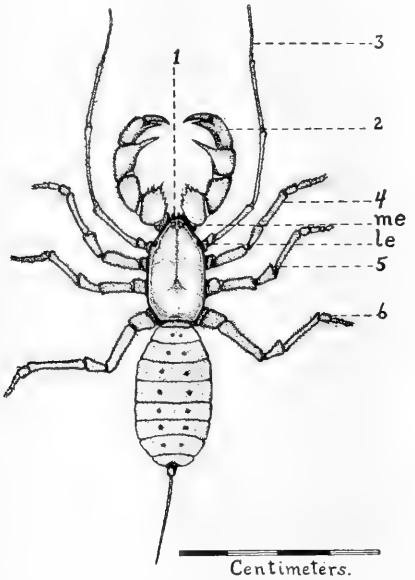

Fig. 1 Mastigoproctus giganteus Lucas: *1*, chelicerae; *2*, pedipalps; *3*, modified anterior legs which serve as feelers; *4*, *5*, *6*, walking legs; *me*, median eye; *le*, lateral eyes.

The big pedipalps are surprisingly powerful and can inflict a considerable flesh wound. A peculiar feature of the action of the pedipalps is their alternate striking and grappling movement which carries the attacked object forcibly toward the chelicerae. Of interest in this connection is the statement of McMurrich ('94, p. 446) that the chelicerae of the Thelyphonids bear the outlets of poison glands on their terminal spines. Börner ('04, p. 12) did not find any poison outlets in the chelicerae of the

Thelyphonids with which he worked, although he speaks of them as being present in others of the Pedipalpi.

Unlike the true scorpions, the Thelyphonids have an attenuated, many-jointed tail with little muscular development and no terminal sting. Although the tail vibrates spasmodically when the animal becomes excited, it appear to be in no wise a weapon of defense.

The position of the median and lateral eyes is shown in figure 1. The median eyes are black, bead-like elevations rising out of small depressions dorsally situated on either side of the mid-line at the anterior end of the head. The lateral eyes, of which there are three pairs, are located considerably more caudad on the dorsolateral margins of the cephalo-thorax. The three lateral eyes of each side are clustered, close together, in a minute triangle. When a small beam of light is focused on them, they reflect the light so that they appear like a single brilliant spot. There is no visible reflection of light when the median eyes are thus illuminated.

While these scorpions were very sensitive to light, at no time during the experiments did I observe any reactions which might be interpreted as visual responses. As often as animals were approached with forceps in handling them, they never made any movement which would indicate that they could see. A particularly striking failure to demonstrate a visual response was given by the following procedure. Animals were thrown into a state of alertness by scratching the table near them. An object could even then be waved back and forth directly in front of the eyes, between the opened jaws, without their making any attempt to strike at it. Pinch one of their spines, however, and they throw themselves entirely clear of the table in a frenzied attack on the forceps. Repeated many times and in many different ways, experiments with moving objects never elicited any activity. While these results, being entirely negative, are not conclusive as to complete lack of vision, there is no reason to doubt that vision in distinction from photosensitiveness, is, at least, very poorly developed.

APPARATUS

The apparatus used in these experiments needs little explanation other than the diagram of figure 2. At one end of a table 3 meters long, a circle 50 cm. in diameter was marked out and graduated in degrees. One axis (pp') was drawn through the

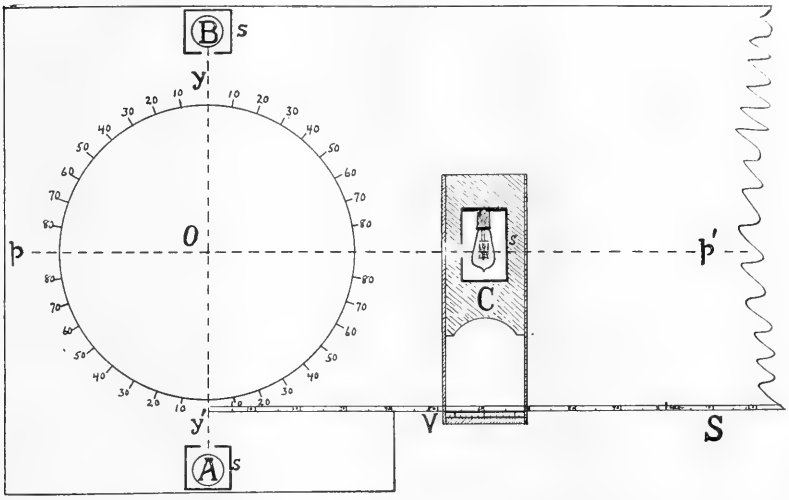

Fig. 2 Plan of apparatus. About O as a center a circle 50 cms. in diameter was described and graduated in degrees. This is termed the 'observation circle.' The axis pp' of the circle is parallel to the edge of the table and the axis yy' is perpendicular to pp'. On the axis yy' two 15 watt Mazda lamps, A and B, were set up, each distant 35 cms. from the center of the circle. On the axis pp' a similar lamp was mounted on a movable frame, C. Affixed at right angles to the base of the frame, C, was a scale, V, reading as a vernier against the scale, S, on the edge of the table. Light proof cases, s, s, s, enclosed each light except for diaphragms 3 cm. by 3 cm. so placed that the beams of light transmitted each centered at O.

center of the circle parallel to the edge of the table, and another axis (yy') at right angles to the first. Fixed lights were located at either end of the axis yy', equidistant (35 cm.) from the center of the circle. A movable light was arranged to slide along the axis pp'. The frame on which this movable light was carried had a scale comparable to a vernier which could be read against a scale on the edge of the table. Each of the three

lamps was enclosed in a case, light proof, except for a diaphragm 3 by 3 cm. The diaphragms were placed so that the central ray of the beam transmitted was directed toward the center of the observation circle. Determinations of the illumination delivered by the lights were made with standard candles and a Bunsen photometer. Each fixed light gave, at the center of the observation circle, an illumination of 120 candle meters. The intensity of the third light was varied by moving it to different positions along the axis. Records of its intensities are given in connection with the experiments in which it was used. The whole apparatus was located in a dark room and, in order to reduce reflected light to a minimum, all parts of it except the scales were painted flat black.

MEASUREMENTS OF REACTIONS TO LIGHT

Eighteen normal and active specimens were available for the experimental work. Identification numbers were painted on the back of each animal so the records of the same individual might be compared under various conditions. Nothing being known as to the nature of their reactions to light, preliminary qualitative observations were necessary. Each animal was therefore started from the center of the observation circle first heading directly toward the source of a single horizontal beam of light, second heading away from the source of light, and, third, heading across the beam of light. The animals without exception moved away from the source of light. Furthermore, the turnings although not always instantaneous, were always direct. There was nothing which could possibly be construed as a 'trial movement'. The turning of unexcited animals was a deliberate, coördinated rotation of the body about the thorax as a center. Turning was sometimes accompanied by locomotion but often orientation was nearly completed before the animal began to crawl. The center of rotation was between the middle pair of the legs functional in walking (i.e., the third pair of legs.) In turning, the anterior walking leg on the side toward which the animal was swinging, pulled, while its fellow on the opposite side pushed. The middle pair of walking legs

remained comparatively passive, while the posterior legs acted in a manner just the reverse of the anterior pair.

During the preliminary experiments various methods of handling the animals were tried. It was found that picking them up with forceps lowered vertically down upon the animals between the walking legs in such a way as to seize them across the thorax gave least disturbance. The matter of handling was of great importance because in animals over-stimulated mechanically the 'fighting reaction' obliterated all other responses. Particularly was this true of the males. The vibration of the tail, however, served as a warning of over-stimulation, so that with care it was possible to eliminate this complicating factor and obtain consistent reactions to light. The preliminary experiments served to demonstrate that Mastigoproctus giganteus is a typical negatively phototactic animal, and that quantitative experiments could be planned on that basis.

As has been already stated, the experiments on normal animals were designed primarily to serve as a basis of comparison for subsequent experiments directed toward the determination of the relative effectiveness of the various photoreceptors. To ascertain the rôle played by each part of the receptive mechanism, the method of progressive elimination appeared to be most practicable. Under this method three general types of interference with the photosensitive organs would be possible: unilateral, bilateral, and a combination of unilateral on one set of organs with bilateral on another. With this scheme of procedure in view, it was attempted to select for measurement the normal reactions which would be most readily, and most recognizably disturbed by the proposed elimination experiments.

We know that a negatively phototactic animal will turn and move away from the source of light, when placed heading across a horizontal beam, or when placed heading into the beam. These responses are initiated in the photoreceptive mechanism, and the amount of deflection, other factors being constant, depends on the sensitiveness of the mechanism. Consequently, if these two reactions are measured for normal animals, and then

for animals with some part of the receptive apparatus eliminated bilaterally, it should be possible to deduce with reasonable accuracy the part played in orientation by the eliminated photoreceptors.

We know also that a negative animal will continue to crawl away from a source of light when headed in that direction, and that when subjected to equal opposed lights, it will move along a norm to the line connecting the two sources. These reactions both result from the tendency of a negative animal to maintain stimulation in bilateral equilibrium. The accuracy of the reactions depends on the sensitiveness, and especially on the bilateral balance in effectiveness, of the receptors. By interfering with the photoceptors of one side only and comparing with the normal, the measurements of the unbalanced reactions thus induced, it should be possible to obtain additional evidence as to the relative importance of any particular part of the receptive mechanism.

We should, therefore, be in possession of the desired data on which to base further work, if, by the use of some reliable quantitative method (such as measuring the angular deflection from an original path of locomotion), we determined the normal reactions, (1) to a single horizontal beam of light of known intensity acting laterally on the animal; (2) to a horizontal light acting on the animal from in front; (3) to a horizontal light acting on the animal from behind; and (4) to equal opposed lights acting on the animal bilaterally.

The measurements first made, were obtained by subjecting animals to a single horizontal beam of light from the side. The illumination at the center of the observation circle was 120 candle meters. As a precaution against the possible distorting effect of unbalanced sensitiveness, the animals were placed at the center of the observation circle heading across the beam first one way, then the other, so that right and left sides were subjected alternately to the light. (For discussion of this method see Patten, B. M. '14, pp. 230–233.) Although no visual response had been indicated, the lights A and B (fig. 2) were each used for half of the measurements so that the animals should be crawl-

ing sometimes away from, and sometimes toward, the observer. Reactions were measured in degrees of deflection from the direction in which the animal was headed when subjected to the stimulus. Four animals were used in this and in each of the following series of measurements. Each animal was given ten trials. With the limited material at my disposal, it was not possible to use greater numbers of animals and cover the ground adequately. The forty reactions measured for each case, however, are sufficiently consistent to indicate that the results are essentially the same as would be obtained with a greater number of animals. The measurements are collected in table 1.

TABLE 1

Reactions of Mastigoproctus to lateral illumination of 120 candle meters. The measurements are recorded in degrees of deflection from an initial path of locomotion. In figure 3, B the same measurements are plotted graphically

TRIAL NUMBER	ANIMAL 9		ANIMAL 10		ANIMAL 11		ANIMAL 12	
	Deflection away from light	Deflection toward light	Deflection away from light	Deflection toward light	Deflection away from light	Deflection toward light	Deflection away from light	Deflection toward light
	degrees		*degrees*		*degrees*		*degrees*	
1	80		70		85		85	
2	70		80		60		85	
3	69		65		80		60	
4	70		50		60		70	
5	63		50		60		60	
6	52		75		50		65	
7	60		80		60		62	
8	65		60		40		80	
9	68		55		40		70	
10	80		60		70		70	
Totals....	677		645		605		707	
Averages.	67.7° away from light		64.5° away from light		60.5° away from light		70.7° away from light	

An inspection of the individual reaction measurements and the averages will give a clear idea of the consistency of the responses. There is not a single positive reaction among the forty trials, and only two negative reactions as low as 40 degrees. When it is considered that to attain, under these experimental condi-

tions, the theoretically perfect response of 90 degrees an animal must come immediately into orientation without progression, the average response of 65.8 degrees is seen to be remarkably accurate and immediate.

Table 2 shows the amount of deflection obtained by starting animals directly toward the source of light. Under these conditions the maximum response possible would be 180 degrees.

TABLE 2

Reactions of Mastigoproctus to anterior illumination of 120 candle meters. The measurements are recorded in degrees of deflection from an initial path of locomotion. In figure 3, D the same measurements are plotted graphically

TRIAL NUMBER	ANIMAL 15, NEGATIVE DEFLECTION	ANIMAL 16, NEGATIVE DEFLECTION	ANIMAL 17, NEGATIVE DEFLECTION	ANIMAL 18, NEGATIVE DEFLECTION
	degrees	*degrees*	*degrees*	*degrees*
1	160	160	170	165
2	145	108	110	152
3	120	135	150	150
4	121	161	95	156
5	120	145	98	150
6	153	150	110	125
7	102	155	100	140
8	110	125	150	110
9	150	140	153	140
10	130	90	165	148
Totals.....	1411	1369	1301	1536
Averages....	141.1	136.9	130.1	153.6

As was the case in the experiments with lateral illumination, the amount by which the experimental measurements under these conditions fall below a theoretically perfect response was due mostly to the progression of the animals while they were becoming oriented. It would be possible to devise a scheme of measurement based on the final orientation which would indicate responses apparently nearer the theoretical. For our purposes, such treatment would be disadvantageous, for it is the abruptness with which orientation is attained rather than the final accuracy of orientation, which shows the results of slight differences in the effectiveness of the receptive mechanism.

The measurements given in table 3 were made on the reactions of animals placed heading away from the source, in a horizontal beam of light of 120 candle meters intensity. They show perhaps more clearly than either of the preceding sets of measurements the accuracy of alignment with the rays of light, which we may expect to find in this form. The individual trials vary rather widely to right or left of the theoretical response,

TABLE 3

Reactions of Mastigoproctus to posterior illumination of 120 candle meters. The measurements are recorded in degrees of deflection from an initial path of locomotion. The same measurements are plotted graphically in figure 3, A

TRIAL NUMBER	ANIMAL 5		ANIMAL 6		ANIMAL 7		ANIMAL 8	
	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left
	degrees	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
1		25		40	10		20	
2		20	23			5	30	
3	10			35	20		60	
4		30	40			25	60	
5	0	0	0	0	40			25
6	10			43	10			60
7		30		43	30		20	
8		20	30			5	20	
9	5			60	40			30
10	25		30			30		30
Totals....	50	125	123	221	150	65	210	145
Averages.	7.5 to left		9.8 to left		8.5 to right		6.5 to right	

but the character of the reaction is never in doubt, and the average of a number of trials conforms very closely to the theoretical reaction.

The reactions which showed least variability were those obtained by subjecting the animals to bilateral stimulation by equal opposed rights. Table 4 gives the measurements in detail. That the variability of these reactions is less than that under any other conditions is, in all probability, due to the fact that in balanced bilateral illumination the shading of the photoreceptors by the animal's own body is reduced to a minimum.

TABLE 4

Reactions of Mastigoproctus to equal bilateral illumination. The opposed beams were each of 120 candle meters intensity. Measurements are recorded in degrees of deflection from an initial path of locomotion. In figure 3, C the same measurements are plotted graphically

TRIAL NUMBER	ANIMAL 6		ANIMAL 7		ANIMAL 8		ANIMAL 9	
	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right
	degrees	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
1	0	0		3		5		15
2		10		11	20			15
3	6			9	5			8
4	1		10			3		9
5	10			18		15	5	
6		8	0	0		7		7
7	15			10		15		11
8	10			5		10		7
9		5	5			5	5	
10		10		8		3		8
Totals....	42	33	15	64	25	63	10	80
Averages	0.9 to left		4.9 to right		3.8 to right		7.0 to right	

The reaction measurements given in tables 1 to 4 have been summarized graphically in figure 3. The circles represent the observation circle of the apparatus. The direction of the light rays is indicated by groups of arrows. At the center of the circles are heavy arrows indicating the initial position of the animal for each series of experiments. Each of the arrows indicating the path taken by the animals in response to stimulation, represents the average point of emergence and approximately the average path for ten trials of one individual.

The reaction measurements presented above, cover the determinations which it was planned to make on normal animals. The results of some of the preliminary experiments with partially blinded animals seem to have a place here inasmuch as they help to establish the reliability of the normal reaction measurements as a basis for future comparisons. Moreover, they are significant in indicating the correctness of our hypothesis that normal orientation depends on the maintenance of stimulation

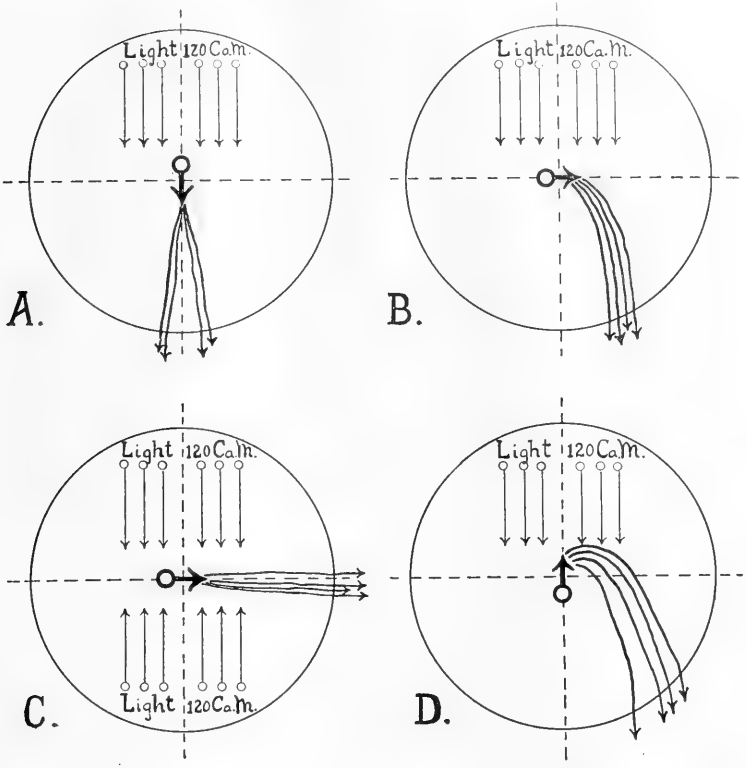

Fig. 3 Graphical representation of the amplitudes of the whip-tail scorpion's reactions under illumination from different directions. *A*, reactions to light from behind; *B*, reactions to light from the side; *C*, reactions to balanced opposed light; *D*, reactions to light from in front. The circles represent the observation circle of the apparatus, the groups of arrows the direction of the light, the central, heavy arrows the position which the animal occupied when subjected to stimulation. Each of the arrows indicating the path taken by the animals represents the average course and point of emergence for ten trials of one individual.

in bilateral equilibrium, and its corollary that unilateral interference with the photoreceptive mechanism will produce an unbalance in the normal reactions proportional to the extent of the interference. Since it is planned to extend these experiments considerably, only a brief summary of them and their bearing on the data of the present paper is given.

In a group of four animals, the left median eyes of two individuals, and the right median eyes of the remaining two individuals, were covered with an opaque cap sealed on with asphaltum varnish. These animals were then subjected to equal bilateral stimulation between opposed lights each delivering an intensity of 120 candle meters. The reaction measure-

TABLE 5

Reactions of Mastigoproctus with one median eye covered, to equal bilateral illumination. The opposed beams were each of 120 candle meters intensity. Compare these measurements with the normal reactions (table 4) to the same conditions of illumination

TRIAL NUMBER	ANIMAL 13—RIGHT MEDIAN EYE BLACK		ANIMAL 14—RIGHT MEDIAN EYE BLACK		ANIMAL 11—LEFT MEDIAN EYE BLACK		ANIMAL 12—LEFT MEDIAN EYE BLACK	
	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left
	degrees	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
1	10		18			25		15
2	8		20			30		10
3	9		17			20		10
4	20		12			30	5	
5	10		10			35		10
6		15	15			30		5
7	0		15			45		10
8	5		7			30		0
9	18		15			31		12
10	40		12			21		15
Totals....	120	15	141			297	5	87
Averages.	10.5 to right		14.1 to right		29.7 to left		8.2 to left	

ments given in table 5 show that this procedure caused a consistent deflection toward the side of the blackened eye.

Similar experiments in which the lateral eye groups were covered on one side of the head, caused an equally consistent and somewhat more extensive unbalancing of the normal reaction (table 6). In forty tests there appeared only one deflection (and that a very small one) toward the normal side. In all other cases the deflections were toward the side on which the eyes had been covered. A comparison of the measurements given in tables 5 and 6 with the measurements obtained from

TABLE 6

Reactions to Mastigoproctus with the lateral eye group on one side of the head covered. Animals were subjected to bilateral stimulation by equal opposed lights, each of 120 candle meters intensity. Compare these reactions with normal reactions (table 4) to the same conditions of illumination

TRIAL NUMBER	ANIMAL 6—RIGHT LATERAL EYES BLACK		ANIMAL 10—RIGHT LATERAL EYES BLACK		ANIMAL 8—LEFT LATERAL EYES BLACK		ANIMAL 9—LEFT LATERAL EYES BLACK	
	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left	Deflection to right	Deflection to left
	degrees	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*	*degrees*
1	30		35			22		20
2	50		22			32		0
3	28		20			28		40
4	38		45			30		30
5	40		25			62		30
6	50		0			33		20
7	46		22		5			10
8	20		50			42		20
9	22		10			38		0
10	40		30			45		20
Totals....	364		259		5	332		190
Averages.	36.4 to right		25.9 to right		32.7 to left		19.0 to left	

normal animals under identical experimental conditions (table 4) can leave no doubt as to the effectiveness of asymmetrical interference with the photoreceptors, in producing a correspondingly unbalanced reaction.

Before leaving the work with normal animals, it seemed advisable to test their reactions to a considerable range of intensities. Aside from the interest attaching to a determination of the threshold of sensitivity to light, there are special considerations in connection with the problems in hand which make desirable the securing of such data. In any opaque animal, owing to the shadow of the body, the illumination actually effective on the photoreceptors varies greatly from the photometrically determined illumination. Furthermore, in experiments which involve the elimination of part of the receptive mechanism, the total effective illumination would be greatly reduced as compared with that received by normal animals under the same experimental conditions. If we should happen to be dealing with an animal

in which the sign of the reaction to light was reversed by lowering the light intensity, it would introduce serious complications. Since such reversals are not uncommon (Frandsen, '01, p. 214; Hadley, '08, p. 180; Parker, '02, p. 119, etc.), the necessity of obtaining data covering this point is apparent.

Since the variations in effective intensity in the present experiments would all be decreases, animals were tested from the highest intensity used (120 candle meters), through a series of

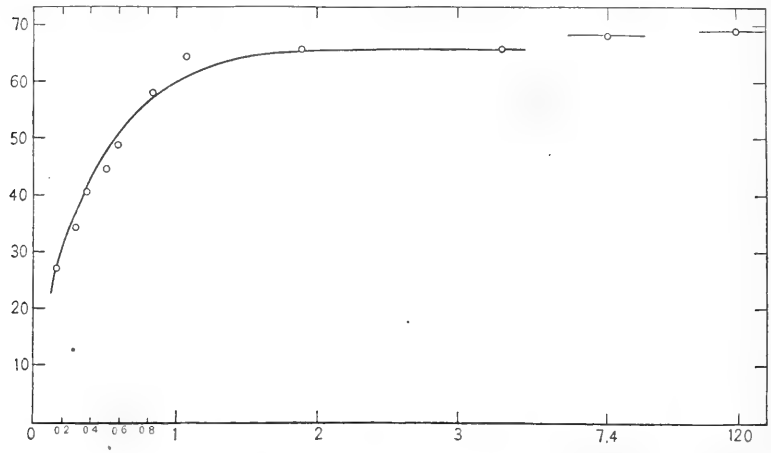

Fig. 4 Graph showing the increase in deflection exhibited by animal 18 under various intensities of lateral illumination. Each point represents the average of ten reactions. The smoothed curve has been added to bring out more clearly the general nature of the rise in reaction amplitude. The breaks in the graph were made to reduce the extent of the horizontal axis. There is no change in scale in the vertical axis.

diminishing intensities. For these experiments the movable light of the apparatus (*C*, fig. 2) was used. The curve plotted for animal number 18 (fig. 4) shows the nature of the results obtained. Each point was located by averaging ten reactions. Deflections induced by lateral illumination were plotted along the vertical axes, and along the horizontal axes were plotted the intensities of the illumination in candle meters. A smoothed curve was drawn in to show more clearly the general nature of the changes in reaction amplitude.

It was found impracticable to measure the reactions to light of less than 0.16 candle meters. Below this intensity, light did not have kinetic effect sufficient to induce locomotion of an extent justifying quantitative treatment. As far as extended locomotion is involved, we may say that the threshold intensity for the kinetic effect of light is about 0.16 candle meters. The fact that the responses were invariably negative to all intensities which induced locomotion, together with the fact that a reaction practically as great as the average reaction to an illumination of 120 candle meters, was induced by illumination as low as 1.18 candle meters, showed clearly that no complications due to the comparatively small changes of intensity met with under the experimental conditions need be anticipated.

DISCUSSION

The way in which the experimental work with normals animals was planned as a basis for future investigations concerning the photoreceptors of the same form has already been outlined. Further consideration of the data from this point of view may more profitably be taken up at a later time in connection with the proposed experiments. There are, however, certain phases of the reaction measurements presented above, which are of interest in regard to methods of orientation. Without unduly adding to the already lengthy discussions of this problem, attention may be called to some points that appear significant.

As to the precise nature, or even the general method of operation of the reflex mechanisms involved in orientation, there is considerable difference of opinion. The phenomena recorded and the animals studied are extremely varied. 'Blanket explanations' have so far led only to personal animosities. There is, as always, helpfulness in a clearly formulated, and frankly tentative, working hypothesis, but particularly is there need of careful work on specific problems, carried out from an unprejudiced point of view, and accompanied by non-controversial analysis. In the following consideration of the orientation of Mastigoproctus, I have earnestly endeavored to throw aside preconceived ideas and approach the problem with an open mind.

It is clear that no reaction such as orientation can result from a stimulus which does not first induce activity on the part of the organism. The kinetic energy liberated by stimulation must be regarded as the basis of any movement due to the influence of the stimulus, however highly modified or definitely restricted that movement may be. There is no reason to think that stimulation itself, or the conditions capable of producing stimulation, differ whether the motor responses initiated are tropic or simply kinetic. The conditions which determine the directive or non-directive character of the response are to be sought rather in the distribution and intensity of the stimulus actually effective on the receptors, and the existence within the organism of transmission paths which limit or direct the kinetic response.

Any analysis of the mechanism of orientation should, therefore, be based on a consideration of the kinetic response to stimulation. From this point only, is it possible to proceed logically to a consideration of the factors which so determine the direction of expression, and intensity of the activity set up by stimulation, that locomotion in a definite direction is established.

Mastigoproctus, at least while confined in the laboratory, exhibits a strong tendency to remain quiet unless stimulated in some way. Illumination above 0.16 candle meters intensity acts as a stimulus inducing locomotion. If animals which are at rest are subjected to illumination from above they begin to move about aimlessly. Activity does not cease immediately after the change in illumination necessarily involved in starting the experiment. On the contrary locomotion persists for long periods of time.[1] When the light is shut off, locomotion ceases. During this procedure the animal being uniformly illuminated from above, there are no appreciable changes in the intensity of the light operative on the photoreceptors. The conclusion is clear,

[1] In many cases already recorded acclimatization to steady illumination is known to result, after varying periods of time, in the cessation of stimulation. The scorpion is no exception. Cessation of activity due to acclimatization bears in no way on the point at issue. The process is one involving so long a time that it can play no appreciable part in an orientation which is immediate.

increasing the illumination above the threshold of sensitiveness produces a kinetic reaction; the initial reaction may be attributed to change of intensity, but the persistence of the reaction is due to the stimulating effect of light of constant intensity.

The experiments made to determine the threshold of sensitivity, brought out another point with regard to the kinetic effect of light. Near the threshold intensity the locomotion induced by light appeared only after a latent period, and even after it began, was very slow. Increasing the intensity resulted in decreasing the latent period and accelerating locomotion. These results are in line with those obtained on other arthropods by Yerkes ('00), Carpenter ('05), and Hadley ('08). We may conclude that the degree of the kinetic response to light is correlated with the intensity of the light.

As a basis for considering the directive reactions of the whip-tail scorpion we have, then, the facts (1) that light induces locomotion; (2) that light operates as a stimulus causing locomotion both through change of intensity and constant intensity; and (3) that the degree of kinetic energy exhibited is correlated with the intensity of the illumination.

Most of the qualitative observations concerning the orientation of Mastigoproctus to horizontal light are so closely in line with results already familiar for many negative animals that they may be dismissed with enumeration.

1. If light of an intensity which induced undirected activity when applied from above, was allowed to act horizontally, it induced orientation.

2. The response to a horizontal light applied from behind the animal was locomotion in the direction of the rays of the light. Individually the reactions varied to right or left of the central ray of the beam; collectively they followed the central ray ccurat ely.

3. Response to light from the side was a deflection away from the light approaching 90 degrees as a maximum.

4. Response to light from in front was a deflection approaching 180 degrees as a maximum.

These reactions serve to establish clearly the sign and the general nature of the reactions of the whip-tail scorpion to light, but they do not serve to give us any information concerning the mechanism of orientation not already available from the recorded reactions of other forms.

The responses of normal and partially blinded animals to balanced opposed lights, however, bring out certain points of interest which are not so apparent in experiments with a single beam of light. The response of normal animals under equal bilateral illumination was locomotion in a direction at right angles to a line connecting the two sources of light. In these reactions there appeared no semblance of anything which could be interpreted as a 'trial movement.' There were no side to side swingings which would produce a rapid sequence of changes of intensity on the photoreceptors. The animals moved between balanced lights with more directness and precision than in any of the other experiments. Under such conditions the distribution of the stimulus on the photoreceptors is bilaterally symmetrical, and the effective intensity is relatively constant. Is the symmetrical distribution of the stimulus merely incidental to an orientation governed by other factors, or is it a critical factor in the maintenance of orientation? The question may best be answered by interfering with the symmetrical stimulation and ascertaining the effect on orientation. In table 5 are given reaction measurements obtained by subjecting animals in which one median eye had been covered, to equal bilateral illumination. If symmetrical stimulation is incidental, we should expect to find no change from the normal reactions to the same conditions of illumination. A comparison of the reactions recorded in table 5 with the normal reactions (table 4) shows that blinding one median eye produced consistently a deflection toward the blackened eye. Table 6 records a similar series of measurements made on animals in which one of the lateral eye groups had been covered. The results are in complete agreement with those obtained by covering one median eye. Furthermore it was demonstrated experimentally that a similar un-

balance of reaction appeared when asymmetrically sensitive[2] animals were subjected to a single horizontal beam of light. The well marked and consistent deflection toward the side on which photoreceptors were eliminated indicates that bilateral balance of stimulation must be regarded, in this form, not as incidental, but as a critical factor in orientation.

If it be granted that the bilateral distribution of stimulation is one of the determining factors in orientation, there still remains the question as to whether light of constant intensity operates as a stimulus, or whether light stimulates only when there are changes of intensity. As far as I am aware, no one questions the conception that a change of light intensity acts as a stimulus. It would seem to be equally clear that where changes of intensity occur the resultant stimulation plays an important part in orientation. The point at issue is whether light operates as a stimulus effective in the attainment and maintenance of orientation only through changes of intensity or both through changes of intensity and constant intensity. From a purely theoretical standpoint it is possible to account for the attainment of orientation on either basis, but we must also take into consideration the maintenance of orientation once it is established.

Let us attempt to account for Mastigoproctus' maintenance of orientation to equal opposed lights on the assumption that light acts as a stimulus only through change of intensity, and that orientation is brought about by a series of changes of intensity which cease to occur when orientation is attained. There are two phases of continued locomotion in a definite direction, persistence of course, and persistence of locomotor activity. Maintenance of orientation would, in this case, have to depend on the combined operation of (1) a tendency to maintain any established direction of locomotion, and (2) the check-

[2] An interesting case in this connection, was that of an animal in which the lateral eye group on one side of the head had failed to develop. This animal exhibited an asymmetry of sensitiveness and of reaction which corresponded qualitatively and quantitatively with that artificially produced by covering one lateral eye group in a normal animal.

ing of movements out of alignment by the operation again of change of intensity. Certain data obtained in other experiments than those under balanced illumination bear on these points. The fact that when animals are moving under the stimulation of light from above the locomotion is aimless, and characterized by frequent changes of direction, would seem to indicate that the tendency to continue locomotion in a given direction is a factor which is so easily overcome that it is not sufficient to account for extended locomotion in a given direction. The same conclusion is strengthened by the haphazard, and non-persistent locomotion exhibited by totally blinded scorpions when induced to move by mechanical stimulation. While the tendency to continue locomotion in a definite direction is not sufficient of itself to account for an accurately maintained orientation, it might be contended that any radical change in the direction of crawling would produce changes of intensity which would throw the animal back into orientation. In the reactions of Mastigoproctus under balanced illumination no wide or sudden variations of direction occurred which would produce the necessary changes of intensity. Inasmuch as the frequent changes in direction characteristic of the animals' locomotion under non-directive stimulation do not appear under bilaterally balanced illumination, it is only reasonable to conclude that there is a factor present which over-rides any 'spontaneous' tendency to change direction, or any minor stimuli encountered in locomotion. The very fact that variations in direction do not occur, precludes the operation of changes of intensity as the factor effective in the maintenance of orientation. On the other hand it is quite logical to attribute the absence of variations from the established direction of locomotion to the directive effect of light of constant intensity.

The unbalanced reactions induced by blackening one eye are also difficult to explain if we deny the effectiveness of constant intensity as a stimulus. Neither right nor left photorective systems are subjected to a series of intensity changes when asymmetrically blinded animals are subjected to balanced illumination. It is hardly possible to explain the curved path

of locomotion exhibited under such conditions on the basis of the single sudden change of intensity produced on both sides of the head, in starting the experiment.

When we attempt to account for the persistence of locomotion on the assumption that light of constant intensity does not operate as a stimulus, we are confronted by other difficulties. It has already been demonstrated that light of constant intensity acting vertically stimulates to kinetic activity. It would be weird physiology to assume that when light of the same intensity acts horizontally, inducing a directive reaction, constant intensity no longer stimulates. Moreover it has been shown (1) that locomotion ceases in the absence of stimulation, (2) that in the reactions to equal opposed lights, once orientation is attained, it is so accurately maintained that no considerable changes of intensity on the photoreceptors are encountered, and (3) that locomotion under such conditions is indefinitely persistent. In view of these facts it is clear that the assumption that changes of intensity are the only effective photic stimuli is not in accord with the facts established concerning the reactions of Mastigoproctus. The conception that light acts as a stimulus both through changes of intensity and constant intensity is in accord with what is known concerning the general physiology of stimulation by light, and with the specific reactions here described.

The conclusions we would seem to be justified in drawing with regard to the orientation of Mastigoproctus may be briefly summarized as follows. The bilaterally symmetrical distribution of the stimulus on the photoreceptors which in all cases exists when the animals are in orientation cannot be regarded as incidental to an alignment of the body dependent on other factors. The behavior of the animals under all the conditions of illumination worked with, is in accord with the hypotheses (1) that the animals so move that stimulation is brought into bilateral equilibrium and there maintained; (2) that the muscular responses which bring about the bilateral balance of stimulation result from a proportional transmission (over paths at present unknown) of the impulses initiated in the receptors to definite

muscle groups; and (3) that light may operate as a stimulus effective in bringing about kinetic and tropic responses, both through changes of intensity and constant intensity.

I wish to express my indebtedness to my father, Dr. Wm. Patten, for procuring the specimens of Mastigoproctus on which this work was done, and to Prof. W. M. Wheeler who identified the species for me. The greater part of the work was done at Woods Hole. It is a pleasure to express my appreciation of the courtesies and facilities extended to me by the Marine Biological Laboratory.

SUMMARY

The photic reactions of whip-tail scorpions were studied with a view to establishing quantitatively, certain characteristic responses. No attempt was made to treat exhaustively all phases of their behavior under the influence of light. The object was rather to obtain such reaction measurements as would best serve as a basis of comparison for subsequent work directed toward determining the relative effectiveness of the various parts of their complex photoreceptive mechanism.

Reactions to photic stimuli of known intensities were recorded in terms of the induced angular deflections from an initial direction of locomotion. The results obtained may be summarized as follows:

1. The threshold for the kinetic effect of light was at about 0.16 candle meters.

2. The response was clearly negative to all directive illumination which induced locomotion. Up to an intensity of 1 candle meter the amplitude of the reactions increased rapidly. In the intensities above 1 candle meter the increase in deflection was much more gradual.

3. When started heading away from the source, in a horizontal beam of light of 120 candle meters, animals continued to move along the path of the rays. In 40 trials the average was within 0.6 of a degree of the central ray of the beam.

4. When subjected to light of 120 candle meters acting on them from the side, the scorpions turned and moved away from the light. The average deflection was 65.8 degrees.

5. When subjected to balanced, opposed lights each delivering an illumination of 120 candle meters, the average trail was within 3.7 degrees of the norm to the line connecting the sources of light.

6. When started directly toward a light giving an illumination of 120 candle meters, the scorpions turned and moved away from the source. The average deflection was 140.4 degrees.

7. Unilateral elimination of any part of the photoreceptive mechanism caused an unbalancing of subsequent reactions.

8. Light operates as a stimulus inducing kinetic and directive responses both through changes of intensity and constant intensity.

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THE SPLEEN DURING HIBERNATION

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FOUR FIGURES

In a study of the ductless glands during hibernation the other organs were observed routinely.[1] The most striking changes noted in the hibernating animal occurred in the spleen.

Polimanti[2] in his recent monograph on hibernation mentions the spleen only briefly and there are few references in his comprehensive bibliography to observations on the spleen during hibernation. It was thought that a note recording our observations might be of value.

These observations were carried out on spermophiles (S. tridecemlineatus). The data in regard to length of time torpid, temperature and other important factors of the hibernating animal have been given in detail in another paper[1] and will not be repeated here. Suffice to state that spleens from 30 hibernating animals and a corresponding number from active animals were studied. Of the active animals 12 were killed after varying lengths of time of activity after awakening. This number does not include the animals used in the special experiments reported in this article. The time of hibernation varied from twelve hours to one hundred and seventy-five days. Specimens of the active animals were obtained at various times throughout the year.

Unless otherwise stated, the active animals were killed by bleeding under light anesthesia. The torpid animals were bled without anesthesia. The specimens were fixed in several fluids, those most used being formalin, neutral formalin, Zenker and Zenker acetic. The sections were stained with hemotoxylin-

[1] Mann, F. C. The ductless glands and hibernation. Am. Jour. Physiol., 1916, vol. 41, pp. 173-188.

[2] Polimanti, O. Il letargo. Rome, Bardi, 1913, 683 pp.

eosin, Mallory's connective-tissue stain and a few other special stains such as Scharlach R.

The structure of the spleen of the spermophile does not differ essentially from that of the spleen of other rodents. The splenic nodules are prominent and the sinuses rather large. The most notable fact in regard to the histology of the organ is the relatively thick trabeculas containing a large amount of smooth muscle. In the gland of the active animal usually only a small amount of blood is found.

Within twelve hours after an animal becomes torpid the spleen presents a very characteristic appearance. Grossly and microscopically the organ is markedly congested. It is greatly enlarged and much darker in color than normal. The capsule is tense and the tissue friable. On section an increased amount of dark blood escapes. Owing to the large amount of blood present it is impossible to recognize any of the finer details of the organ grossly. Microscopically the organ presents a most intense congestion. The sinuses and venous capillaries are distended to their fullest extent with blood. In some organs red corpuscles were found in the germ centers. It appeared as if the congestion was so great as to force the cells into these centers.

The hibernating spleen reaches its maximum state of congestion within a few days after the animal becomes torpid and maintains this condition until after about forty days of hibernation. After the animal has been torpid for seventy-five days the amount of blood contained in the spleen is not greatly in excess of that found in the organ of the active animals. The blood seems to begin to decrease at the periphery of the organ first. This is probably due to the fact that the effect of the contraction of the intrinsic muscles is first exerted on the surface of the organ.

Besides the marked congestion there seems to be very little other change in the spleen. In some animals which had been torpid for many days there seemed to be a slight proliferation of the connective tissue, especially around the splenic nodules. Specimens of the spleens of both active and torpid animals were

fixed in formalin and the section stained with Scharlach R for fat. Rarely were fat droplets found in any of the cells of the organs of active animals. In several of the spleens of torpid animals, certain cells were found containing fat droplets. These cells, usually few in number, were large lymph cells which were always found in the germ centers. We have never observed fat-containing cells in any other part of the spleen.

In the spleen of the active spermophile there can always be found a considerable number of phagocytic endothelial cells containing red blood corpuscles or blood pigment. In the congested spleen of the animal which has been torpid for only a short length of time there seems to be a marked decrease in these cells. Owing to the marked congestion it may be that this is only a relative decrease or the cells are masked by the large amount of blood present. However, in some of the organs of animals which had been torpid less than forty days we were not able to find any of these cells. They reappeared in the animals which had hibernated longer and were still more numerous in the active animals which were killed within a short period after awakening. However, the largest number have always been found in the active animals.

Some of the torpid animals were killed without bleeding. The congestion of the spleen did not seem to be greater in these animals than in those which were bled.

Some experimental procedures were employed in the attempt to reproduce in the active animal the picture of the spleen found in the torpid animal. Some animals were killed with ether and about fifteen minutes after death the spleen was carefully removed and fixed. Other spermophiles were asphyxiated either in a closed jar or with illuminating gas. Some animals killed by the latter method were asleep. In some experiments the venous outflow was impeded for a short period before death. While most of the spleens in animals subjected to these experimental procedures showed congestion, it was impossible to attain completely the intense congestion noted in the organ of the animal which had been torpid for only a short period of time.

The adult spermophile usually withstands operation very well but removal of the spleen proved to be an exception. Most of the animals died within a few weeks after splenectomy, but a few lived for several months. Careful observation showed that these splenectomized animals hibernated under the same conditions as the controls.

The intense congestion of the hibernating spleen is probably due to a loss of tone and relaxation of the intrinsic muscles of both the spleen and the blood vessels. A consideration of the part this plays in the phenomena of hibernation must be purely speculative. It is possible that the spleen acts as a store room for the red blood cells in the early stages of hibernation and allows them to be added to the circulation as needed. The fact that the corpuscles in the congested sinuses appear perfectly normal and that phagocytosis of the red cells seems to be decreased and not increased as one would anticipate, strengthens this idea. However, the fact that splenectomized animals hibernate normally shows that this is only a secondary factor.

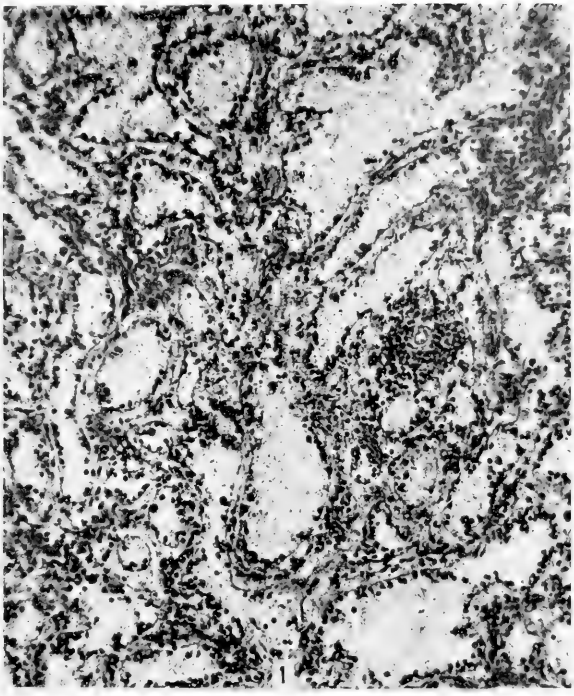
PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Photomicrograph of spleen of Spermophile 260, a female captured in the spring of 1915. It had been torpid continuously for fifteen days, although it had hibernated for a short time also earlier in the season. Did not have access to food. Killed January 1, 1916, by bleeding. At this time it weighed 85 grams and the rectal temperature was 12°C. The photomicrograph shows the marked distention of the venous sinuses with blood. × 100.

2 Photomicrograph of same section as that shown in figure 1. × 280.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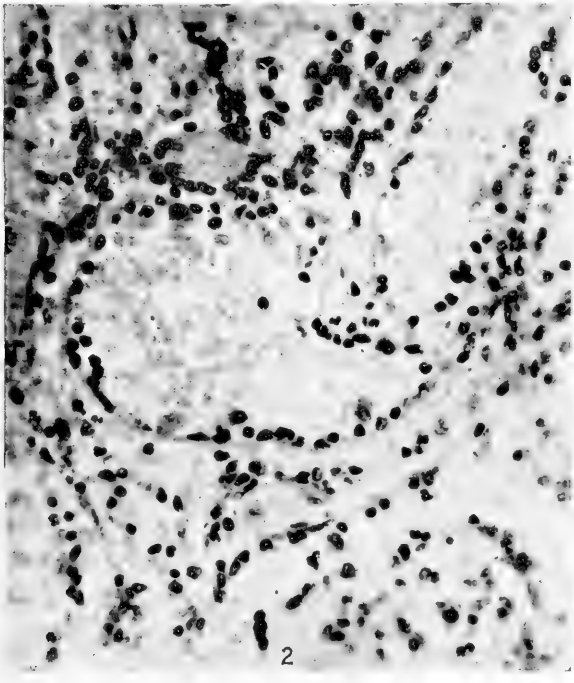
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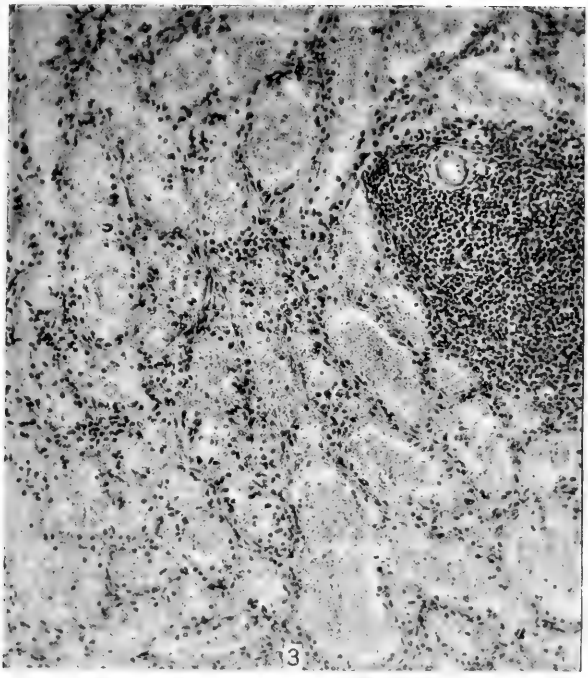
PLATE 2

EXPLANATION OF FIGURES

3 Photomicrograph of spleen of Spermophile 117, a male captured in the spring of 1915. It had been torpid for thirty-five days. Had access to food. Killed January 10, 1916, by bleeding. At this time it weighed 165 grams and the rectal temperature was 14°C.

4 Photomicrograph of spleen of Spermophile 266, a female captured in June, 1914. It hibernated when kept in the cold during the winter of 1914–1915. Placed in hibernating room and food withdrawn the latter part of September, 1915. Killed January 27, 1916, after hibernating about one hundred and twenty-five days. Rectal temperature at time of death 17°C. Daily observations were not made. The spleen is practically normal.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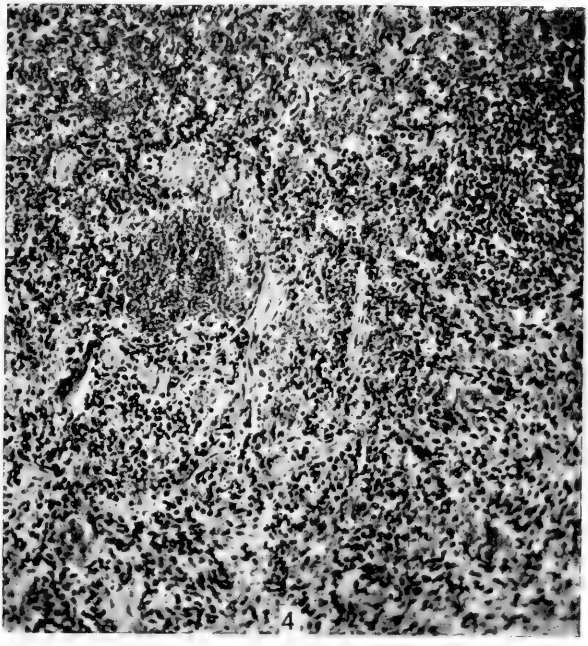
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STUDIES ON A RACE OF PARAMOECIUM POSSESSING EXTRA CONTRACTILE VACUOLES

I. AN ACCOUNT OF THE MORPHOLOGY, PHYSIOLOGY, GENETICS AND CYTOLOGY OF THIS NEW RACE

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THREE PLATES AND TWELVE CHARTS

CONTENTS

I. INTRODUCTION

While examining a number of paramoecia taken from a laboratory culture in the early part of January, 1915, one individual was seen to have three instead of the usual two contractile vacuoles. Subsequent examination of the stock from the original culture showed that a large percentage of the animals possessed the extra organs. Several animals with three vacuoles were therefore isolated and pure lines started from them. It soon became evident that the potentiality for one supernumerary vacuole was not only inherited but that the offspring might possess even more than one extra contractile organ. A preliminary account of the behavior of these organs in heredity was published some months ago (2).

The discovery of such a marked variety of a form which has served as a basis for so much valuable study opens an interesting field for investigation. Parts of this report must necessarily be of the most preliminary sort and various portions will be completed from time to time and published separately. The finding of the new race of paramoecium affords opportunity for comparison with the common race and for a study of the physiological activities of the two varieties. The following points will be considered more or less completely in the following paper:

1. The extra vacuole gives a definite character in the Protozoa whose inheritance can be traced. The existence of two races differing as far as is known only in this one character gives an opportunity of crossing the two races by conjugation and of determining whether inheritance in protozoa is Mendelian as it is in the metazoans.

2. A comparison of the two races may be helpful in the analysis of the function of the contractile vacuole.

3. Some light may be thrown on the process of conjugation through a study of the behavior of both races.

II. TECHNIQUE

A separate pipette was used for each culture to guard against cross contamination and was hung in a special holder attached

to the jar when not in use (3). In following the offspring of a single individual for several generations each of the daughter cells was isolated in a watch glass and as fast as the individuals divided a record was made of the number of vacuoles in each and they were again separated. The syracuse watch glasses that were used were sterilized in boiling water to which a little clean paraffin had been added. This deposited an invisible coating on the glass which was, however, sufficient to prevent the drop of hay infusion from spreading over the surface as it would on perfectly clean glass. On the paraffined surface the fluid rounds up and presents the minimum surface for evaporation (3). It was found that the most satisfactory pipette for this work had a short tip of almost hairlike fineness. The method of making these has been described (3).

It is rather difficult to find a method of slowing down the animals for the purpose of making accurate observations without killing them. The first method used was to place an individual in a small drop of water and over this a cover glass was lowered with the aid of a fine pair of forceps. The cover had a drop of hardened balsam at two corners which had been filed down to the proper thickness and which prevented it from crushing the animal. The excess fluid was drawn off with a piece of filter paper. To recover the animal the slide was tilted over a watch glass and by means of a stream of hay infusion the cover and whatever was under it were flushed into the crystal. This method was very laborious and slow, and the chances of losing the paramoecium great. The following method has proved to be the better: An individual is placed on a slide in a fairly large drop of the medium. The liquid is drawn off with a pipette under the dissecting microscope, the animal being kept as nearly as possible in the center of the drop. Finally, enough of the fluid is removed so that the adhesion of the surface film to the slide exerts sufficient pull to hold the animal quiet. In the center of the drop the pressure is least and it seldom causes a paramoecium to burst. Toward the edges, however, the chances are very great that the animal will succumb almost instantly to the greater pressure. In such a preparation the

pressure at the center tends automatically to lessen as the surface tension slowly draws the liquid from its close adhesion to the slide and rounds it up. This takes a sufficient length of time, however, to allow accurate observations to be made under the compound microscope. Occasionally the pressure is relieved too quickly and the paramoecium is given enough fluid to move about in, which makes observation with the high power impossible and the processs has to be repeated. As soon as the examination has been made, a drop of fresh hay infusion is added. To drop this liquid immediately on top of the animal after it has been under pressure may frequently crush it and the safer way is to allow the fresh fluid to flow from the side into the drop containing the animal. The paramoecium may then be picked up in a pipette and placed in its respective watch glass. When examining paramoecia in pure lines a separate pipette is used for each line.

Under the 16 mm. lens when the animal is compressed, the vacuoles stand out with diagrammatic clearness appearing as so many holes punched through the cell and as a rule are to be found close to one side and almost invariably lying in a straight line. This latter characteristic was one of the criteria for differentiating between the contractile vacuoles and food vacuoles when these were so numerous as partly to obscure the former. The comparative lack of refraction of the pulsating organs was another basis of distinction. When, however, the refraction of the food vacuoles was almost identical with that of the contractile vacuoles under the low power, the 4 mm. lens was swung into place and the contraction of the supposed contractile vacuoles was watched for. It has been observed that the vacuoles in animals raised in watch glasses are more difficult to see than they are in animals living in larger amounts of medium. In the watch glasses the food vacuoles have a lack of refraction which gives them an appearance very much like the contractile vacuoles whereas the food vacuoles in animals raised in the large culture jars frequently appear very nearly black.

Cytological technique. For measuring and drawing the whole paramoecium Worcester's fluid was used (10 per cent formalin

saturated with mercuric chloride). Animals fixed in this way can be immediately cleared in glycerine which brings out many of the internal parts very well, and the vacuoles, if they happen to be expanded at the time of fixation, are visible. For whole mounts and sectioning, paramoecia were fixed in Schaudinn's, Gilson's, Flemming's and Worcester's solutions. As comparatively little time has been devoted to cytological studies I am not prepared at present to offer any criticism of the fixatives.

To Dr. M. H. Jacobs I am indebted for his constant interest, advice and criticism during the progress of the work recorded here and I also feel under great obligation to the other members of the Zoological Department of the University of Pennsylvania whose many kind suggestions have been of great assistance to me.

III. MORPHOLOGY

a. General description

In general form, the animals of this race are apparently identical with the common slipper-shaped Paramoecium caudatum. The newly discovered individuals average rather larger than any of the representatives of the two-vacuoled race I have found about Philadelphia. Chart 1 shows a curve plotted to illustrate the range in size. The peak of the curve is at 233 μ and is very nearly midway between the two extremes. On this same chart are plotted the ends of two curves formed by animals taken about Philadelphia. The range of one race is from 153 μ to 207 μ and in the other from 106 μ to 173 μ. In the collection of slides belonging to the Department I have found paramoecia somewhat larger than the new race ranging from 197 μ to 325 μ. The extremes of all the races studied by Jennings were 50 μ and 332 μ.

In certain cultures where the individuals were unusually favorable for study a band slightly darker than the surrounding protoplasm could be seen across the center of the animal in the region where the constriction appears at the time of division (figs. 4 and 6). It is interesting to note that in these cultures

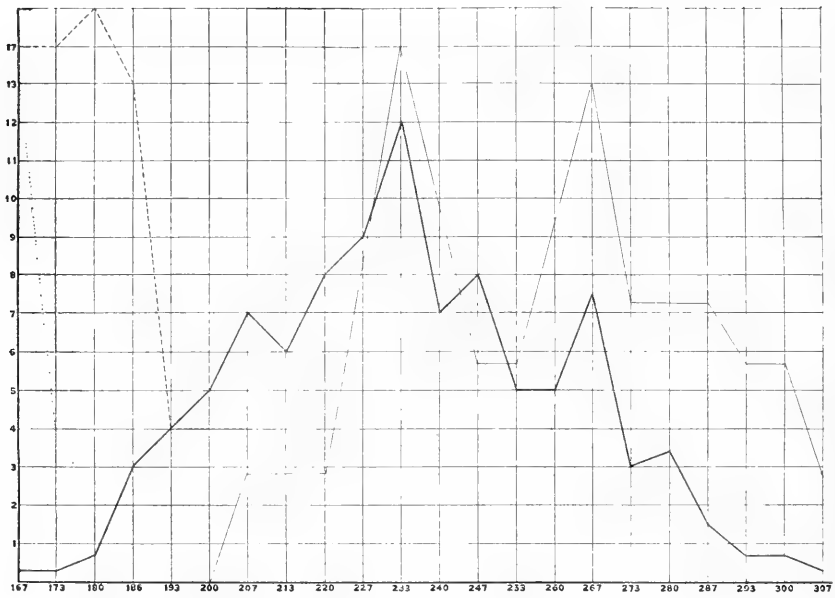


Chart 1 On the axis of abscissas are marked the various sizes in μ while on the axis of ordinates are placed the percentages. The heavy solid line represents the multivacuoled race of paramoecium while the light broken line indicates the larger sizes of a small two-vacuoled race and the light unbroken line is based on figures obtained from fixed preparations of two-vacuoled animals in the Laboratory collection.

the percentage of animals with three and four vacuoles was very high. The consideration of this phenomenon will be left for future work.

b. The vacuoles

1. Location. So exactly are the contractile vacuoles located that a single straight line parallel to the outline of the animal might be drawn passing through the center of each. In a very few cases I have found one or two vacuoles slightly displaced from the usual straight row condition (fig. 9). In the majority of individuals the extra vacuoles are located in the posterior part of the animals as can be seen from the drawings. In a few

instances, however, one extra vacuole was found in the anterior end (fig. 3), but I have never found more than two vacuoles located in the foremost half of the body. It is a curious fact that, rare as this condition is in the normal medium of hay infusion, in cultures to which a little sea salt has been added paramoecia with two vacuoles in the anterior end have been found to be much more numerous. As yet no plausible explanation for the increase of this rare arrangement has been found—if, indeed, it is not merely a coincidence.

There can be no doubt that the contractile vacuoles are very definitely located organs and fixed in position for a given individual. When an animal is swimming the vacuoles can be seen to turn with the cell and always maintain the same position in relation to each other. As far as I have been able to determine, both the radiating canals and what appears to be the excurrent pore or tube aid in holding the vacuoles in position and particularly the latter. That there is a connection with the outer wall of the animal can be seen when a paramoecium bursts directly opposite to a fully expanded vacuole, when the vacuole, drawn by the outflowing protoplasm, can be seen straining at some retaining fastening.

2. The vacuoles. Under normal conditions the vacuoles in this race measure on the average about 10 μ in diameter with a capacity of 500 cubic micra. There may be some variation in this size due to the age of the vacuole, i.e., the length of time since it had appeared, but I think that it is safe to say that the diameter of fully formed vacuoles is very nearly the same. There can be no question that these vacuoles originate separately as they generally lie at some distance from each other and no cases have been seen when a vacuole appeared as though it had been separated by division from another vacuole. The average size of the vacuoles in the common race which I have observed is 11 μ with a cubic content of 664 cubic micra. In my fixed preparation the vacuoles are surrounded by what appears to be a definite morphological membrane (fig. 16). When animals have been kept at a temperature a few degrees above freezing for a few hours the vacuoles expand and do not contract and it is

interesting to note that the anterior vacuole becomes from three to four times the diameter of those in the posterior end. This, in the cases studied, was not true for the common two-vacuoled race, neither vacuole, exceeding the other in size.

IV. OBSERVATIONS ON THE BEHAVIOR OF THE VACUOLES

To sum up briefly our knowledge of contractile vacuoles:

The contractile vacuole is a pulsating organ surrounded by a membrane of physiological importance, belonging to the ectoplasm. These vacuoles form an osmotic system and the evidence at hand shows them to function in part as excretory and respiratory organs.

a. Percentage of variation in contractile vacuole number

After the pure lines that had been started had multiplied considerably examination was made of a number of individuals in each line. Apparently these high numbers of vacuoles were not in every case passed on to the offspring for in all of the cultures, whether started with three or four vacuoled paramoecia, there were considerable numbers of two vacuoled forms. But on the contrary in the pure lines started with three vacuoled forms there were found individuals possessing four contractile organs. Observations made on one culture in the early days of the work showed a range of variation in vacuole number in the following percentages.

	per cent
Two vacuoles	8.6
Three vacuoles	65.7
Four vacuoles	25.7

Later and more accurate observations were made for a period of thirty days on one culture (covering 663 individuals) and the results for that time averaged showed the proportion to be:

	per cent
Two vacuoles	16.13
Three vacuoles	66.48
Four vacuoles	16.43
Five vacuoles	0.904

In both of these tables, and in all of the following work, the three vacuoled animals show themselves to be in the excess in nearly all cases. The statement made in my first note on this race that there was an apparent tendency for the vacuole number to settle down with three as the standard must be retracted. When the note was written I was not familiar with the response of the vacuole number to various conditions (rate of division, age of medium, etc.).

The last table shows that a five-vacuoled paramoecium appeared. Animals with five vacuoles, while not rare, seldom have been found in a percentage greater than the one indicated. The cause of the limited production of these extreme forms will be discussed under Section VI. Recently four paramoecia have been obtained with six contractile vacuoles and one with seven (figs. 10 and 12). It is a striking sight to see a paramoecium with six or seven vacuoles in a row all swelling and contracting regularly. The six vacuoled condition is very rare and in over a year I have not seen more than a half dozen animals thus equipped, and the majority of these I have found recently. The only cases seen in 1915 were very doubtful as the contractile vacuoles were hidden by food vacuoles of similar refractiveness.

The above results were obtained on cultures maintained under relatively similar conditions and it is interesting to compare them with the average of the results obtained from a number of cultures under observation for a much longer time and under much more varied conditions. The data from which charts 2, 3, 4 and 5 were made served as a basis for these calculations. These data were obtained on paramoecia under exceedingly varied conditions—in cultures ranging from freshly made up infusions to cultures that were very old, in cultures where plenty of medium was present and in cultures that were undergoing rapid evaporation, in culture during conjugation and after the process had stopped. The averages given below are based on an examination of 9818 animals.

	per cent
Two vacuoles	23.5
Three vacuoles	61.3
Four vacuoles	13.4
Five vacuoles	0.22
Six vacuoles	0.042
Seven vacuoles	0.0001

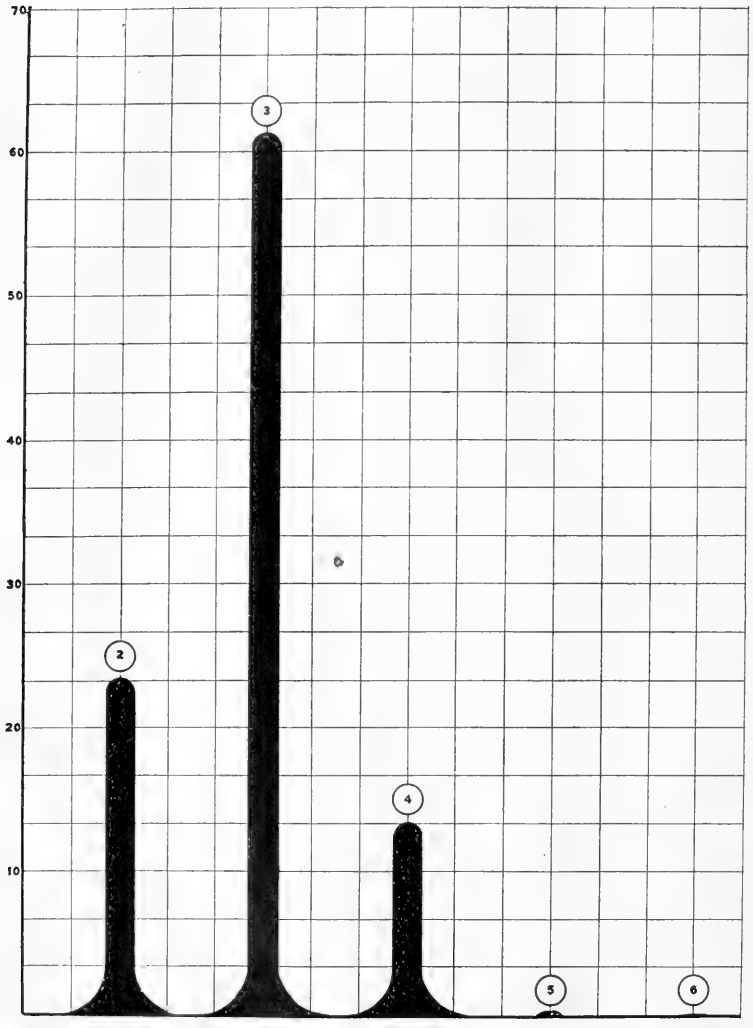
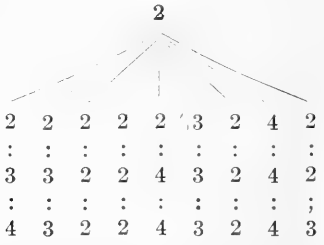


Chart 2 This chart gives a graphic illustration of the comparative numbers of two, three, four, five and six-vacuoled animals. This chart was prepared from data on ten thousand animals living under all conditions of medium.

Chart 2 illustrates these percentages graphically. In comparison with the twos, threes and fours, the fives and sixes, and especially the latter, scarcely appear above the base line.

b. Variability in vacuole number in the life cycle of the individual

While making the above studies it became evident that the time of day apparently had some influence on the various percentages. This suggested that the period in the life cycle of the individual at which the observation was made might play a rôle in determining the general percentage of the various vacuole numbers. (In this paper reference to the life cycle of the paramoecium always refers to the period between transverse divisions.) A single individual showing two vacuoles was therefore isolated and allowed to divide several times. Examination was then made of all the offspring and each was isolated in a separate watch glass. At intervals of from three to five hours the animals were again examined with the following results.



As can be seen, paramoecia possessing two contractile vacuoles increased this number in several cases before division. In some of the individuals the number was not increased. However, continued work with the two vacuoled forms has demonstrated that they have not lost their power of producing multi-vacuoled daughters. In one case I have followed individuals through three generations (representing fifteen animals in all) without a three vacuoled form appearing and then the extra vacuoled animals were produced in just a large percentages as in other lines. This point will be more concretely illustrated under 'selection,' page 316.

The potentiality for the higher number of contractile vacuoles is maintained although several generations may be passed before it exerts itself or, better, before the conditions are such that it must exert itself.

c. The number of contractile vacuoles in the offspring

When normal individuals get ready to divide, and when the beginning of the constriction has appeared, two new vacuoles are added so that immediately after division the daughter cells are fully equipped with excretory organs. In this new race, as a rule, two vacuoles are added at this time, although cases appear when only one seems to be formed. In a certain percentage, apparently more than two have been added before division but whether the vacuoles in excess of two were added before or after division cannot be ascertained from my data, as the observations were not, as a rule, made until some time after separation had taken place, in which case the extra vacuoles might have been formed after division. In a few cases division occurred without the formation of any new vacuoles.

For 225 animals in:

15.11 per cent—one vacuole was added
54.66 per cent—two vacuoles were added
30.22 per cent—more than two vacuoles were added

From this it is evident that in at least 50 per cent of the cases the usual normal addition of two new vacuoles prevails as in the common race.

With these facts in hand, the number of vacuoles possessed by the offspring of the individuals of this new race may be understood. It is practically impossible, however, to predict with certainty what number of contractile vacuoles the offspring of any particular paramoecium will have immediately following division. Some of the possibilities observed up to date are illustrated below.

(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)	(m)	(n)
2	2	2	3	3	3	3	4	4	4	5	5	5	6
∧	∧	∧	∧	∧	∧	∧	∧	∧	∧	∧	∧	∧	∧
2 2	2 3	3 3	2 2	2 3	3 3	4 2	2 3	4 2	3 3	4 2	3 3	5 2	4 2

As stated above, as a rule two vacuoles are added at the time of division (one to the anterior end and one to the posterior end). a, e, i, j, and m represent cases where presumably such an addition has taken place. In b, c, f and g more than two vacuoles have been added although as pointed out above this may be due to post divisional additions rather than to additions at the time of constriction. d, h, and l represent cases where only one vacuole, has been added, while n indicates that there has been no increase whatever.

d. Effect of various conditions on the vacuole number

Division rate. Early in the course of these observations it appeared that in newly started cultures of fresh hay infusion where the animals were increasing rapidly the percentage of two-vacuoled forms was frequently very high. On the other hand, in older cultures where the division rate was presumably slower the percentage of multi-vacuoled animals outnumbered the twos. To test this point more accurately, a number of cultures were set up to see whether this condition was the rule. From the time the cultures were well established records were made of the percentage of individuals possessing two, three, four or five vacuoles in each jar every day, or every few days, extending over a period of from two to three months. These records supplied the data for drawing up the accompanying graphs. The results of these experiments have been plotted in various ways to show conclusively that the mode of behavior is essentially always the same.

In all the charts the three-vacuoled animals are represented by a heavy broken line, the twos by a heavy unbroken line and the fours by a light unbroken line and when the fives are drawn in a light broken line is used. The figures placed across the various curves in all the charts except No. 5 indicate the number of vacuoles in the form which the curve represents. Chart 3 represents a single culture It can be seen at a glance that although the twos are at first more numerous their line drops rapidly until it meets the axis of abscissas on March 10 while

the threes and fours rise to a certain point which is maintained with more or less fluctuation to the end. Chart 4 is drawn from figures based on the average of three cultures and shows the various curves to follow practically the same paths that they did in the preceding graph, and although the twos in this case

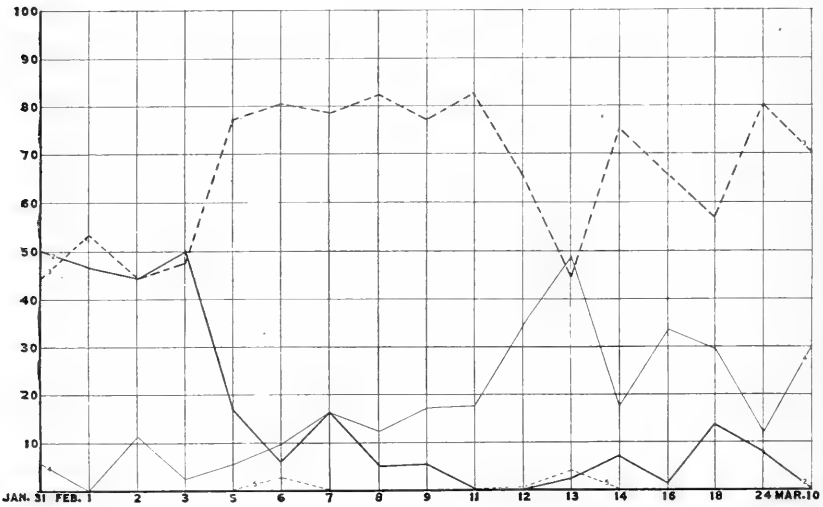


Chart 3 In this chart and in all succeeding ones the axis of abscissa represents time in days and the axis of ordinates represents percentages. The description of this chart is in the text, page 299.

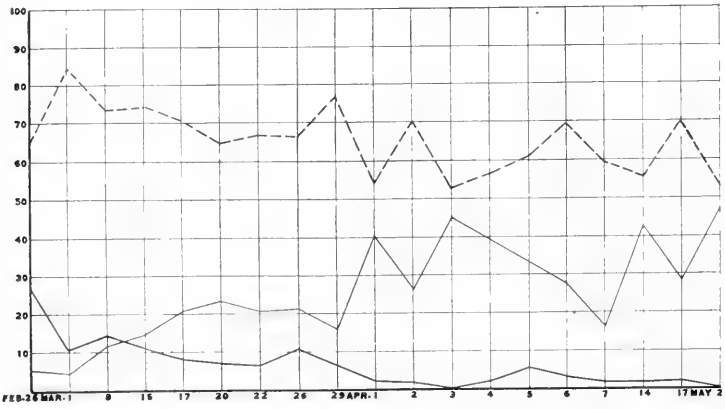


Chart 4 This graph is described on page 300.

did not begin in the majority, they nevertheless drop toward the base line noticeably. Chart 5 is a striking confirmation of the two previous charts. Six cultures were followed and the results plotted on the one chart. The fours play a comparatively unimportant part and will not be discussed. The X formed by the crossing of the unbroken line representing the two vacuoled animals falling from high on the axis of ordinates to the axis of abscissas by the dotted curve of the three-vacuoled paramoecia rising from low percentages to higher ones is very marked.

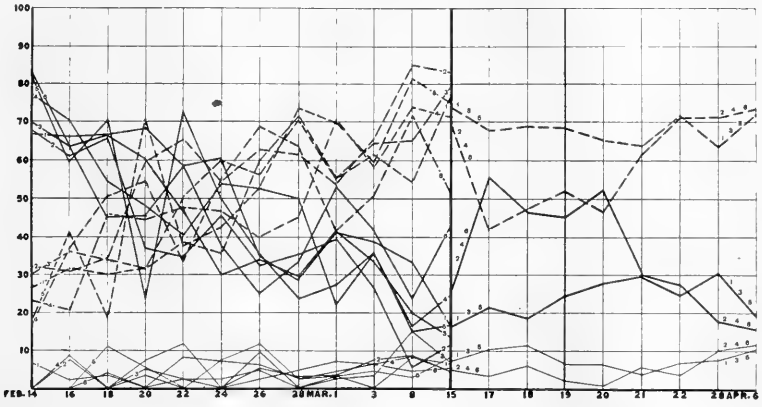


Chart 5 The description of this chart is on page 301.

The numbers on the various curves indicate the number of the culture and not the number of the vacuoles as on the other charts. The same method of designating the various forms is used as on the other graphs.

Still more interesting are the results obtained from March 15 on and represented on this graph. Cultures 1, 3 and 5 were undisturbed while fresh hay infusion was added to cultures 2, 4 and 6. The results for each three cultures were averaged and plotted. 1, 3 and 5 continued as before while 2, 4 and 6 due to the acceleration of the division rate caused by the addition of fresh infusion show a rapid increase of two-vacuoled paramoecia with a corresponding drop in the threes. By April 6, however, the rate of increase had slowed down and the curves of the one set of cultures were very close to the corresponding curves of the other set. Chart 12 while drawn primarily to illustrate the

effect of conjugation shows conditions similar to the above. Chart 6 illustrates graphically the relation between the division rate and the percentages of animals possessing various numbers of vacuoles. The very heavy line represents the division rate and it can be readily seen how the percentage of two-vacuoled

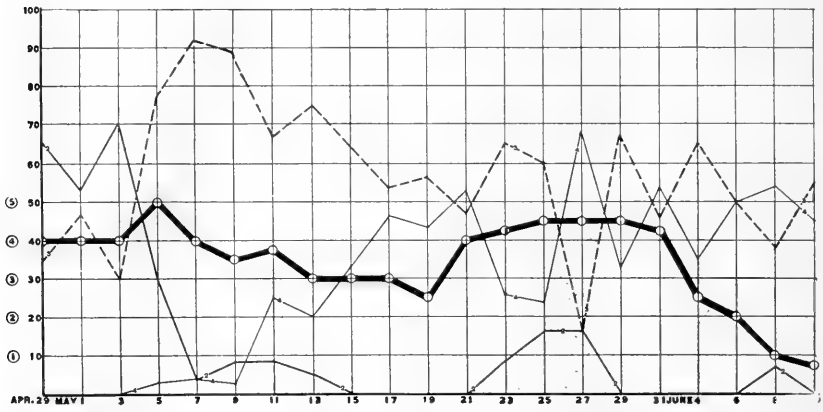


Chart 6 This graph compares the fluctuations of the number of two, three and four-vacuoled paramoecia in a single culture for a period of nearly two months with the daily division rate. The division rate was obtained by isolating a number of single animals in separate watch glasses in fluid filtered from the main culture. At stated intervals an average of the number of divisions that had occurred in all the watch glasses was struck. The figures in circles at the extreme left of the chart indicate the number of divisions and the broad black band traces the division rate across the graph. The other figures are the same as have been used on previous charts. It is evident from this chart that in general while the threes and fours increase as the division rate decreases there is apparently another factor at work that cannot be accounted for on this basis.

animals rises and falls with the increase or decrease of the fission rate.

Age of the culture. To determine whether paramoecia divide more rapidly in fresh hay infusion than in an old, worn out medium, liquid was filtered from a culture nine months old. A few drops of this fluid was placed in several watch glasses and a paramoecium taken from the same culture as the liquid was isolated in each. From this same old culture single paramoecia were placed in similar amount of fresh hay infusion. In six

days all of the animals isolated in the fresh medium had divided twice producing four animals, while one of the animals in the old culture had divided once, one had not divided at all, and two had died. Even in the new medium the division rate was apparently very slow, but the animals were so starved that it took them several days to return to the normal condition. After starved paramoecia have been abundantly fed I have always found their division rate to increase to normal.

This experiment was repeated using animals that were not starved. The division rate for a period of five days was 2.7 in the old infusion against 5.6 divisions of sister paramoecia in freshly made hay medium. A more complete account of the effect of various media will be given under Physiology.

V. PHYSIOLOGY

a. General

It has been over two years since the original animals of the new race were discovered and they not only show no indication of dying off but have given much evidence of being a line superior in vitality to any of the two-vacuoled paramoecia with which they have been compared. Cultures of this new race have been kept in various laboratories of the Zoological Department of the University of Pennsylvania and in the course of time, since no particular care was taken to prevent the mixture of various cultures, the multi-vacuoled animals found their way into the general cultures and it was not long before they had displaced the common form. For some time this year it was impossible to find a normal two-vacuoled paramoecium in any of the cultures maintained in this laboratory. This extra vacuoled race is made up of rather large animals and under many adverse conditions these paramoecia surpass the common races in viability and vigor.

One of the characteristics of the race in question that was first observed was the extreme resistance it seemed to possess to culture media which were so old that they would probably not have supported the common race of caudatum. Several cultures made up at the beginning of the summer recess came

through the hot weather in a very healthy condition although the cultures had remained in a room and in which the temperature was at times quite high. Cultures are being carried along at present that are nine months old and although most of the animals have died there are still a few very active paramoecia remaining.

The division rate for this race has a somewhat wider range than I have observed in the case of other races. Under normal conditions (fresh or nearly fresh hay infusion) these animals divide from one to three times in twenty-four hours. When the culture medium is older the rate may drop below this. There have been times, however, when the animals under observations divided as rapidly as five times in twenty-four hours and this rate was maintained for six or seven days. This rate of division is much more rapid than is usual for the two-vacuoled races.

In an attempt to determine whether or not the rapidity of division is the sole agent in the production or inhibition of the formation of extra vacuoles, several single individuals were isolated in watch glasses containing hay infusions that differed widely in age and condition. Three sets of experiments were set up in each kind of infusion and in three days the number of divisions that had occurred and the percentage of two, three and four-vacuoled animals that were present was recorded for each of the three sets of experiments and for each type of medium. The results obtained in each infusion were averaged and are given below. All the experiments were repeated to determine whether the results of the first set would be constant. The cultures from which the animals for this experiment were drawn was started from a single individual. The number of divisions given is for a period of three days.

1. Medium filtered from culture nine months old

NUMBER OF DIVISIONS	EXPERIMENT A	NUMBER OF DIVISIONS	EXPERIMENT B
3	Two vacuoles, 44 per cent Three vacuoles, 48 per cent Four vacuoles, 8 per cent	2.7	Two vacuoles, 31 per cent Three vacuoles, 61 per cent Four vacuoles, 8 per cent

2. Medium filtered from culture from which paramoecia were obtained to conduct these experiments. Culture was about one week old

NUMBER OF DIVISIONS	EXPERIMENT A	NUMBER OF DIVISIONS	EXPERIMENT B
4.3	Two vacuoles, 81 per cent Three vacuoles, 18 per cent Four vacuoles, 1 per cent	3.7	Two vacuoles, 30 per cent Three vacuoles, 70 per cent

3. Hay infusion freshly made up

NUMBER OF DIVISIONS	EXPERIMENT B
5.6	Two vacuoles, 82 per cent Three vacuoles, 18 per cent

4. Medium filtered from culture containing many animals which had been allowed to evaporate (concentrate) and had been refilled with fresh hay infusion four times. The concentration of the metabolic products present was doubtless quite high and the culture had a very strong odor. At the time this experiment was started fresh infusion had been added to the culture about twenty-four hours

NUMBER OF DIVISIONS	EXPERIMENT B
4.5	Two vacuoles, 37.5 per cent Three vacuoles, 50 per cent Four vacuoles, 12.5 per cent

The foregoing data point strongly, I think, to the fact that rapidity of division plays a very important rôle in influencing the production of the extra vacuoles. Apparently one of the chief requirements for the production of the extra vacuoles for which this race possesses the potentiality is sufficient length of time between divisions. When the rate of division is rapid the two-vacuoled animals tend to predominate as illustrated by Experiment A, 2; and Experiment B, 3. With a slower rate of multiplication there appear more three and four-vacuoled paramoecia as the results of Experiments A, 1 and B, 1 and 2 show. Moreover, in old cultures where the division rate is low two-vacuoled animals are very scarce when not absolutely lacking. While there is some slight variation in the results of the corresponding sets of experiments I am not at present inclined to

believe that this fact interfered with any of the above statements, as the variability of individuals may readily account for this. In general all of the results indicate that extra vacuoles appear as a rule when the rate of fission is relatively slow. Chart 6 compares graphically the division rate with the percentages of two, three and four-vacuoled, animals.

There are, however, enough exceptions to the general rule to suggest the probability of some other factors playing a part and very likely, not an unimportant part in determining the number of vacuoles. As yet, I am not certain what this factor or these factors may be, but Experiment B, 4 gives at least the basis of a working hypothesis. In this particular experiment although the division rate was high the number of two-vacuoled paramoecia developed was 43.5 per cent under the number produced in Experiment A, 2 where the rapidity of division was approximately the same. The difference between the experiments lay in that the fluid used in Experiment B, 4 contained many times the amount of metabolic waste products present in Experiment A, 2. From this result the tentative suggestion may be made that although the rate of division seems to be the primary agent in influencing the vacuole number, the presence of waste products in the environment has an activating effect on the production of extra vacuoles. Indeed if the amount of the waste products of metabolism is sufficient it may in part offset the effect of a rapid division rate.

Merely slowing down the rate of division is not sufficient, as this was tried by placing six paramoecia in a constant temperature room at 12°C. In four days two had divided only once and the rest not at all while the controls at room temperature had each divided three times. When the animals in the cold room were examined it was found that the vacuole number had not increased at all. Under these conditions although division did not occur for four days, the temperature lowered the metabolic activities of the paramoecia and made the production of extra vacuoles either impossible or unnecessary.

b. Resistance

This race has proved itself to be more resistant to all the abnormal conditions it has been subjected to than have the common animals.

Heat. Dr. Jacobs has found that under conditions where the ordinary paramoecia are killed at temperatures of 40° to 42° C. the race in question may survive an exposure to 44°. Animals that have been living in a hay infusion to which a small percentage of sea salt has been added resist high temperatures for very much longer periods of time than are paramoecia taken from pure hay infusion. My experiments along this line are, as yet, not sufficiently extensive to base conclusions.

Cold. A few animals of the multi-vacuoled race withstood an air temperature of

1° to 2° for 45 hours
−3° to −4° for 2½ hours

The common race was killed in the first case between five and thirty hours and in a trifle over an hour in the second experiment. In this time, although ice crystals were formed, the fluid did not freeze solid at this low temperature.

Distilled water. Mr. Mitchell Carrol, of the Zoological Laboratory of the University of Pennsylvania, has maintained this race of paramoecium in distilled water for fifteen generations, covering a period of forty-four days. In similar experiments of my own these animals showed the same ability to live in distilled water.

c. Rate of vacuole contraction

A few observations on the rate of vacuole contraction under varying conditions have been made on this new race of Paramoecium to determine whether there is any essential difference between this and the common stock. Two methods have been employed. In the first method each vacuole as it contracted was called off to an assistant who noted the time. Several observations were made on each vacuole and then the records obtained for each vacuole were averaged. In all forty-four animals were studied at 23°C. with an average of three observations per vacuole per animal.

	FIRST VACUOLE	SECOND VACUOLE	THIRD VACUOLE	FOURTH VACUOLE
Two vacuoled animals	9	9		
Three vacuoled animals	10	13	13	
Four vacuoled animals	11	12	14	9

The second method employed, though not so accurate as the first since only one observation per vacuole per animal was obtained, gave results which are approximately the same as the first. The time of contraction was taken with a stop watch from the instant the vacuole disappeared until it disappeared again. With animals possessing three or more vacuoles two watches were frequently used as the paramoecium would often dry up if each contraction was timed separately. The results obtained at each temperature are given below. The figures are based on observations of thirty-five paramoecia.

The well known fact that the rapidity of vacuole contraction increases with the temperature is again brought out in these tables. It is evident in the multi-vacuoled race that there is a

Multi-vacuoled race. Time in seconds; temperature in Centigrade

	FIRST VACUOLE	SECOND VACUOLE	THIRD VACUOLE	FOURTH VACUOLE	TEMPERATURE
					deg. C.
Two vacuoled animals	8	13			22.0
	9	10			23.0
	10	9			24.0
Average	9	11			
Three vacuoled animals	13	12	12		20.0
	11	12	13		20.5
	8	8	10		24.0
	10	12	12		22.5
Average	10.5	11	11.7		
Four vacuoled animals	12	14	13	12	20.5
	10	9	13	12	23.0
Average	11	11.5	13	12	

Common two-vacuoled race

FIRST VACUOLE	SECOND VACUOLE	TEMPERATURE
10.7	10.9	20.5
10.2	9.8	22.0
10.0	10.6	25.5
Average....10.3	10.4	

progressive lessening of rate of pulsation from anterior to posterior end. This is not so evident in the records of the two-vacuoled race as both vacuoles appear to beat in almost identical rate. While the observations were not taken at the same temperature, the relative frequency of the various vacuoles in each case would remain approximately the same.

An interesting condition appears in the first table by averaging the average time of contraction of the vacuoles in each group. The result is:

	seconds
Two-vacuoled group	10
Three-vacuoled group	11
Four-vacuoled group	12

There is a steady slowing down of the rate of pulsation as the number of vacuoles increases. Whether this will be borne out by more extensive studies remains to be seen.

1. The effect of salt solutions on the rate of contraction. The work of other investigators has shown that marine protozoa seldom possess contractile vacuoles and that when fresh water forms are subjected to saline solutions the rate of contraction decreases greatly and in some cases the vacuole disappears entirely (6). This race was subjected to salt solutions of various strengths to determine whether it was possible to cause some of the extra vacuoles to disappear permanently. Enough sea salt was added to hay infusion to bring the percentage of salt to 0.06 per cent or to 0.1 per cent. The animals could not withstand an immersion in a solution as strong as 0.5 per cent immediately but they survived very well if the percentage was allowed to in-

crease slowly through evaporation.[1] The salt slowed the contraction rate but did not in any way influence the number of vacuoles. The most marked effect in this direction was while the animals were still unaccustomed to their environment. After they had become somewhat acclimated, to the new conditions after which the rate of pulsation became more normal although it never regained the usual rapidity. This may account in part for the varying results noted below.

	FIRST VACUOLE	SECOND VACUOLE	THIRD VACUOLE	TEMPERATURE	CONCENTRATION OF SALT
				deg. C.	*per cent*
Two vacuoled animals.....	25	25		20.0	0.06
	27	23		21.5	0.1
Three vacuoled animals.....	34	23	45	20.0	0.06
	14	19	18	21.5	0.5
	26	19	18	21.5	1.0

2. The effect of cold on the rate of contraction. Cold slows the rate of contraction markedly and extreme cold, i.e., 1° to 2°C. apparently brings contraction almost to a complete stop.

3. The volume of the vacuoles of the two races. In making this comparison the volume of the animals was calculated in the following manner. The percentage of difference in weight of a paramoecium modeled in clay and a cylinder of the same length and diameter made of the same material was determined. The volume of a cylinder the length and diameter of a living paramoecium was calculated and the volume of the paramoecium was obtained by reducing the volume of the cylinder by the determined percentage of difference between the clay models.

[1] It might be noted here that cultures containing a small percentage of salt thrived wonderfully and the infusion seldom became as murky or as bad smelling as the untreated cultures. It seems likely that the salt prevents the great growth of bacteria producing putrefaction, but it seems to interfere in no way with the supply of food for the paramoecia. Animals treated in this way are more tough and when they burst under pressure the protoplasm oozes out in a thick mass instead of the usual thin stream. Frequently the animal is able to clear itself of the extruded mass and the mutilated area is covered over. Under normal conditions the ejection of the protoplasm is so rapid that there is no possibility of this recovery. This treatment might be of assistance with individuals to be operated on.

The diameter of the vacuoles was obtained from a number of fixed preparations some of which were sectioned.

Multi-vacuoled race

Average length..... 233 μ
Average width..... 51 μ
Volume..... 284, 493 cubic μ
Capacity of each vacuole..... 500 cubic μ (diameter, 10 μ)

To deliver themselves of a quantity of liquid equal to the volume of the animal, at a temperature of 22°C., the

2 vacuoled animals contract 284 times in 47 minutes at a 10 second rate
3 vacuoled animals contract 190 times in 35 minutes at a 11 second rate
4 vacuoled animals contract 142 times in 28 minutes at a 12 second rate
5 vacuoled animals contract 114 times in 25 minutes at a 13 second rate
6 vacuoled animals contract 95 times in 22 minutes at a 14 second rate

The rate for the five and six vacuoled animals has been based on statistics given above (page 309).

Common race. Large individuals

Average length..... 265 μ
Average width..... 53 μ
Volume..... 397, 690 cubic μ
Capacity of each vacuole..... 664 cubic μ (diameter, 11 μ)

At the same temperature these animals require forty-nine minutes to pump out a quantity of water equal to the volume of the animal.

Small individuals

Average length..... 180 μ
Average width..... 53 μ
Volume..... 194, 260 cubic μ

This lot would require twenty-three minutes for the same operation.

It is evident from these figures that the new paramoecia are superior to the larger forms in the quantity of liquid they can excrete in a given time but in general are not quite equal to the smaller races in this respect. I have been unable as yet to obtain a wild race as large as these new animals to compare their respective resistances and this will form an interesting part of future work. I have not attempted as yet to determine whether there is any correlation between resistance to various substances and the number of vacuoles but the addition of vacuoles in the individuals as the culture ages and the conditions are presumably becoming more unfavorable is a response that is very suggestive.

d. Conjugation

A cycle of conjugation was awaited for over a year and then a method of bringing it about at will was hit upon accidentally. Two cultures which had been started with 200 cc. of hay infusion were allowed to stand uncovered. In seven days the fluid had evaporated to one half and a strong epidemic of conjugation was observed. The experiment was repeated successfully eight times, conjugants always appearing when the fluid had been reduced to approximately one half its original volume. With the containers used this usually required a week.

From these results it seemed probable that conjugation had been produced by the rapid concentration of metabolic products. To test this theory further a number of cultures were set up. Some jars were covered and others were allowed to evaporate. The jars used were much smaller than the original containers and the liquid surface exposed was not more than one third as great. Consequently the evaporation was much slower and three weeks were required to reduce the liquid to the point that one week produced in the larger jars. In three jars that contained (1) pure hay infusion, (2) hay infusion plus 0.05 per cent sea salt, and (3) hay infusion plus 0.1 per cent sea salt, although evaporation did not take place conjugation began in (3) in thirty-six days, in (2) in thirty-eight days and in (1) in thirty-nine days. These cultures had been started in drinking glasses. Three cultures similarly made at the same time were allowed to evaporate. No conjugants appeared. When the medium became very low in the container it was replenished with fresh infusion and again allowed to evaporate. This was repeated five times and no sign of conjugation was observed.

During the year previous to these experiments cultures had been made up using 500 or 600 cc. of liquid and in this time as has been stated no conjugation took place. Conjugation has been obtained in eighteen cultures when the exposed surface of the fluid was sufficient to permit rapid evaporation at room temperature or when the quantity of fluid was small and the multiplication of the animals rapid.

It is of considerable interest to note that when conjugation did take place in cultures to which a minute quantity of sea salt had been added the epidemic did not last nearly as long as in the untreated infusions. In one set of experiments conjugation lasted for twelve days in the unsalted culture while in the culture containing 0.1 per cent sea salt it died out in three days. Another time an untreated medium maintained conjugation for thirty-three days against thirteen and nineteen days in two salt cultures. The length of one of the periods of conjugation is figured on chart 7.

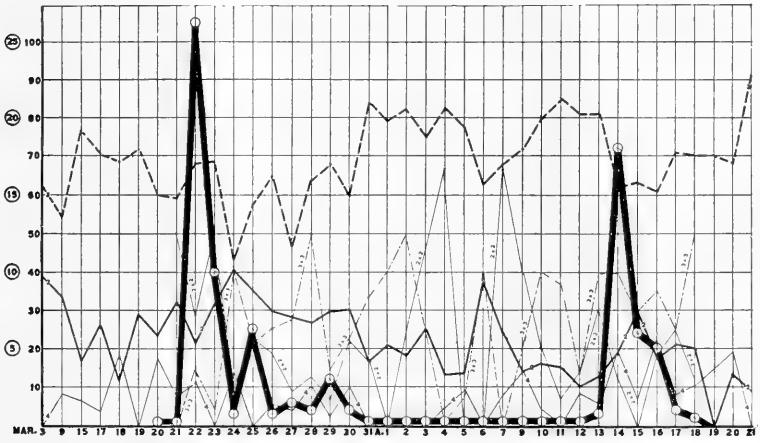


Chart 7 This chart was drawn from records obtained from a single culture and illustrates the percentage of conjugants to unfused forms found during a cycle of conjugation. The figures in the circles at the extreme left of the chart indicate the percentage of conjugants and the heavy black band traces the fluctuations of the percentage of conjugants across the graph. The other curves represent either individuals or conjugants and are labeled on the chart. It will be noted that when the number of conjugants is high the percentage of three and four-vacuoled forms decrease and increase as conjugation dies out.

In brief, conjugation has been obtained in cultures that have been started with single individuals under the following conditions.

1. Conjugation appeared in practically all cases when the culture was evaporated rapidly causing the metabolic products to concentrate considerably.

2. Conjugation appeared in cultures having a comparatively small amount of fluid supporting a large number of paramoecia.

3. No conjugations were seen in some cultures undergoing the same treatment as 1 and 2 particularly when the concentration was not rapid.

4. No conjugation took place in cultures made up with a large amount of fluid.

5. When a slight amount of sea salt was present conjugation sometimes occurred a little before the control of plain hay infusion showed signs of this condition and in all cases the epidemic of conjugation in the saline solutions died out before it did in the pure hay media.

As a discussion of the various theories of conjugation in relation to the results given above is somewhat foreign to the main subject of this paper a full consideration of the phenomena will be published separately.

VI. GENETICS

The account that precedes this section has dealt with the general behavior and inheritance of the contractile vacuoles. In this section will be considered the inheritance of the extra organs for several consecutive generations and the effect of selection and of conjugation on the race.

While these experiments might have been carried on for more generations the technical difficulties were so great as to make further pursuance of this line of investigation unpracticable. In this part of the work the animals had to be examined with the 4 mm. objective in many cases to be certain of the number of vacuoles they possessed, and to do this the animals could be allowed so little fluid to move in that many times I have lost them through drying thereby breaking the continuous line of succession. Indeed, dozens of experiments were started before the results illustrated below were obtained.

a. The inheritance of the contractile vacuoles for four generations

Lines were started with one two, one three and one four-vacuoled paramoecium. As rapidly as they divided the num-

ber of vacuoles in the daughter cells was recorded and the animals were separated and again allowed to divide when the operation was repeated. The results of these experiments are shown in the charts 8, 9 and 10. The last generations on chart 8 and 9 show practically 50 per cent of twos and threes. The same generation on chart 10 shows a much higher percentage of twos. A study of these charts will, I think, convince the reader that

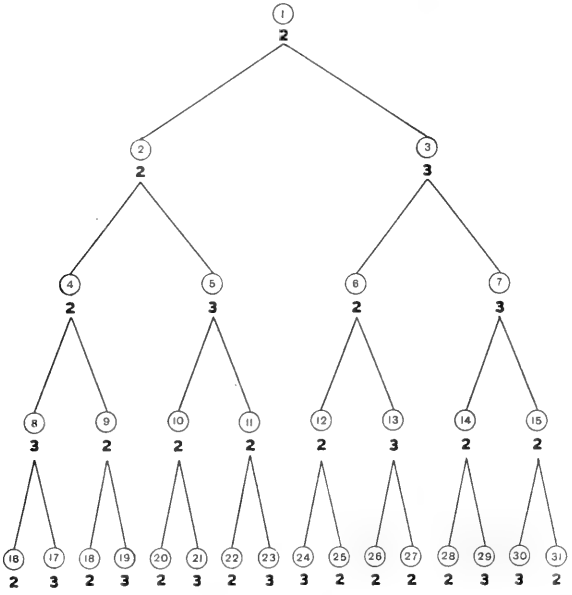


Chart 8 This figure illustrates the mode of inheritance for four generations from a two-vacuoled progenitor. The heavy figures indicate the number of vacuoles for any descendant.

the number of vacuoles any one individual may possess is no indication of its potentialities—that the fewer-vacuoled paramoecia may give rise to animals with more vacuoles and vice versa. The last statement was demonstrated by starting cultures with several of the animals possessing different numbers of vacuoles from the fourth generation of these lines. After the lines had multiplied considerably examination showed the percentages to be in no way different from those given throughout this paper. In the case of experiments such as these the two-

vacuoled forms tend to predominate, as fresh hay infusion was used for each generation. As has been pointed out before, fresh medium causes the animals to divide more rapidly and that rapidity of division may be said in a general way to keep the number of vacuoles low.

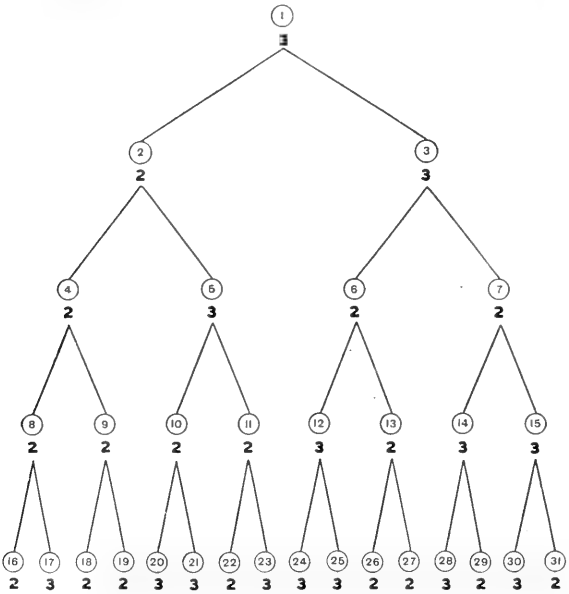


Chart 9 In this case the line was started with a three-vacuoled paramoecium.

b. Effect of selection

Selection has been carried on in three ways.

First method. Individuals possessing two, three, four and five vacuoles were isolated. When they had divided, the number of vacuoles in each was recorded and, in the case of the two-vacuoled line, the paramoecium with the lowest number of vacuoles was allowed to continue while in the three, four and five-vacuoled lines the highest vacuoled animals were always selected. The individuals not selected for continuing the lines were discarded. This plan was carried on for a period of from nine to fourteen generations and the results are given on chart 11. Here the vacuole number of the progenitor is placed at the

top of the line in a diamond-shaped frame. Directly beneath it is the number of vacuoles in the selected descendents. To the left of this line is placed the number of vacuoles in the sister cell. At first sight it would seem that selection has some effect but figuring the percentage of animals with twos, threes, fours and fives in each line it is found that they vary little more than 10 per cent.

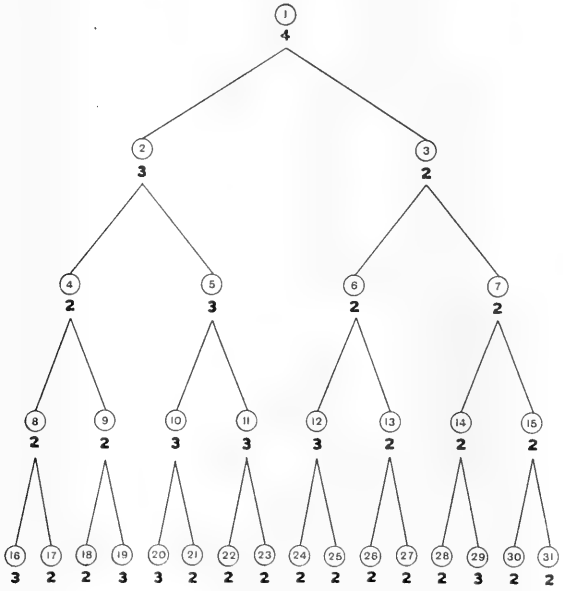


Chart 10 A four-vacuoled paramoecium gave rise to paramoecia possessing the number of vacuoles indicated on this chart.

Second method. The same difficulty was experienced with selecting by the first method as was found in following all the animals for several generations, i.e., the danger of losing an important individual through drying. By the second method each animal was allowed to multiply for a considerable number of generations. A large number would then be examined and the percentage of twos, threes, fours and fives noted. One would then be isolated to start a new line. In an experiment where selection was carried on for nearly three months according to

this method at the time of writing no tendency to produce more high-vacuoled animals is evident.

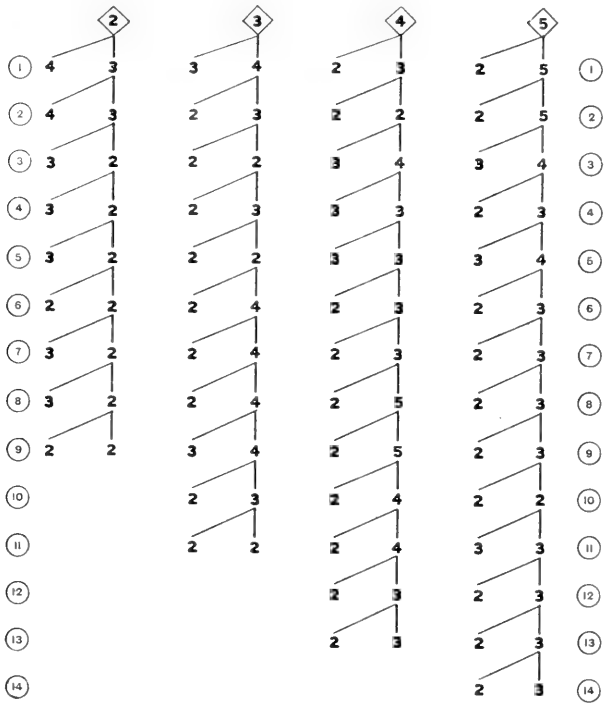


Chart 11 This figure illustrates the effect of selection and is described on page 316.

Analysis of Chart 11

	TWO VACUOLES	THREE VACUOLES	FOUR VACUOLES	FIVE VACUOLES
	per cent	*per cent*	*per cent*	*per cent*
Line 2	53	37	10	
Line 3	50	27	23	
Line 4	40	37	15	7
Line 5	41	41	7	10

Experiment A was started with a two-vacuoled paramoecium and selection was always made of animals with the least number

of vacuoles and the line carried on with them. The last examination of this culture showed the percentages to be as follows:

	per cent
Two-vacuoled paramoecia	13
Three-vacuoled paramoecia	78
Four-vacuoled paramoecia	9

Experiments B and C were carried on with selection for animals with the most contractile vacuoles.

Experiment B started with a three-vacuoled paramoecium gave rise to:

	per cent
Two-vacuoled paramoecia	31
Three-vacuoled paramoecia	69

Experiment C started with a four-vacuoled paramoecium produced:

	per cent
Two-vacuoled paramoecia	43
Three-vacuoled paramoecia	57

In Experiment A where the selection was for the animals having the least number of vacuoles the percentage of multi-vacuoled paramoecia produced was greater than in B and C. It might seem that the two-vacuoled animals of Experiment A were possessed of a potentiality to produce more multi-vacuoled offspring than were the paramoecia of B and C. As far as is known the culture conditions in all three experiments were the same. As has been stated several times before, the extensive data that have been gathered on this race under all conditions leads me to feel confident that in experiments such as A the greater number of multi-vacuoled animals were produced because of some characteristic of the culture medium which either slowed down the division rate (allowing more time for vacuoles to develop) or had some other influence on the animals which produced extra vacuoles and which as yet I have been unable to discover. In B and C this factor was presumably absent or was not so potent.

c. Immediate and temporary effect of location of the vacuoles in the parent form on inheritance by offspring

When a paramoecium possessing the most common arrangement of vacuoles (the supernumerary vacuoles located posteriorly) divides, the anterior half receives fewer vacuoles than the posterior half. For instance, an animal with three vacuoles—one anterior and two posterior—divides. Just before division a new vacuole is added to each end which results in a daughter paramoecium from the anterior half with two vacuoles while the daughter arising posterior to the constriction has three vacuoles. The paramoecium springing from the posterior half starts its life cycle with more vacuoles than does its anterior mate but the anterior animal, however, as had been emphasized before, has not lost the power of producing paramoecia with the higher number of vacuoles.

d. Effect of conjugation

When conjugation was first observed it seemed that there was what might be termed a selective mating or more exactly that there were more two-vacuoled animals conjugating at the beginning of the epidemic than multi-vacuoled individuals. Further work and the final plotting of the results showed that this was not the case. Neither was there a tendency for threes to pair only with threes or fours with fours but there was a heterogeneous mating in approximately the same percentage as the frequency of the various number of vacuoles in the individual units. Chart 12 illustrates these points. Conjugants were found paired in nearly all possible combinations: 2×2, 2×3, 2×4, 2×5, 3×3, 3×4, 3×5, 4×4 and 4×5. During an epidemic of conjugation the proportions of these various combinations on any one day followed in a fairly close manner the percentage of single animals having two, three and four vacuoles in the same culture. This relation becomes clearer when graphically represented as on chart 12. Here the lines representing the various combinations in conjugation may be seen to follow rather closely the rise or fall of the lines rep-

resenting the vacuole number in the single paramoecia. More of the multi-vacuoled paramoecia conjugate at the end of the epidemic than at the beginning, apparently, not because of different physiological characteristics but because more of these forms appear as the culture ages.

The period of conjugation was awaited with great interest as furnishing an opportunity for determining the nature of this

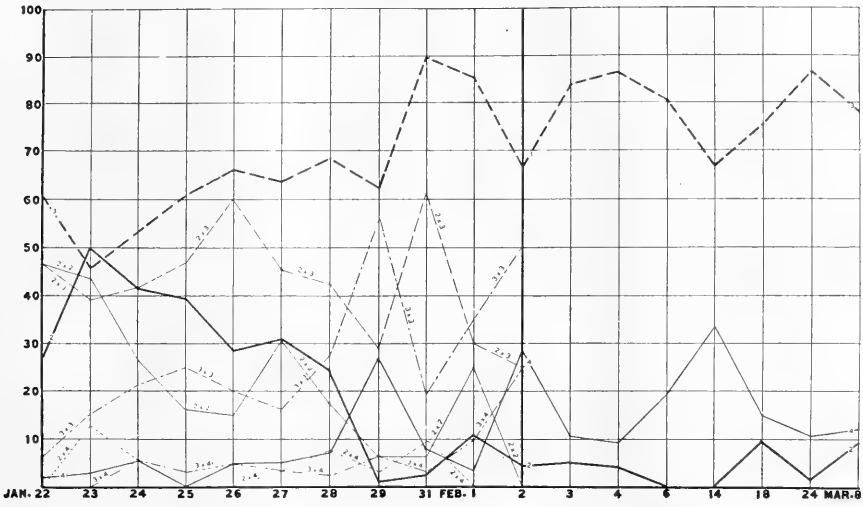


Chart 12 This graph is reproduced to show the manner in which the various combinations of paramoecia in conjugation follow closely the percentages of two, three and four-vacuoled paramoecia on any day. Note that as conjugation ceases on February 2 the percentage of three and four-vacuoled paramoecia increase. Further discussion is to be found on page 320 under "Effect of Conjugation."

new character. If the paramoecia were homozygous then we should expect no change in the inheritance of the potentiality for extra organs. But should they be heterozygous, individuals might result which were pure for the lower number of vacuoles. I have used the words 'might result' in the last sentence, since, as far as we know, conjugation between heterozygous paramoecia would present equal possibilities for the offspring of the exconjugants to be heterozygous as it would for a homozygous line

to appear. However, this line is evidently pure for this character since exconjugants when separated produced offspring which showed no variation in the percentage of vacuoles from the number possessed before conjugation began as is indicated by the following observations. Single conjugants of the various combination of vacuole number were isolated and allowed to separate and divide a number of times and then examined. The results were not different from those obtained on unconjugated forms. To take two typical cases in which the conjugants were isolated, and allowed to separate and divide for a week or more.

	per cent
Conjugant 2 × 3:	
Two-vacuoled paramoecia	31
Three-vacuoled paramoecia	69
Conjugant 2 × 4:	
Two-vacuoled paramoecia	32
Three-vacuoled paramoecia	69

The number of two-vacuoled forms here is slightly higher than the average due probably to the rapid division stimulated by fresh hay infusion.

In one instance the exconjugants were allowed to divide twice, producing eight animals in all, and then each animal was isolated in a separate watch crystal and allowed to multiply for some time. This was done to determine whether any difference in potentiality would crop out due to the redistribution of the micronucleus. No change was observed.

No effect can be ascribed to conjugation other than a temporary increase in the percentage of two-vacuoled forms and a corresponding decrease of the threes and fours. This may indicate that conjugation speeds up the division rate somewhat as it has been shown before that increased rate of division results in individuals with fewer contractile vacuoles than is the case when the division rate is slow.

Chart 5 beginning with March 19 when conjugation began in cultures 1, 3 and 5 shows a slight rise in the 'two' line (marked 1–3–5) and a fall in the 'three' line for the same cultures both, however, returning to very nearly the same point on April 6

as they were on March 15. Conjugation had by this time dropped to less than 0.5 per cent of the population.

e. Attempts to cross the two races

One of the most interesting points to be developed in connection with this new race of paramoecium is the effect of crossing with the common form. This is the first time to my knowledge that two forms of protozoa with definite individual characters have been available for crossing. I have made one hundred and fifty attempts to cross the two forms with no success. The difficulties that beset the mating of these two paramoecia are many. Cultures of both races have to be entering into a period of conjugation at the same time. Single individuals from both races must be placed in as small amount of fluid as possible so that they will have every opportunity of coming together. The chances that either or both of the mated paramoecia are in a condition which makes conjugation necessary are small. The matings must be made up fresh at least, every twenty-four hours for when either of the individuals divide there is no way of telling to which race an animal belongs. In spite of these obstacles I am confident that the crossing will ultimately be achieved after sufficient number of matings have been made and perhaps after certain methods of technique have been determined which are not evident at present.

VII. CYTOLOGY

My studies on the cytology of these paramoecia are not sufficient as yet to permit a detailed account. There is one micronucleus present (fig. 15) which indicates that this race is a variety of Paramoecium caudatum. In all the preparations made I have had great difficulty in staining the micronucleus and in the majority of cases so far I have been unable to make the stain remain in it. The depression in the macronucleus where the micronucleus usually lies is frequently visible but it appears empty. I do not believe that this appearance is due entirely to faulty technique, as the same methods give excellent results when used on the common race.

VIII. DISCUSSION

In the course of some experiments by Dr. M. H. Jacobs on the effect of high temperatures on paramoecia, various lots of these animals were ejected into hay infusions at temperatures ranging from about 36° ot 42°C. Those that survived this treatment were thrown into a battery jar containing hay infusion. In this jar the new animals were discovered. I am still of the opinion (stated in my preliminary note in Science (2)) that these new paramoecia may have been produced as the result of the high temperature, although I have been unsuccessful as yet in a number of attempts to reproduce this race artificially. It would be a most striking coincidence if this multi-vacuoled character has been in existence for any length of time that its discovery should be delayed until found in the culture made up of the descendents of heat treated animals.[2]

In this new paramoecium we have a form which lends itself to a study of the processes of heredity in the protozoa. The character is seemingly not at all comparable to those reported for Difflugia by Jennings (5). Since the vacuoles are, however, very definite structures and a marked variation from the common form, their study and comparison is exceedingly interesting and offers many possibilities. The data possessed at present are insufficient to make a discussion of these hereditary processes of value. Selection experiments, though incomplete, seem to indicate as far as they have been carried that the character cannot be affected by selection in either direction. It will be impossible to determine whether the tendency or potentiality for extra vacuoles is germinally controlled until we have succeeded in crossing the two strains. There is some evidence at present that points to the cytoplasm as the portion of the cell

[2] Since this paper was sent to press I have received Shumway's paper (11) in which he has reported the formation of extra contractile vacuoles in thyroid fed animals. None of his specimens had more than three vacuoles and the inheritance of the extra vacuole in the form studied has not been demonstrated. The percentage of extra-vacuoled animals among his thyroid fed paramoecia (20 to 30 per cent) is considerably less than I have found in my race (77 per cent).

that controls the production of the vacuoles since their production seems to be controlled by so many conditions. Whether these conditions act on the cytoplasm directly or on the cytoplasm through the nucleus there is no possibility of determining with the knowledge available at present.

IX. SUMMARY

1. This race which is a variety of Paramoecium caudatum is considered possibly to have arisen under experimental conditions when certain animals were subjected to high temperatures.

2. The animals of this race range in length from 167 μ to 307 μ and are unusually strong and resistant.

3. A slightly denser portion extends across the middle of some of the individuals as a band.

4. The extra vacuoles are generally located in the posterior end of the cell.

5. The number of vacuoles possessed by individuals of this race varies from two to seven.

6. The number of vacuoles may increase during the life time of the individual.

7. Various conditions affect the number of vacuoles present in the individual, such as the rate of division and the age of the culture medium.

8. Though extra vacuoles may not appear for several generations for the above reasons, the potentiality for this organ has not been lost.

9. The division rate ranges between one and five times in twenty-four hours.

10. Besides being influenced by the rate of division the vacuole number may apparently be increased in the individual by the presence of katabolic products in the environment.

11. The resistance of this race to heat, cold, distilled water, etc., is very high.

12. The rate of pulsation of the vacuoles is slightly slower than is that of the vacuoles in the common race but the new animals are in general superior to the common race in their powers of excretion.

13. Conjugation is believed to be caused by the concentration of katabolic products in the environment.

14. It may be said that a potentiality for *extra* contractile vacuoles rather than for a definite number of the organs is passed on from generation to generation and that this potentiality if effected by various conditions during the life time of the individual.

15. Selection has no effect in raising or lowering the vacuole number.

16. The location of the vacuoles in the parent has an immediate but temporary effect on the number possessed by the offspring.

17. Conjugation has no effect upon this new character.

X. BIBLIOGRAPHY

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PLATE 1

EXPLANATION OF FIGURES

The figures are reproduced at a magnification of 480 × with the exception of figure 16 which is 1120 ×. Figures 3, 4, 5, 6 and 8 were drawn from living animals slightly flattened under pressure. The others were outlined from fixed preparations.

In preparing these figures an attempt has been made to reproduce all the various characteristics of the contractile vacuole observed. In general when the vacuoles are expanded the radiating canals are not visible but in favorable specimens or in animals under pressure they appear as thread-like rays as shown in figures 2, 3, 4, 5 and 10. Note that in most cases the vacuoles lie in a line parallel to the outline of the body. The anterior end of the paramoecium is toward the upper part of the page in all the plates. In all the figures the contractile vacuoles are not stippled.

1 Paramoecium with three contractile vacuoles arranged in the most common manner.

2 Paramoecium with two vacuoles.

3 Paramoecium with extra vacuole in the anterior end—a rare grouping.

4 Paramoecium with four vacuoles in the usual position but with one vacuole slightly displaced to the right.

5 Paramoecium with four vacuoles in common arrangement.

6 Paramoecium with four vacuoles in the unusual grouping of two in the anterior end and two in the posterior end. Note the slightly darker area across the middle of the animal.

PARAMOECIUM POSSESSING EXTRA VACUOLES

ROBERT T. HANCE

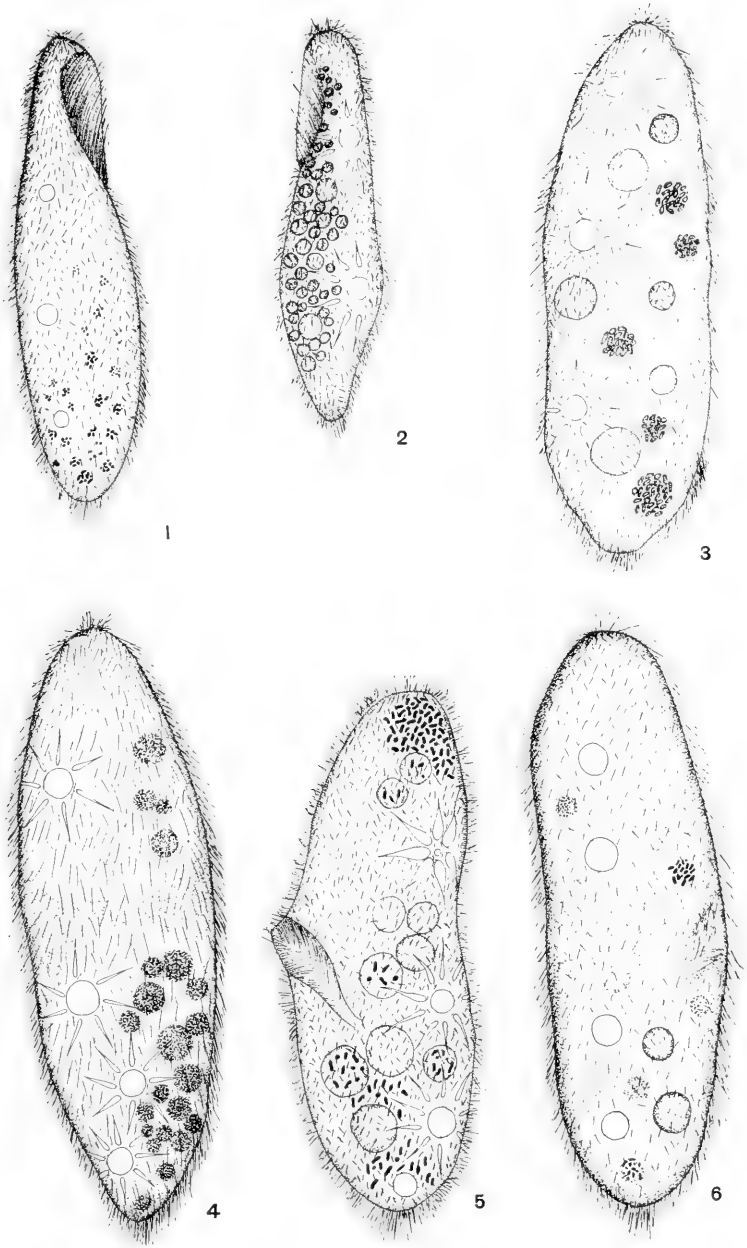


PLATE 2

EXPLANATION OF FIGURES

7 Paramoecium with five vacuoles.

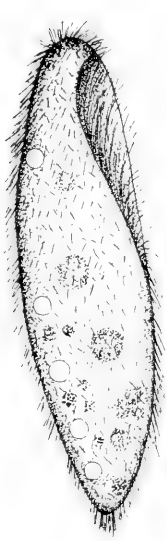
8 Paramoecium with five vacuoles two being located in the anterior end.

9 Paramoecium with five vacuoles, two of which are slightly displaced to the right. This displacement is rare.

10 Paramoecium with six vacuoles.

11 Paramoecium dividing. Before constriction occurred this was a three-vacuoled animal. A new vacuole has been added to each half.

12 Paramoecium with seven vacuoles.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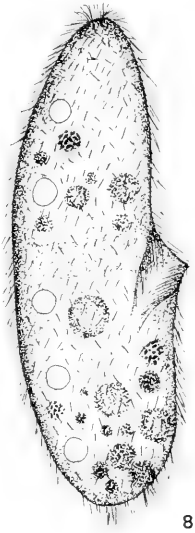
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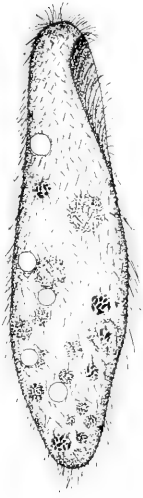
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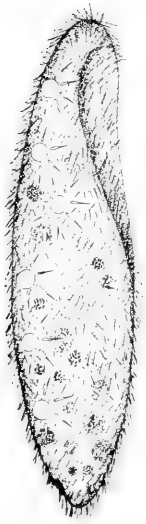
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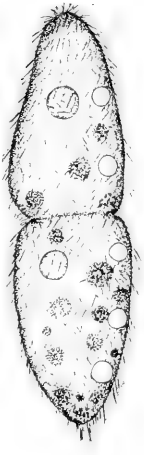
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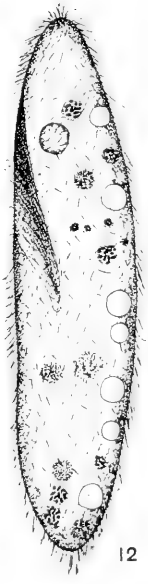
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PLATE 3

EXPLANATION OF FIGURES

13 A paramoecium dividing. This animal before division had four vacuoles arranged as in figure 6. A new vacuole has been added to each end.

14 A conjugating pair of paramoecium. One animal has three and the other two vacuoles.

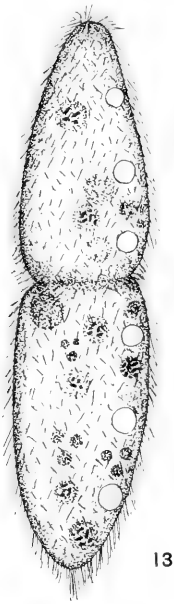
15 Drawn from a fixed and stained preparation showing the macronucleus and the single micronucleus.

16 Drawing made with the aid of an oil immersion lens showing the vacuoles to be surrounded by a morphological membrane.

17 A paramoecium dividing. Before division began this animal had five vacuoles arranged as in figure 7. A new vacuole has been added to either end.

18 A paramoecium with a greatly enlarged vacuole. This condition has been rare in my cultures. Occasionally the animals recover from this condition but more frequently they die with the vacuole still distended.

19 The mouth of this paramoecium has been forced out under pressure.



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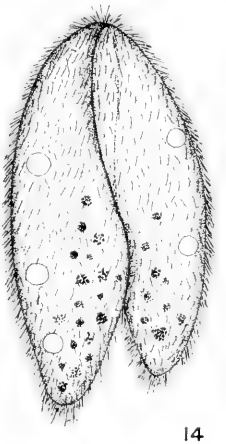

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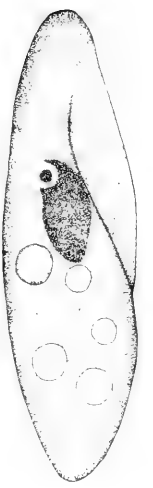

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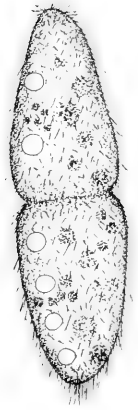

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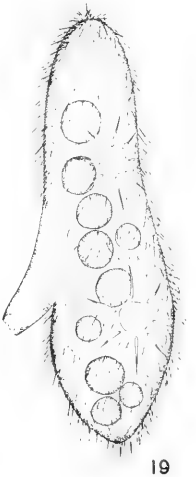

19

CONJUGATION AND ENCYSTMENT IN DIDINIUM NASUTUM WITH ESPECIAL REFERENCE TO THEIR SIGNIFICANCE

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CONTENTS

INTRODUCTION

That conjugation is a rejuvenating process of some sort or another has been very widely held. Engelmann ('76), Bütschli ('76), Maupas ('88 and '89), Hertwig ('89), Calkins ('06, '09, '13) and many others support this idea. Not all of these investigators agree with reference to the details concerning the rejuvenating effect, but practically all hold that if conjugation is prevented the race dies out, indicating that the progeny of a single individual passes through periods of vitality corresponding somewhat to youth, maturity and old age in metazoon individuals.

Calkins has very ably championed this view. He says ('09, p. 103) referring to the offspring of an exconjugant:

> If at any given period [we] could combine them in one mass of cells, we should have the analogue of a metazoon and would find that the protoplasm represented by the aggregate cells would manifest the same successive periods of vitality as those of youth, adolescence, and old age in Metazoa. We would find that the young cells divided more

rapidly than they do later in the cycle; we should find that after a certain time they become sexually mature and are able to conjugate and so to perpetuate the race; and we would find that, ultimately, evidences of weakened vitality and degeneration appear in the aggregate of cells, and that they finally die of old age.

Calkins consequently holds that conjugation causes an increase in the rate of fission and he supports this view by experimental evidence which will be presented later. In this contention, however, Calkins stands practically alone. Neither Maupas nor Hertwig, both of whom carried on very extensive experiments on various protozoa, were able to find any evidence indicating an increase in the rate of fission after conjugation. In fact, Hertwig actually found a decrease. But both found in their pedigree cultures that the race died out after a certain number of vegetative divisions and they maintain that this must have been due to the absence of conjugation. Consequently they concluded that conjugation, although it does not cause an increase in fission-rate, is a rejuvenating process which prevents the race from dying out.

This view received very serious opposition in the results obtained by Woodruff in an extensive series of experiments on Paramecium ('11, '12, '13, '14). These results prove conclusively that certain races of Paramecium can continue to reproduce indefinitely without conjugation.

Jennings has recently ('13) with characteristic thoroughness, investigated the problem of the significance of conjugation in Paramecium. He maintains (p. 293) that "Conjugation decreases the rate of fission, causes a great increase in variation in the fission rate, brings about many abnormalities, and greatly increases the death rate." And he concludes (p. 378)

> Conjugation does not produce rejuvenescence, for after conjugation most of the animals are less vigorous than before. *What conjugation does is to bring about new combinations of germ plasm, just as is done in the sexual reproduction of higher animals. One result of this is to produce biparental inheritance; another is to give origin to many variations; in the sense of inherited differentiations between different strains. Some of the new combinations are better adapted to the existing conditions than others; these survive while the others die out.*

Thus we find that Maupas obtained no increase in the rate of fission after conjugation and that both Hertwig and Jennings obtained a decrease. Calkins however still retains his original idea regarding the effect of conjugation on the rate of fission. He says ('13, p. 523)

> Experiments here re-described show that the vitality of a given race is increased by conjugation. An ex-conjufant from a pure line that had lived for 369 generations in culture, divided 376 times after conjugation in nine months, while the pure line control that had not conjugated, and from which the ex-conjugant was obtained, divided only 277 times in the same period.

Following Fermor ('13) Calkins has recently ('15) extended to encystment the idea that conjugation causes an increase in the vitality of the race. He maintains that there are two kinds of cysts, one kind serving merely to tide over periods of unfavorable environmental conditions, the other serving in the process of nuclear reorganization, structurally and physiologically. Thus he maintains that encystment at times has essentially the same function as conjugation, that it is a rejuvenating process resulting in an increase in the rate of fission. He presents experimental evidence in support of this contention and maintains that it is also supported by the discovery of Woodruff and Erdmann ('14) that in Paramecium reproducing vegetatively there are periods of depression ending in nuclear reorganization which is followed by increased vigor.

The experiments described in this paper were continued, with some intermissions, from April, 1910 to May, 1914. They were undertaken primarily to ascertain for Didinium, the relation between conjugation and the rate of fission and to ascertain in general the effect of preventing conjugation. Aside from the evidence bearing directly on these problems there was, however, considerable evidence obtained which has important bearings on other problems, notably, the function of encystment, vitality of cysts, mutations, the relation between conjugation and death-rate, and variability in the rate of fission. Questions concerning the vitality of cysts and mutations will be discussed in separate papers, which will appear elsewhere; all of the other problems will be considered in the following pages.

MATERIAL AND METHODS

Throughout the entire period covered by the experimental work connected with this paper numerous observations were made on didinia collected in various places and on mass cultures kept in the laboratory under various conditions. These observations concerned primarily the life history and the general physiology of the organisms, especially those phases of it which are associated with the processes of encystment and conjugation and the relation between these processes and the environment. The main part of the experimental work consisted, however, of the study of a series of groups of pure lines which were started and maintained as follows.

Four pairs of conjugating didinia were taken from a vigorous culture which had been in the laboratory for some time Each of these four pairs was then put into a rectangular watch-glass containing a few drops of solution and a considerable number of paramecia The watch-glasses were now piled one upon the other, placed into a damp chamber and later examined daily. Six of the ex-conjugants died within a few days. The other two divided and from each, two lines were started and labeled (conj.) indicating that conjugation had occurred. Thus there were four lines started with individuals which had conjugated. Simultaneously four lines were started with individuals taken from the same culture, but which had not conjugated. From this group of lines other groups were started from time to time as indicated in table 1.

All of the cultures were kept continuously in the same damp chamber and all were treated as nearly alike as possible They were fed throughout the entire experiment practically exclusively on paramecia, which were obtained from four cultures kept in liter jars These cultures were continuously maintained in the most uniform and vigorous condition possible During the early part of the experiment one-half of the solution in each of the four jars was replaced by standard timothy hay solution (100 cc. water + 1 gram hay, boiled ten minutes) at given intervals but that in each jar at a different time. During the latter

part the hay and water were added directly at various intervals depending upon the rate of development. This method was found to maintain the cultures more nearly in a uniform condition than the one first used. Moreover, in feeding the didinia, an equal amount of solution was always taken from two or more of the paramecia cultures and mixed in a separate dish, thus increasing the uniformity of the culture medium. Two drops, sometimes three, of this mixture which contained numerous paramecia were put into each of the required number of clean watch-glasses. A drop of solution containing one didinium was then taken from each of the pedigree cultures and put into each of the watch-glasses supplied with fresh food. After recording the number of fissions the remaining didinia were either destroyed or used in making further studies of conjugation and encystment. Nothing was sterilized in these experiments but the same pipet was used in transferring all of the didinia. Moreover, from time to time a drop of solution was taken from each of the pedigree cultures mixed and then a drop of the mixture added to each of the new preparations. Thus all were inoculated with the same bacteria and all were the same in other respects.

Throughout all the experiments the cultures were transferred and the number of generations recorded daily, twice a day, or every other day. During a greater part of the summer, when it was relatively hot, the fission-rate was so high that it was found advantageous to transfer twice a day, for eight or even nine generations, over 1000 individuals, were sometimes produced in twenty-four hours. During the rest of the year they were usually transferred daily. They were transferred every other day only during a few short periods, when the temperature was exceptionally low.

We shall present the results obtained in the experimental observations under several headings, as follows.

NATURE AND CAUSE OF ENCYSTMENT, VITALITY AND ACTIVATION OF CYSTS

When didinia are about to encyst they become quiet, loose their cilia, mouth and seizing apparatus and secrete a heavy wall about themselves. The wall usually adheres firmly to the substratum. Thus the cysts become fastened to various objects usually near the surface of the solution in which they live. Under certain conditions practically all of the didinia in a solution suddenly encyst, so that cultures having countless numbers of active didinia at a given time may have practically none a few hours later.

Encystment can usually be induced at any time by cutting off the food supply. But it frequently occurs when there is an abundance of food present and sometimes it does not occur when there is none. This is especially true for cultures in which conjugation has been prevented for a considerable number of generations. In fact, in lines which had passed through 1500 to 1600 generations it was almost impossible to induce encystment.

To induce the didinia to come out of the cysts it is usually only necessary to add a vigorous culture of paramecia, or the solution from such a culture. The paramecia are not absolutely necessary. Active didinia are ordinarily found twenty-four hours after the solution has been added; but at best only a very small percentage of the cysts develop. Some can, however, live a long time and drying under atmospheric conditions is not fatal. In an experiment described elsewhere, cysts kept sealed air-tight in a 10 cc. vial for nearly five years were still found to be viable.

Didinia are consequently very hardy and they are, as far as known, destroyed by only two organisms; a small rhizopod which probably develops inside of the cysts, devours the protoplasm and then bores its way out through the wall and a hypotricate which swallows them whole in considerable numbers.

Encystment clearly serves to bridge over unfavorable environmental conditions, and it unquestionably facilitates distribu-

tion, especially when the cysts are dry. Calkins maintains that it also functions as a rejuvenating process. Our results do not, however, support this contention, as will be demonstrated later.

NATURE, CAUSE AND DURATION OF CONJUGATION

Didinia that are about to conjugate divide in rapid succession two or three times without taking any food. During this process they become much reduced in size; the protuberance at the anterior end bearing the mouth becomes considerably enlarged and somewhat flattened; and when they are ready to conjugate they have a peculiar jerky movement. Such individuals are consequently readily recognized. They are usually found in restricted areas in the cultures. In watch-glasses having numerous didinia fairly uniformly distributed, I have repeatedly seen numerous individuals, some conjugating and others ready to conjugate, all crowded together in one small spot and none elsewhere. Whether these aggregations are due to a restriction of the preliminary processes to specimens in these regions or to reactions in the preconjugants which result in the aggregation, I am unable to say. However, the fact that they tend to remain in the region seems to indicate that they aggregate. They probably secrete some sort of substance, when they are in the conjugating state which acts on them as a weak acid acts on paramecia, but which does not affect individuals which are not ready to conjugate. The preconjugants in the restricted area swim about rapidly, continuously stopping and turning in various directions. Thus they frequently collide and if in these collisions they chance to meet mouth to mouth, they usually remain united owing to the adhesive character of the mouth during this period. Frequently the mouth of the two conjugants is not directly opposite and occasionally three individuals become united. Thus we see in these simple creatures, special provisions which facilitate the unions of sexually mature individuals, provisions which are in some respects similar to those found in the higher animals.

At first union is very weak so that the conjugants can readily be separated by squirting them out of a pipet. Later they become so firmly united that they cannot be separated without injury. The conjugating pairs continue to swim about, but their movements are, owing to their relative position, necessarily more or less uncoördinated. Usually one proceeds forward for a time and then the other. Thus they rarely get any considerable distance from the place where union occurred. The duration of the union varies greatly. It depends largely upon the temperature. Occasionally, pairs are found which never separate. But at room temperature, they usually remain united only about twenty to thirty hours. During this time there is nuclear transfer similar to that found in Paramecium (Prandtl, '06). All of the steps involved in this process, degeneration of macronucleus, divisions of micronucleus, etc., occur after the union of the conjugants takes place. Whether or not there is anything in the nature of nuclear reorganization in conjugants which are artificially separated is not known.

The ex-conjugants usually begin to feed about one hour after they separate. Then, after growing rapidly for about two hours, they divide, after which they proceed as usual. If they are not allowed to feed after conjugation occurs they do not divide. Nor do they encyst. Some of them live a week or more but all invariably die without fission if they are not fed. This was repeatedly observed and it was found to hold without exception.

The discovery that ex-conjugants die if they are not fed greatly facilitated the process of continuing, for a long time, pedigree cultures without conjugation; for such cultures were at different periods maintained in the encysted state, and it was often difficult to obtain cysts without at the same time having conjugants which were difficult to eliminate before it was known that they die if they are not fed.

The elimination of food, subjection to optimum temperatures and the presence of numerous individuals in a small space facilitate conjugation just as it does in Paramecium (Jennings, '10). This process, however, often occurs in the presence of much food with very few (4 to 8) individuals present, and it

frequently does not occur in the absence of food, no matter how many are present or what temperature is employed. In fact I was quite unable in many instances to induce conjugation. It is evident, therefore, that conjugation depends upon internal as well as upon external factors, but what these factors are is as yet unknown. The results of my experiments seem to indicate that conjugations can be induced more readily in lines in which it has been prevented for long periods than in lines in which it has recently occurred. They indicate, however, that there is no specific relation between the tendency to conjugate and the number of consecutive asexual generations produced in a line; for in several instances I obtained conjugation in abundance in lines which had produced less than 100 consecutive asexual generations and in others I was unable to induce conjugation in lines which had produced more than 1000 such generations. Moreover, in certain lines in which conjugation occurred among the accessory specimens at a given time it was not possible to induce conjugation during certain later periods, i.e., after more asexual generations had been produced. Furthermore, Calkins ('15, p. 238) obtained conjugation in individuals which had produced only two asexual generations; and Jennings ('10) obtained similar results in Paramecium.

THE EFFECT OF CONJUGATION AND ENCYSTMENT ON THE RATE OF FISSION

All of the evidence obtained on the relation between fission-rate and conjugation and encystment is presented in table 1. This table contains a brief history of all of the groups of pure lines that were studied. In it are given the time each group was isolated in relation to the time of conjugation and encystment, the ancestry and duration of each, the number of lines each contained and the average fission and death-rates.

An examination of table 1, with some explanatory additions, shows the following: In April, 1910, two groups of four lines each were isolated, one immediately, the other some time after conjugation. After these two groups had continued for thirty days the latter was closed. During this time the fission-rate

in the conjugating group was slightly lower than it was in the non-conjugating group and the death-rate was also somewhat lower. On May 20 all of the dishes containing the didinia belonging to both groups were returned to the damp chamber after removing one specimen from each to continue the lines. In all of the dishes returned the didinia soon became very numerous and two days later conjugation occurred in one dish of each group. Fifteen conjugating pairs (30 individuals) from the non-conjugating group were isolated. Twelve of these individuals died without dividing. From the remaining individuals, four were selected to start a new group of five lines as represented in the table. During the next ten-day period the rate of fission in these lines, which had just conjugated was somewhat lower than it was in those which had conjugated forty days earlier, but during the following period it was a trifle higher. At the close of this period, June 11, all of the didinia were left in the dishes. They became very numerous and soon consumed all of the paramecia after which many of them encysted. The dishes containing the cysts were then properly labeled, sealed with paraffin and vaseline and stored away in the damp chamber. Toward the last of April, 1911, vigorous paramecia cultures were added to all of the dishes, and the following day active didinia appeared in one belonging to the more recently conjugating group. None appeared in any of the other dishes.

TABLE 1

Effect of conjugation and encystment on fission-rate and death-rate in Didinium

The large numbers in the columns represent the total average number of fissions in a group of lines for the period given above. The small numbers directly below the large ones indicate the number of lines in the group present at the beginning of the period; wherever these are missing the number of lines in the preceding period has been maintained. The small numbers followed by 'd' indicate the number of lines which died out and those followed by 'c' the number which encysted during the period. The brackets and arrows indicate the origin and ancestry of the different groups of lines. *Conj., Conj. sep., small, large,* signifies that the numbers following each refer to a group of lines which originated respectively from ex-conjugants, conjugants separated before nuclear transfer occurred, small individuals ready to conjugate, and large individuals not ready to conjugate. *Cyst.* signifies that the group of lines represented by the preceding numbers were encysted during the period indicated above.

TABLE 1

TABLE 1—Continued

The didinia soon became numerous and in a few days many conjugating pairs were discovered. In the meantime, they had been examined at intervals short enough to make it certain that conjugation had not previously occurred. Similar precautions were taken throughout the entire series of experiments.

From the culture containing the conjugating specimens five groups of lines were started May 2, 1911, as indicated in the table. The lines in two of these groups contained individuals which had conjugated normally (ex-conjugants); those in one group contained individuals which had been mechanically separated immediately after they had joined in the process of conjugation (conjugants separated or 'split pairs'); those in another group contained individuals which showed by their size and action that they were ready to conjugate (small); and those in the fifth group contained individuals which showed no indication of preparation for conjugation (large).

The table shows that these five groups in the beginning consisted respectively of 70, 20, 20, 13 and 10 lines, and that during the first ten-day period the fission-rate was somewhat lower in the two conjugating groups than it was in the three non-conjugating groups but that for the following three periods it was remarkably nearly the same in all.

At the close of these periods all of the active didinia died owing to excessively hot weather during which the temperature reached 35° several times. Cysts, however, which had formed in some of the dishes retained for special study after isolating individuals for propagating the lines, were not injured, and from one of these, four new groups of lines were started.

Thus, the experiment was continued, groups of new lines being started from time to time, some from individuals which had recently conjugated, some from conjugants separated, some from individuals ready to conjugate, some from large specimens which were not ready to conjugate and some from specimens which had been encysted for various periods of time, as indicated in the table. Consequently there were present throughout the entire experiment continuously, several groups of lines which differed markedly with reference to the number of generations since

encystment or conjugation had occurred in them. And since these groups all originated in the same individual and were carried along parallel with each other and treated alike, it is obvious that the rate of fission ought to differ in them if conjugation or encystment has any effect on it.

The detailed description, given above, of the earlier periods shows that if conjugation had any effect on the rate of fission during these periods it was a retarding effect which, however, continued for but a short time after conjugation. An examination of the table shows that the same may be said regarding all of the other periods. It shows also that encystment has no appreciable effect on the rate of fission. Take for example, the five day period (9/22–9/26, 1911) or better still, the twenty day period (9/7–9/26, 1911); here we find that the seven groups of lines produced during this period the following average number of generations respectively: 76, 75, 74, $74\frac{3}{4}$, 76, 73, $75\frac{2}{3}$, a maximum difference of only three divisions in twenty days, while the number of generations since conjugation had occurred in these groups of lines varied from 118 to 524 and the number of generations since encystment from 75 to 236. Moreover, the rate of fission is not greatest in the groups in which the period considered is nearest the point of conjugation and encystment or least in those in which it is farthest from these points.

The sixty day period immediately following (6/1–7/30, 1912) shows even a more striking similarity in the rate of fission in lines which have conjugated compared with those which have not. Here we find that a group of lines in the sixty days immediately after conjugation produced an average of 248 generations and that during the same period lines from the same stock but in which conjugation was prevented produced an average of $249\frac{3}{5}$ generations. Again, near the close of the experiment we find that in a twenty day period (5/9–5/29, 1913) lines immediately after conjugation produced 32 generations, while the stock from which they were isolated, after having passed through over 1,500 generations without conjugation, produced in the same period and under identical conditions, $33\frac{3}{5}$ generations. There is consequently no evidence whatever indicating that conjuga-

tion causes an increase in the rate of fission in accord with Calkins' contention and the same may be said with reference to encystment.

Calkins maintains that in Didinium the rate of fission is relatively high immediately after encystment; that after continuing for about 100 generations it becomes lower and continues to decrease for 30 to 50 generations when encystment again occurs, during which there is a nuclear reorganization resulting in rejuvenescence and an increase in the rate of fission. There is not the slightest evidence indicating that anything of this sort occurred in our experiment. If encystment affects the rate of fission there ought to be some evidence of it in the periods following (9/6, 1911) but there is none, as an examination of the results presented in the table for these periods clearly shows. Moreover, at the close of the last part of the experiment, three groups of lines had passed through 1035, 831 and 850 generations respectively, without encystment or conjugation, proving conclusively that if there are cycles with reference to encystment in accord with Calkins' contention, they are very different from those he describes.

It is true that toward the close of the experiment, after a long period without conjugation or encystment, the didinia seemed to lose their accustomed vigor, but neither conjugation nor encystment served to remedy matters as a comparison with the rate of fission in the wild groups during these periods shows. In reference to this the following extract taken from my notes is illuminating. It refers to the last periods of 1913, and gives a good idea of the condition of the didinia toward the close of the experiment.

All individuals not used to continue the pedigree lines were retained in their respective dishes. These were all examined from day to day. Conjugation occurred very freely in all but the wild lines, encystment very rarely. The didinia in all of these cultures eventually died out except a few cysts. Conjugation did not save them. Paramecia from five different cultures were tried as food also some paramecia fed on malted milk but the didinia still died. The individuals in the old lines were much smaller than those in the wild lines, the cysts were also relatively small and there was a strong tendency to produce monsters. There was no observable difference regarding this between the

lines which had passed through 1559 generations and those which had passed through only 850. Various means were employed in attempting to induce encystment, but except in the wild lines in which it occurred freely, only a few were secured, none at all in the 850 generation group.

All of the cysts produced in the old lines were carefully preserved. The following year vigorous cultures of paramecia were as usual added to all of the dishes. Active didinia appeared in only one of twelve dishes containing cysts of the old culture, while in those containing the cysts of the wild culture, treated precisely the same, they appeared in practically all. From the didinia thus obtained two new groups of lines were started as indicated in the table. In both groups the rate of fission and the condition of the didinia was practically as it had been before encystment. There was no appreciable improvement in the old culture. The death-rate was not very much higher in this than it was in the culture from the wild race but the fission rate was much lower and there was no noticeable change in the condition of the specimens produced. These lines were carried along for forty days and then abandoned. They did not die out, but it is, of course, impossible to say whether or not they would have recuperated, if the experiment had not been closed.

As stated above, individuals recently isolated from a wild culture were much more vigorous than those in the lines long continued without conjugation, but the individuals in lines started from another wild culture were even less vigorous. These lines continued from 1/17 to 5/29, 1913. By referring to table 1 it will be seen that in them the fission-rate was considerably lower and the death-rate was much higher than it was in the lines long continued without conjugation, and that they died out at the close of the period, owing to the fact that it was impossible to induce them to encyst.

Thus we see that in these experiments there is no support for Calkins' contention concerning the effect of conjugation and encystment on the rate of fission. Nor is there any support for the contention of Maupas and others that protozoa must conjugate from time to time in order to continue the race, that is, that there are cycles consisting of vegetative and sexual reproduction

alternating; for an organism that can produce 1646 vegetative generations can in all probability continue reproducing asexually indefinitely. There is, in fact, no evidence of cycles anywhere in the entire experiment. Toward the close there was to be sure a loss in vigor, but the loss was quite as great in the group of lines which had produced only 850 generations as it was in the one which had produced 1559 generations.

But if conjugation and encystment do not cause an increase in the rate of fission, how can the following results obtained by Calkins be explained? And if conjugation is not a rejuvenating process, what is its function?

Calkins in 1904 cultivated on slides a line of paramecia for 369 generations; then he started a new line from some of the individuals of this line, after they had conjugated, and carried it along parallel with the old line for nine months. During this time the former (ex-conjugant) produced 396 generations and the latter (non-conjugant) only 277 (Calkins and Gregory, '13, p. 517).

These results seem strongly to support Calkins' conclusion that conjugation causes an increase in the vitality of protozoa. The support is, however, not so strong as it seems. In the first place, both lines were not treated identically. "The ex-conjugant was treated for twenty-four hours with beef extract on December 9th, the A series (non-conjugant) with beef extract on December 14th, January 8th and 15th." It is not possible to say what effect this different treatment may have had, and it is, therefore, evident that it may have caused the observed difference in rate of fission. In the second place, Calkins and Gregory maintain that in different lines originating in the same individual there is a marked difference in the rate of fission. They say ('13, p. 477) concerning four such lines, A, B, C and D "In one hundred days a typical representation of A would have divided 65 times, of B, 90 times, of C, 81 times and of D, 95 times." Accordingly, if D had produced 396 generations, the number produced by Calkins' ex-conjugants mentioned above, A would have produced only 270.9 generations, that is, six generations less than Calkins' non-conjugants. It is con-

sequently evident that here greater variation was found in four non-conjugant lines than was found in the ex-conjugant and non-conjugant lines mentioned above. Obviously then, the fact that the rate of fission differed in these lines adds extremely little support to the conclusion that conjugation causes an increase in vitality in protozoa.

Concerning the significance of the evidence which Calkins presents in support of the contention that encystment at times causes an increase in the rate of fission, the following may be said: Calkins kept didinia continuously in spring water which was frequently changed. To this he added from day to day a few paramecia. He maintains that, under such conditions, the rate of fission decreased while the rate of encystment increased until after the production of 84 to 148 generations all were encysted. He then poured off the old water and added "fresh water and Paramecium." This caused the didinia to come out of the cysts after which, he maintains, the rate of fission was again practically the same as it had been in the beginning. And he holds that this shows that encystment results in rejuvenescence.

The result of Calkins' experiments seem to show fairly clearly that there is a decrease in the rate of fission under the conditions stated and that there is an increase after encystment, but they do not seem to me to show that this increase is necessarily due to encystment. To recover the race from encystment Calkins, as stated above, replaced the solution by fresh water (he does not say what kind) and paramecia (presumably many). Thus he subjected the organisms to a marked change of environment. Now, Calkins and others have shown that just such changes cause definite increase in the rate of fission in Paramecium under conditions similar to those in which the didinia were before encystment. Is it not therefore reasonable to assume that the increase in the rate of fission in Didinium noted by Calkins was due to the change in the environment rather than to encystment? At any rate, we have demonstrated that Didinium can produce over 1000 generations without any apparent periods of depression except at the close and that after having produced

many generations thus it was almost impossible to induce encystment. Calkins obtained even a more marked effect of continued culture on encystment. He says ('16, p. 266): "In my experiments with Didinium the race apparently lost its power to encyst and ultimately died out after six months' culture without encystment." If there really are in Didinium, cycles ending in encystment as Calkins maintains, they are very long and if encystment results in better adjustment to the environment during periods of depression, if it is a rejuvenating process, why was there not a strong tendency to encyst near the close of our experiment when the vitality of the didinia was low, instead of no such tendency whatever?

Calkins also obtained other results which do not seem to be in harmony with his conclusions. He says ('15, p. 238):

> During the first cycle no conjugating pairs were observed in any of the stock dishes although such material is prepared daily and always watched for at least five days. During the first week of the second cycle, epidemics of conjugation appeared in the stock dishes. This period of conjugation lasted about ten days, after which not a pair was seen. Conjugation epidemics appeared again in the third cycle and at a corresponding time. The first pairs were seen in the stock dishes on the third day after recovery from encystment (March 12) and pairings occurred in great numbers until March 20th, after which not one pair could be obtained from the material. During the height of the epidemic in the stock material two cases of conjugation occurred in the isolation cultures. One of these pairs (March 16) was the union of two individuals out of eight derived from one individual isolated the day before. The second case occurred on March 17 between two individuals among sixteen derived from an individual isolated the day before.

In the first cycle referred to the didinia produced 131 generations and then encysted; in the second they produced 148 generations. In each case conjugation occurred shortly after the didinia came out of the cysts. In my work I observed repeatedly a strong tendency to conjugate shortly after encystment, thus confirming the results obtained by Calkins. But if encystment and conjugation have the same function, if both are rejuvenating processes, why was there such a strong tendency to conjugate soon after "recovery from encystment?" And why was

there no tendency to conjugate later in the cycle, especially at the close when all of the individuals encysted?

An examination of table 1 shows that in Didinium there was great variation in the rate of fission in different periods. These variations were largely due to changes in temperature. Woodruff maintains that there are similar variations in the rate of fission in Paramecium, but he holds that there are certain fluctuations in this rate in the absence of any variation in temperature or other environmental factors. These he calls rhythms. He says ('11, p. 353): "[Rhythms] are due to fundamental factors in cell phenomena and not to extraneous causes." For all that is known to the contrary there may have occurred in Didinium variations similar to those called rhythms by Woodruff. But if there were any such fluctuations they were either exceedingly small or they occurred simultaneously in all of the lines running parallel, regardless as to the difference in the number of generations produced by these lines since conjugation or encystment had occurred. Consequently, if there were such fluctuations of any considerable magnitude they could not have been specifically related to conjugation or encystment with reference to time.

By referring to table 1, it will also be seen that in 1912, 7/15–7/20, one of the groups of lines divided into two groups having different rates of fission. These two groups of lines originated from a single individual during a period of abnormally high temperature. They were carried for 315 days during which time one group produced a total average of 838 generations ($2\frac{2}{3}$ per day) and the other a total average of 634 (2 per day). The difference in the two groups was practically the same throughout the whole period and it appears to have been permanent. The origin and significance of this mutation will be more fully discussed in a separate paper.

As indicated in the table the pedigree culture continued with certain intermissions from April, 1910 until May 1914. During this time there were produced in one of the groups of pure lines an average of 1646 generations without conjugation and in the same group of lines 1035 generations without encystment. The

stock became very weak toward the close but it did not die out, and, of course, it is not known how much longer it would have survived. The fact that it continued so long without conjugation or encystment seems to indicate that neither of these processes is necessary for continued existence.

This conclusion is in perfect harmony with what is known regarding the life-history of many of the lower plants and with the results obtained by Woodruff in very thorough and extensive experiments on Paramecium. Woodruff's conclusion is, however, open to criticism. He says, after having obtained 3029 generations without conjugation ('12, p. 123): "I believe this result proves beyond question that the protoplasm of a single cell may be self-sufficient to reproduce itself indefinitely, under favorable environmental conditions, without recourse to conjugation, and clearly indicates that senescence and the need of fertilization are not primary attributes of living matter." That the protoplasm of certain single cells is self-sufficient to reproduce itself without recourse to conjugation has, in my opinion, long since been known, for nothing in the nature of conjugation has ever been discovered in bacteria and in a considerable number of algae. If this is true, Woodruff's results merely support a well established conclusion regarding the necessity of conjugation for the continued existence of protoplasm; they make it possible to add Paramecium to the list of organisms in which conjugation has been found not to be necessary for continued reproduction.

THE EFFECT OF CONJUGATION ON DEATH-RATE

Jennings ('13, p. 293) found, in extensive and very thorough experiments with Paramecium that "Conjugation decreases the rate of fission, causes a great increase in variation in fission rate, brings about many abnormalities, and greatly increases the death-rate." He concludes (p. 378), as previously stated, that conjugation does not produce rejuvenescence but that it functions in the production of new combinations which are better adapted to the existing conditions than others and that these serve to perpetuate the race while the others die out.

The results obtained in our experiments with Didinium do not support these contentions. We have already demonstrated that conjugation in Didinium causes only a slight decrease in the rate of fission, if any at all, and in this and the following sections

TABLE 2

The effect of conjugation on death-rate

Conjugants separated signifies that conjugants were mechanically separated before fertilization; *small* signifies that the individuals were ready to conjugate; and *large* that they were not ready to conjugate. The numbers in all cases refer to individuals, not to pairs. The number died includes all individuals which did not divide after conjugation or after they were isolate, in case there was no conjugation.

DATE	EX-CONJUGANTS		CONJUGANTS SEPARATED		SMALL NON-CONJUGANTS		LARGE NON-CONJUGANTS	
	Number isolated	Number died	Number isolated	Number died	Number isolated	Number died	Number isolated	Number died
1910, 2/28	12	11						
1910, 4/17	8	6						
1910, 4/25	40	29						
1910, 5/23	12	5						
1911, 2/10	14	6						
1911, 4/30	70	48						
1911, 5/1	20	12	20	12	15	9	10	0
1911, 6/18	54	11	36	19	10	5	8	3
1911, 6/27	36	2	20	0			5	0
1911, 7/29	20	0						
1911, 8/2	24	9	2	1	5	0		
1911, 8/10	24	24					5	0
1911, 8/26	2	0	6	0			3	0
1912, 6/3	44	0	22	0			5	4 encysted
1912, 6/6	20	0	28	0			5	4 encysted
Total.....	400	163	134	32	30	14	41	3

it will be demonstrated that it causes no appreciable increase in variation in the rate of fission or in death-rate.

The results obtained on the relation between conjugation and death-rate are, as previously stated, presented in brief form in table 1. A portion of these results is given in detail in table 2. This table contains a record of all isolated ex-conjugants which died without fission and of all other isolated specimens which

died during the same periods. By referring to the table it will be seen that a total of 400 individuals which had conjugated were isolated and that 163, or 47.5 per cent of these died without fission; that a total of 134 conjugating individuals in which nuclear transfer was prevented were isolated and that 32, or 23.8 per cent, of these died without fission; that a total of 30 small individuals ready to conjugate were isolated and that 14, or 46.6 per cent of these died; and that a total of 41 large individuals not ready to conjugate were isolated and that only 3, or 7.3 per cent of these died.

These results show that the death-rate in the individuals not ready to conjugate was very much lower than in those ready to conjugate and they also show that the death-rate in the ex-conjugants taken as a whole was considerably higher than it was in the separated conjugants and those ready to conjugate. A further study of the table shows, however, that conclusions based on a consideration of the experiments taken as a whole are misleading. This is due to the fact that in a number of experiments in which only ex-conjugants were isolated, the death-rate was on an average higher than in the others. It is obvious, therefore, that these experiments should be omitted in the calculations. When this is done it leaves a total of 200 ex-conjugants of which only 34, or 17 per cent died without fission. That is, 5.8 per cent less than the percentage of deaths in the conjugants which were separated without nuclear interchange and 26.9 per cent less than that in the small individuals ready to conjugate. These experiments, therefore, indicate that conjugation produces a decrease rather than an increase in death-rate. They indicate, however, that the preliminary steps in the process of conjugation do cause an increase in death-rate, for in the large specimens in which no preparation for conjugation had occurred the death-rate was much lower than it was in those in which such preparations had occurred.

In the ex-conjugants and the small individuals ready to conjugate or separated immediately after conjugation the variation in death-rate was very large. In some experiments all died, in others none. In the large individuals not ready to conjugate

it was much smaller. I believe the great variation in the death-rate of the former was largely due to the fact that in the process of isolation the individuals were transferred to solutions consisting of various degrees of dilution of the culture solution in which conjugation occurred. The results of observations made on this point, seem to show that the less the dilution of the solution which induces conjugation the lower the death-rate. If conjugation results in better adaptation to the environment in which it occurs in accord with the contention of Calkins then this is just what would be expected. My observations were, however, not extensive enough to settle the question. I refer to them here merely to indicate that the problem is subject to experimental analysis with the hope of encouraging someone to undertake its solution.

THE EFFECT OF CONJUGATION ON VARIATION IN THE RATE OF FISSION

All the essential data obtained in regard to the relation between conjugation and variation in the rate of fission are brought together in table 3. This table shows the interrelationship of the different groups of lines; the number of lines in each group the coefficient of variation to three decimal places and the dates of the different periods. The standard deviations were also calculated for all of the periods. They were, however, in such close agreement with corresponding coefficients of variation that their reproduction in the table would have been superfluous.

By comparing the coefficients of variation for the ex-conjugants with the corresponding coefficients for the non-conjugants and conjugants separated, it will be seen that while in some instances the variation is greater in the ex-conjugants than in the

TABLE 3

The effect of conjugation on variation in the rate of fission in Didinium

Each of the large numbers in the columns is the coefficient of variation for the fission-rate in the different lines of a given group of lines. They represent the variation in the total number of fissions produced by the different lines in the groups during the periods indicated above. The small numbers directly below each coefficient represents the number of lines involved in the calculation of the coefficient. The brackets and arrows show the origin and the ancestry of the different groups of lines. *Conj.*, *Conj. sep.*, etc., signify the same as in Table 1.

TABLE 3

DATE ......

1910

Wild Culture		4 14-?8	4 29-5 ?	5 8-18	5 ?4-?	6 ?-?1	
	Conj	4 563 4	0 2	11 111 2	5 856 4	4 923 3	
	Large	4 757 4	3 090 4	6 666 [illegible]			
				Conj ↓	10 933 4	4 856 4	Cyst

1911

	5 2-11	[illegible]	[illegible]	6 1-10	
Conj ...	9 372 18	6 093 8	6 ?0? 6	3 182 6	→ Cyst..
Conj ...	55 328 8	10 136 3	0 [illegible]	0 500 [illegible]	
Conj sep ...	4 273 7	9 649 7	? 603 [illegible]	5 494 2	
Small	0 4	3 749 3			
Large	3 117 16	2 381 5	2 477 [illegible]	1 774 3	

	6 18-27	6 28-7 7	7 8-17	7 18-27	
Conj	3 327 8	5 884 6	0 900 2	0 2	
Conj sep .	8 205 4	4 164 4			
Small ...	3 970 4	7 033 5	0 1	1 032 3	Cyst
Conj		4 521 4	0 407 3		
Large	4 902 3	4 367 6	3 992 4	3 370 2	Cyst
Conj sep		2 268 4	9 090 2	0 1	Cyst ...

	8 3-7	8/8-12	8/13-17	8 18-22
Conj ..	6 133 2	10 846 3	2 040 2	2 439 2
Conj sep	7 317 2	1 754 [illegible]	0 3	4 561 3
Small	2 439 2	1 886 2	1 960 2	0 1
Conj	4 347 2	3 448 2	1 860 3	4 761 2
Cyst	—	—	—	—

	8 23-27	8 28-9/1	9 2-6	9/7-11	9 12-16	9 17-21	9 22-26
Conj	6 666 2	2 702 2	2 222 2	2 040 2	0 2	0 2	2 564 2
	Conj	0 2	0 2	2 040 2	3 333 2	3 030 2	0 1
	Conj sep	17 647 2	2 127 2	6 666 2	3 225 2	0 2	5 263 2
	Cyst	—	→	4.166 4	2.035 3	5.103 3	0 3
	Cyst	—	→	1.911 3	0 3	2 886 3	0 3
	0 1	0 2	2 222 2	2 127 2	6 666 2	0 3	2 702 2
	—	—	→	1 911 3	6 148 3	2 828 3	0 2

1912

		6 6-10	6/11-15	6 16-20	6 ?7-?5	6 26-30	7 1-5	7/6-10	7/11-15	7/16-20	7/21-25	7 26-30
	Conj ...	3 402 5	2 815 5	2 564 4	1 990 4	1 724 5	1 834 5	2 631 5	5 194 5	2 898 5	3 535 4	2 325 2
Cyst	Conj sep ...	0 5	2 196 5	2 020 5	2 280 5	2 093 5	2 874 5	2 631 5	0 4	1 506 4	2 401 5	0 2

others, e.g. (1910, 5/24–6/2), (1911, 5/2–11), (1911, 6/28–7/7) the opposite is true in others, e.g. (1911, 5/22–31), (1911, 6/18–27).

Numerous other similar instances appear throughout the whole table. I would call attention in particular to the results obtained in 1912, 6/6–7/30. In this whole series there is very little difference between the coefficients of variation in the ex-conjugants and the conjugants separated. In the first three periods it is a trifle larger in the former, in the next three a trifle larger in the latter, etc. Five lines were maintained in both groups throughout and there were very few deaths in either. This is, consequently, a very good test case. Taken as a whole the table shows conclusively that ex-conjugants in Didinium are certainly not consistently more variable in respect to the rate of fission than are the non-conjugants.

The evidence presented in the three tables seems to show clearly that conjugation in Didinium has no appreciable effect on the rate of fission or on variation in the rate of fission, and that if it has any effect on death-rate it is a retarding effect. In these respects there is a marked divergence between our results and those obtained by Jennings on Paramecium. It seems quite remarkable that in two closely related organisms the effect of an apparently fundamental process should differ so greatly.

The results taken as a whole seem to show conclusively that neither conjugation nor encystment are rejuvenating processes, at any rate, not in the sense in which Calkins has used the term: namely, to indicate a nuclear reorganization in which accumulated waste materials are eliminated. And they are clearly not in accord with the results obtained by Jennings in experiments on Paramecium. They do not, however, overthrow his theory of the functions of conjugation.

Jennings assumes that the production of favorable characters is due, in the process of conjugation, to the union of nuclear substances which differ in potency. If therefore, the nuclear potency of the conjugants is the same, one would not, in accord with Jennings' contention, expect any favorable effect. The didinia used in the experiments described in the preceding pages were very closely related. It may, consequently, be maintained

that the failure to obtain any effect by conjugation was due to the similarity of the nuclear potency of the conjugants. If this is true it is obvious that our results do not militate against the contentions of Jennings as set forth above.

SUMMARY

1. Various groups of pure lines of didinia, all but the first two originating from a single individual, were propagated in parallel series and studied, with certain intermissions, from April, 1910, till May, 1914. During this time there were produced without conjugation in one of the groups an average of 1646 generations per line and without encystment an average of 1035 generations per line. At the close the stock was very weak but it did not die out. It is, therefore, not probable that either of these processes is necessary for continued existence in Didinium.

2. From time to time throughout the entire experiment new groups of lines were started from old ones, some after conjugation, others after encystment and still others without either conjugation or encystment. There were, consequently, continuously present a number of groups of lines which differed in the number of generations produced since conjugation or encystment had occurred. In some instances this difference was very great.

3. The rate of fission varied greatly throughout the experiment, owing largely to changes in temperature but at any given time it and also the death-rate were practically the same for all of the groups of lines regardless of the distance removed from conjugation or encystment. There was, therefore, no evidence indicating the presence of cycles related to these processes.

4. There was no evidence obtained indicating that conjugation or encystment has any appreciable effect on death-rate, fission-rate or variation in fission-rate. This would indicate that neither of these processes is a rejuvenating process, at least not in the sense in which Calkins has used the term.

5. In one of the groups of lines, 721 generations after conjugation and 197 generations after encystment, some of the offspring suddenly began to divide more rapidly than others. The difference in the rate of fission in these two sets of individuals

remained fairly constant throughout the remainder of the experiment, 315 days. During this time one set produced an average of 838 generations per line, $2\frac{2}{3}$ per day, the other an average of 634 generations per line, 2 per day.

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THE PHOTOKINETIC REACTIONS OF FROG TADPOLES

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While one of us (Cole) was studying the problem of functional regulation following grafts over the eyes of frog tadpoles (to be reported later), it was noticed that the animals became more active when being examined under strong illumination. The increase of light intensity seemed to cause an increase in the rate of swimming. A review of the literature, however, revealed no evidence that the frog tadpole is sensitive to light. In fact Franz ('10 and '13), and Laurens ('14) state that it is indifferent, showing no reaction whatever. Since the adult frog is positively phototropic (Parker, '03), and since the young stages of other Amphibians are either positive or negative to light, (Banta and McAtee, '06, for Sperlerpes; Eycleshymer, '08, for Necturus; and Laurens, '14, for Amblystoma), it is natural to suppose that the tadpole of the frog would be sensitive to light, at least during some stages of its development. We therefore undertook the experiments reported here to determine this question.

The reactions of adult frogs and other Amphibians to light have been studied by several investigators, and a review of the literature is given by Pearse ('10). Suffice it to say here that most Amphibians are either positively or negatively phototropic, some of them, especially Cryptobranchus (Pearse, '10), being also photokinetic. The larval stages have been studied by Banta and McAtee ('06), who reported that Sperlerpes larvae are much more sensitive to light than the adults; by Eycleshymer ('08), who demonstrated a negative phototropism for Necturus larvae; by Franz ('10 and '13), and Laurens ('14), who stated that frog tadpoles are not sensitive at all. In 1905 Parker reported the 'photodynamic' reactions of ammocoetes,

the larva of Petromyzon. The photokinesis of other animals has been studied, the most recent report being that for Paramecium by Walton ('16). The results of our experiments show that frog tadpoles are sensitive to light during the mid-larval stages, the reaction becoming a phototropic one just previous to metamorphosis.

We wish to express our sincere thanks to Prof. M. W. Eddy for extending to us the privileges of the Zoölogical Laboratory of the Pennsylvania State College, and to Prof. G. H. Parker of Harvard University for a careful reading and criticism of the manuscript.

All the tadpoles used were between 40 and 60 mm. long, a stage later than that used by Laurens. A definite determination of the species was not made, but since all the adults found in the same pond where the tadpoles were taken, were Rana clamitans, Latreille, it is very probable that the larvae were of the same species. They were collected in the fall and kept in large aquaria throughout the winter. As far as one could tell, the animals remained in a normal condition, feeding on algae, water cress and the remains of small crustacea. Individuals used in the trials were kept in separate jars, so that repeated trials could be made and compared. The results have been grouped together, however, since the individual differences in the reactions were small.

APPARATUS AND PROCEDURE

For the trials a blackened rectangular glass jar 260 by 200 by 150 mm. was used. It was partitioned longitudinally with a blackened glass plate making the smaller section 50 mm. wide. Light was admitted through a circular unblackened area at one end on a level with the bottom.

Light of three intensities was furnished by a small arc lamp of 500 candle power, and two Spencer microscopic lamps, with condensing lenses, of 100 and 48 candle power. The size of the beam was controlled by slips of blackened cardboard containing various sized openings. A blackened 8 candle power lamp, giving just enough light to enable the observation of the animals'

movements, was used for the periods of 'darkness.' With the aid of a dark box and suitable black cloth screens all other light was prevented from entering the jar.

In order that the animals might become adapted to the new conditions they were placed in the jar singly at least an hour previous to experimentation. The temperatures of the water in the aquaria and in the jar were the same. The animals were observed for periods of fifteen minutes each, in 'darkness,' followed by illumination from one of three intensities of light, except in the last few trials where this order was reversed. A trial, therefore, consisted in observing an animal under two or more conditions of illumination. The time that the animal was in motion was recorded and the per cent of time in activity determined. The per cent of photokinesis, then, is the difference between the per cents of activity of the same animal in light and in 'darkness.' An observation period of fifteen minutes was plenty long enough, since a response to photic stimulation was always noticed within at least four minutes. During the first series of trials normal animals were used; in the second series, animals adapted to darkness; and in the third, animals adapted to light. The dark adapted animals had been inclosed in a light-proof box for periods varying from two to thirty-two days, while the light adapted animals had been subjected to the illumination of a 32 candle power light for periods varying from 78 to 148 hours. During all the experiments, care was taken to prevent any mechanical stimulation.

OBSERVATIONS

1. Photokinesis. The majority of animals were found to be decidedly photokinetic. Although the per cent of photokinesis is small the activity observed in strong light was distinctly different from that observed in 'darkness.' The movements in 'darkness' were slow, short, indefinite as to direction, and many times were only tail movements; as compared with those when the animal was subjected to a beam of light. In the latter conditions the swimming was rapid, of longer duration, and in many directions, apparently caused by the presence of light.

Whether light or 'darkness' was used first in the trials seemed to make no difference in the per cent of photokinesis.

The skin was observed to be very sensitive, especially the tail region, for a beam of strong light directed against the tail caused a more vigorous response than one directed against the body. It was also observed many times that when a small beam of high intensity was directed on the tail the animal would do one of two things: remove its tail from the light, or swim vigorously about the jar for several minutes.

The results of seventeen trials with fifteen normal animals showed decidedly photokinetic reactions. In four of these trials positively phototropic reactions were also evident, the results of them being discussed under 'Phototropism.' A record of a typical trial with a normal animal is given in table 1, showing a photokinesis of 23 per cent.

TABLE 1

ANIMAL 15	AMOUNT OF ILLUMINATION	
	100 c. p. Spencer	'Darkness'
Amount activity		
Number of seconds	450	242
Per cent	50	27
Number of movements	23	44
Average in seconds	19.5	5.5

Contrary to our expectations, the per cent of photokinesis of dark adapted animals was found to be about the same as that of the normal animals. The type of movements was also similar, except that the difference between the movements in 'darkness' and those in light was much more marked than in normal animals.

The melanophores of the dark adapted animals observed with the binoculars were found to be fully expanded, a condition existing after short periods of adaptation, as well as after longer periods. There was no discernible increase in the per cent of photokinesis with the increase in the amount of adaptation, even for the longest period of thirty-two days. This ani-

mal (A) was decidedly black due to the expansion of the pigment cells, some of which had even invaded the cornea. This was also noticed in other Amphibian larvae by Laurens ('15), who reported that "after a blind of black shellac or celloidin was placed over the eye for long periods, the cornea was found thickened and invaded by pigment cells." It was again observed that the skin of the tail was the most sensitive, for when a beam of light was directed upon it the response was more vigorous than when directed against the body. The record of an animal adapted to darkness for seven days is shown in table 2, the per cent of photokinesis being 17.2.

TABLE 2

DARK ANIMAL B	AMOUNT OF ILLUMINATION	
	100 c. p. Spencer	'Darkness'
Amount activity		
Number of seconds	159	4
Per cent	17.7	0.5
Number of movements	34	4
Average in seconds	4.6	1

Animals adapted to light were tried at different periods of adaptation, and the per cent of photokinesis and type of movements were substantially the same as those obtained with normal animals. It was observed that the per cent of photokinesis did not decrease with the length of the period of adaptation as much as would be expected. The record of an animal adapted to light for 148 hours is given in table 3. The per cent of pho-

TABLE 3

LIGHT ANIMAL C	AMOUNT OF ILLUMINATION			
	500 c. p. arc	100 c. p. Spencer	16 c. p. Spencer	'Darkness'
Amount activity				
Number of seconds	508	448	332	244
Per cent	57	50	37	27
Number of movements	20	22	35	29
Average in seconds	15.4	20.5	10.6	12.4

tokinesis for this animal under an illumination of a 500 candle power arc lamp is 30.

The results of this trial not only agree with those of other light adapted animals, but also with those from normal animal 15, trial 18, where one intensity was used, and also trial 34, where three intensities were used.

SUMMARY OF PHOTOKINETIC REACTIONS

Because of their similarity all of the trials which gave photokinetic reactions have been grouped together, and the averages of photokinesis for the three kinds of animals calculated. This was done by adding the per cents of photokinesis of all the animals in each series and dividing by the number of animals. These results are given in table 4.

TABLE 4

ANIMALS	NUMBER TRIALS	ACTIVITY		PHOTOKINESIS
		'Darkness'	Light	
		per cent	*per cent*	*per cent*
Normal	17	17.3	39.3	22
Dark adapted	4	5.2	27.7	22.5
Light adapted	11	9.6	26.6	17

2. Phototropism. Six large normal animals showed a distinct tendency towards positive orientation to light; that is, they would come to rest with their heads close to the source of light, or with their heads close against the jar move their tails swiftly back and forth. It was observed that when the light fell on the tail a few seconds, the animal would make two or three short movements, not going out of the light but rather towards the source of light.

Animals 1 and 9 were the least marked as far as positive orientation was concerned, the tendency being manifested in their swimming about nearer the source of the light rather than away from it. Hence their records were included with the photokinetic animals.

Phototropism was especially marked in animals 13 and 14, which were much larger and further developed than the others.

After a few seconds of exposure even to weak light they would orient themselves positively and remain in that position for long periods. When a beam of high intensity was used, vigorous swimming brought the animal towards the source of light, the head being pressed against the side of the jar.

During the first half of the trials with animals 5 and 8 they seemed photokinetic but later phototropic, their reaction time for the former kind of response being much longer than that for animals 13 or 14. None of the trials with animals 5, 8, 13 and 14 were included in the summary of the photokinetic animals, because their positive orientation was very evident.

DISCUSSION

From these observations it seems clear that frog tadpoles with a length of at least 40 mm. or more, are sensitive to light, showing a photokinetic response at all stages, and a positively phototropic one towards the end of the larval stages. This suggests the idea that the nervous mechanism controlling the characteristic phototropic reaction of the adult frog may not be fully developed until metamorphosis is completed. In the mid-larval stages, the animals show a photokinetic reaction, or in other words, the rate of swimming increases with an increase of light intensity, while in the later larval stages the reaction becomes a positively phototropic one, which is the only one observed in the adult. This condition gives evidence contrary to the statement of Pearse ('10), that "the photokinetic quality is apparently little developed in frogs and toads, though they are strongly phototropic. Generally speaking there seems to be no correlation between the photokinesis and the phototropism of Amphibians." Our evidence indicates that there is a relation between the two kinds of reactions the phototropic one being a further development of the photokinetic one.

Laurens ('15) found a similar condition in the response of the melanophores of Amblystoma larvae. These do not react to photic stimulation until the animals are at least 16 mm. long. Babak ('10) noticed that Axolotl larvae, 17 mm. long and less, do not respond to light as the older ones do, and thought that

the explanation for this lay in the incomplete development of the retina. Laurens ('16) stated that "the expansion of the melanophores is due primarily to the direct stimulation of the pigment cells themselves, and not to the inhibitory action of the nervous system." In the case of the frog tadpole, which does not respond at all to photic stimulation in the very young stages, (Laurens, '14), but later shows a photokinetic reaction and finally a phototropic one, there must be some relation between these reactions and the development of the nervous system. The only alternative is that the melanophores in the skin of the tadpole are in control of this reaction and until they are far enough developed along the line of direct irritability, their reactions to light do not appear. Laurens has not reported anything regarding the melanophores of the young frog tadpoles and it is not known whether or not they pass through the same stages as those of Amblystoma larvae. If they do, we would expect a response to photic stimulation as soon as the melanophores become sensitive to direct stimulation. Since these young stages do not respond, we must conclude either, that the melanophores are not yet fully developed, or that a nervous mechanism controls the response, but it is not yet fully developed. We are inclined to believe that the latter condition exists in the frog tadpole.

Similar to the conditions in other Amphibians (Parker, '03 and '05; Reese, '06; Pearse, '10; Laurens, '14, and others), we found that the skin of the tadpole is sensitive to light, being the important receptor in the photokinetic reaction. The tail is the most sensitive region, shown both by the reaction time being shorter than for any other region, and by a more vigorous response.

The reactions of those animals subjected to strong light or total darkness for a long time previous to experimentation showed that such adaptation has very little effect on the response to photic stimulation. We had expected that dark adapted animals would show a much greater per cent of photokinesis, but such was not the case. The melanophores of these animals were fully expanded, and since this condition did not

intensify the photokinetic response, we conclude that the state of contraction or expansion does not affect primarily the reaction to photic stimulation. The light adapted animals showed almost the same per cent of activity as normal animals, which gives further proof that the reaction is probably due to a nervous mechanism and not to direct irritability of the melanophores.

SUMMARY

1. Frog tadpoles of Rana clamitans, Latreille, are sensitive to light when a length of at least 40 mm. or more is attained.

2. The skin is the important receptor for photic stimulation, the tail region being the most sensitive.

3. The reaction to photic stimulation varies with the age or stage of development of the tadpoles; the youngest stages being indifferent, the midlarval stages being photokinetic, and the final stages being positively phototropic.

4. Light and dark adaptation have very little influence on the intensity of the reaction.

5. The reaction is probably controlled by a nervous mechanism and not by direct stimulation of the melanophores.

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THE FREE-MARTIN; A STUDY OF THE ACTION OF SEX HORMONES IN THE FOETAL LIFE OF CATTLE

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TWENTY-NINE FIGURES

I. INTRODUCTION

In a preliminary paper (Lillie '16) the author has briefly discussed the theory of the sterility which is the rule, subject to a few exceptions, in the female of two-sexed twins of cattle. In such females, commonly known as free-martins, the internal organs of reproduction are usually predominantly male in character, and the external organs are usually, at least, of the female type; there are however considerable variations as will appear. The conclusion was reached that the sterile free-martin is zygotically a female, modified by the sex hormones of the male twin, which circulate in both individuals during foetal life owing to secondary fusion of the chorions and anastomosis of the foetal circulation of the two individuals. The conditions thus established in this natural experiment enable us to draw far-reaching conclusions as to the origin and the differentiation of sex-characters in mammals. A somewhat extended treatment of the data, and analysis of the facts is therefore herewith presented.

Previous studies have dealt mainly with the anatomy of the free-martin and have furnished important, though incomplete data on this part of the subject. These will be considered in their appropriate connection. A study of L. J. Cole's ('16) supplies some important statistical data referred to beyond. We shall introduce the subject by a consideration of the various theoretical points of view from which the discussion must start. In considering these it will be important to define more precisely the condition with which we are dealing. The phenomenon of sterility of the female of different sexed twins is usually consid-

ered to be peculiar to cattle;[1] it has been stated to occur in sheep rarely, but I cannot discover on what evidence. There has also been a superstitition,[2] which reappears in the press

[1] According to E. J. Davies ('13) 'hermaphrodites' are common in certain breeds of goats, for instance Toggenburgs and Anglo-Swiss. He even estimates that in 1913 "Among the total birth of kids eligible for entry in the Herd Book it is believed that at least 2 per cent belonged to the class under discussion—and the proportion is probably much greater." This statement is understood from the context to apply to the Toggenburg breed. He goes on to say that "malformed kids have recently come (a) singly, (b) as twin with a normal male, (c) as twin with a normal female, (d) as one of triplets the normal kids being male and female, (e) as one of triplets the normal kids being both males."

Assuming the malformation to mean genital abnormalities we are justified in explaining cases b, d and e on the same principle as the free-martin of cattle. As regards case a we can only say that this phenomenon is not uncommon in other mammals, whatever may be the explanation; it could be explained as due to two-sexed twin association *in utero* with early death and absorption of the male twin. Case C, an 'hermaphrodite' twinned with a normal female, is of unusual interest, for we have no evidence that this condition ever occurs in cattle; but it is to be expected theoretically in any case of blood community of male and female embryos in which the female is decidely in advance of the male in development. The male would then be subject to action of the female sex hormones, and its development would tend to be intersexual. The case cited in illustration of this association seems to be well authenticated. Unfortunately the anatomy of the malformed individual was not studied, as a kid it appeared as a female externally except for the much enlarged phallus; but it grew to male size and even developed very strongly the characteristic smell of the male goat.

[2] I do not know how wide spread this superstition may be; but that it still exists is undoubted. It has been reported to me as a common belief in certain farming communities, and it has been gravely discussed in the correspondence of the Chicago Tribune as recently as March, 1916. Formerly the idea was more wide spread, and was even countenanced by medical men as reported by Dr. James Y. Simpson of Edinburg who quotes from Burn's "Principles of Midwifery" 1843, p. 236, "It is a popular opinion, and I do not know any instance to discountenance it, that if twins be of opposite sexes the female is sterile." Simpson ('44) made an investigation of the subject which may be regarded as disposing forever of the superstitition. He investigated the family history of 123 married women born twin to males of whom 112 had families and 11 had none; he found also that this proportion of childless marriages was not greater than in the general population. Ninety-four of these cases in which he had complete histories had 409 children, an average of 4.2, which was about the same as for the general population of that time and place. He concluded that females born co-twin with males are, when married, as likely to have as many children as other females belonging to the general community. There is no basis for belief in the sterility of such females; the superstition works cruel hardship to innocent people, and it cannot be too strongly stated that there is no basis whatever for it.

from time to time, that it applies to human twins, but this is certainly not the case. In cattle, in about 87 per cent of apparently different-sexed twins, as nearly as I can ascertain, the female is sterile; about 13 per cent are normally fertile (see data beyond p. 381). We must therefore distinguish sterile and fertile free-martins, and it is important to note that the fertile individuals are not known to be in any respect inferior to other females in respect of breeding. The sterile individuals have the external organs of a female, usually, but the internal organs of reproduction are more or less of the male type. Their general bodily appearance is more or less intermediate between a male and a female—it has been compared to that of an ox or spayed heifer—so that an experienced cattle man can usually distinguish them from normal heifers. The bull twin is always normally fertile, and does not exhibit any anatomical peculiarities so far as is known.

It is essential to recognize the fact that the sterile free-martin condition is found only in association with a bull twin.[3] The

[3] Numan ('43) is, so far as I know, the only author who has questioned this; his study is by far the most extensive, and in many respects the most thorough, that has been made on the free-martin. The publication is exceedingly rare, but I have been able to study a copy from the library of the Smithsonian Institution, and as his conclusions are so often quoted from author to author, it seems worth while to give the evidence on which his dissenting statements are based. (D. Berry Hart has published an abstract of Numan's paper (Hart '12). Numan states "The anomaly occurs not only in twins of different sex, but also in female and male pairs, though more rarely." In these cases he refers first to an individual about two years old, judged by external signs alone to be a sterile free-martin; the owner stated that it was born twin to another female, which however was sold shortly after birth about two years previously. This would appear to be slender evidence upon which to base a unique exception, for it was not positively certain that the individual was a sterile free-martin, nor could Numan know unquestionably that it was born twin to another female. It is known also that cystic degeneration of the ovaries may lead to extensive assumption of male secondary sex characters in the cow (Pearl and Surface, '15); the case may belong in this category. This was the only case he had on the female side, and no others have been recorded since. In the case of male pairs he cites also a single case which is of great interest, but wrongly interpreted by him. It was a case of twins one being a normal male and the other a sexually abnormal individual. He judged the abnormal individual to be male on account of the presence of testes in the groins, and malformation of the external parts. The scrotum was absent. This is almost certainly an extreme case of modification of the

association with a male *in utero* is, therefore, in some way a necessary condition of the phenomenon. It is also an invariable rule that the male twin is normal; the reverse condition of a normal female with a defective male probably does not occur. All of my own cases were found in association with a normal bull twin; in the other possible twin combinations both individuals are normal. Of course genital abnormalities may occur apart from twinning, so that the possibility exists that in a sufficiently large collection of twins an individual might be found with genital abnormalities not due to its association with a twin. In this connection Numan ('44) notes that malformation of the sexual organs causing infertility had not yet been observed in single born heifers in his experience; but, in single born males, individuals with incomplete formation of the organs of reproduction had

female similar to one of my own cases (no. 44), and hence no exception; it is discussed *in extenso*, p. 413 of the present paper.

Numan also states, "In two-sexed twins the malformation is not confined exclusively to the heifer; but may also occur in the case of the bull, in which case the heifer is normal. However such examples appear to be very rare." This also refers to a single case viz., that of a two year old bull sent him by a veterinarian who stated that it had been born twin to a female. According to the veterinarian there was not the least abnormality discernible in the external organs of the heifer; the internal anatomy was not studied. The bull exhibited a hypospadiac condition; it possessed a split scrotum and the testes were in the abdominal cavity against the inguinal rings. Numan himself points out that such abnormalities are not rare in bulls born single. Since the actual condition of the female twin was not known, and the condition in the bull is a rather common anomaly the evidence is entirely inadequate to support the idea that the condition was due to twinning.

Numan's emphasis of these doubtful cases appears to be due in part to his fundamental objection to considering free-martins as hermaphrodites. He classified them therefore either as females or as males with defective organs of reproduction; in two-sexed twins the female was classified as male if the modification of the reproductive organs proceeded beyond a certain degree, thus establishing in his mind the occurrence of a profound genital anomaly in one individual of exceptional male pairs; this seemed to render probable a similar occurrence in the opposite case of female twins, which may seem to account for his uncritical acceptance of the case cited above, and his equally uncritical interpretation of modification of the bull as due to twinning with a female.

The entire basis for Numan's statements concerning exceptions to the rule is untrustworthy, and the exceptions cannot be accepted seeing that they are supported by no other writers.

often been found. This observation is of interest in connection with the problem stated in footnote 5, p. 389.

The theoretical interpretation of the free-martin must be based on one of two assumptions, either (1) that it and its partner, are identical, i.e., monozygotic twins, or (2) that they are fraternal, i.e., dizygotic twins. Under the first assumption, as the sex of monozygotic twins is undoubtedly identical, the free-martin would necessarily be interpreted as a modified male. Under the second assumption it would be almost equally a matter of necessity to interpret the free-martin as a female, for there is no possibility that the association of two males *in utero* should cause the transformation of one of them into a free-martin. On the first assumption the explanation of the modification must be found in the twinning process itself, i.e., in the division of the single zygote that *ex hyp.* formed the two twins. But on the second assumption no one has hitherto attempted to explain how the association of male and female *in utero* could lead to sterility of the female with a more or less pronounced male organization of the internal organs of reproduction, nor why certain females should escape the defect, nor why the phenomenon should be peculiar to cattle. It is, therefore, natural that the first assumption should have been the one followed in all previous theoretical interpretation of the free-martin, and that it formed the working hypothesis with which my own work began.

The first theoretical view that we shall consider is that of Spiegelberg ('61) who said of cattle twins: "If the twins are both female or of opposite sex, the organs of reproduction are as a rule well formed; if they are both male, it very frequently happens that one of them is an hermaphrodite." This conclusion was based on the examination of two pairs of different sexed twins in cattle; he made anatomical examination of the free-martins and found one of them a normal female, but in the other the female internal organs of reproduction were mostly absent, and were replaced by rudimentary seminal vesicles, rudimentary vasa deferentia, and a rudimentary gonad on one side which he interpreted as probably a small testis with more or less separated epididymis. The internal organs were accepted

as diagnostic of sex, and the case was interpreted as simple male transverse hermaphroditism.

D. Berry Hart ('10) also interprets the free-martin as a male, basing his interpretation on a comparison of the anatomical descriptions of thirty cases given in the literature, and on an original histological examination of the gonads of John Hunter's specimens which had been in alcohol for one hundred and forty years.

> The special fact that emerges is that all the sexual glands are testes in Hunter's cases, that adjacent structures are epididymis, and that in none of the sexual glands are ova present. The characteristic testicular tissue is in the form of tubuli semeniferi, and in only one are spermatozoa present. It seems to me, therefore, fully established that the free-martin, when the co-twin is a potent male, is a sterile male, and not a sterile female, i.e., they are identical male twins except in their genital tract and secondary sexual characters.

It will be observed that Hart accepts the conditions of the internal organs, and more especially of the gonad, as decisive criteria of sex. This raises a point that we shall discuss later on. Continuing, Hart then proposes a 'Mendelian' theory as follows: He distinguishes potent and non-potent elements in the genital tracts of both sexes, the latter being the undeveloped rudiments of the opposite sex; in the twinning process of a male zygote he supposes we may either get identical male twins, or "the potent and non-potent complex of the genital organs may be divided so that the potent part goes to the potent bull calf, the non-potent to the free-martin." More specifically, "A free-martin with a potent bull twin is the result of a division of a male zygote, so that the somatic determinants are unequally divided, the potent going to one twin, the potent bull, the non-potent genital determinants to the free-martin." He supposes the potent organs to be dominant in the Mendelian sense, the non-potent recessive.

The entire argument is based on the unsupported assumption, which it is quite possible to decide definitely by the facts, that the free-martin is co-zygotic with its male mate. I shall show immediately that this is not the case; so that it is hardly necessary to point out that if the gonad of the free-martin is a testis, as Hart

maintains, it can hardly be classed as a non-potent part of the genital tract, i.e., by definition undeveloped parts of the opposite sex, nor can the vasa deferentia of the free-martin be so classed; nor yet the external genital parts which are usually pure female. The theory moreover implies that as the free-martin receives the 'non-potent' genital parts, the bull twin must lack them, and must continue to propagate male individuals lacking them, for neither of which deductions is there the slightest evidence, or any attempt to produce evidence. Finally, there is not any attempt to explain why the twinning process should be attended by such extraordinary results in cattle, and involve nothing of the kind in other mammals. Hart notes that the theory implies the possibility of a similar defect in the twinning of a female zygote, and he refers to Numan's case cited before (footnote 2) as an example.

Bateson ('13, pp. 44–45) also attempts an explanation of the free-martin on the basis that it is co-zygotic with its twin,

> For it is impossible to suppose that mere development in juxtaposition can produce a change of this character. It is conceivable that we should interpret it by reference to the phenomenon of gynandromorphism, seen occasionally in insects, and also in birds as a great rarity. In the gynandromorph one side of the body is male, the other female. A bullfinch for instance has been described with a sharp line of division down the breast between the red feathers of the cock on one side and the brown feathers of the hen on the other. In such cases neither side is sexually perfect. If the halves of such a gynandromorph came apart, perhaps one would be a free-martin.

The interpretations of Hart and Bateson are based on the theory that the free-martin and its twin are monozygotic, and they involve the conclusion that the free-martin is derived from a male zygote. They may be called anatomical interpretations because they are based exclusively on anatomical evidence. Cole has also come to the same conclusion on statistical grounds, which I discuss in the second part (pp. 380).

The literature on this subject is scanty, and it would serve no good purpose to continue with the few incidental citations that might still be made. Embryological evidence that would alone give a basis for correct interpretation has been entirely lacking;

my principal work has been to secure this evidence, which is herewith presented. The order of presentation is decided by the theoretical considerations, which obviously require that we should first of all determine whether we are dealing with a phenomenon of division of a single zygote, or with development of two zygotes in juxtaposition. Other considerations follow immediately from this, and the anatomical side is considered last. In conclusion the more fundamental theoretical questions come up for consideration.

II. ARE THE STERILE FREE-MARTIN AND ITS MATE MONOZYGOTIC OR DIZYGOTIC?

The first question that confronts us, therefore, is whether the free-martin and its male twin are monozygotic or dizygotic? This question can be answered decisively only by the embryological data; contributing evidence may be furnished by study of the degree of resemblance of the twins to one another, and by a statistical study of the sex ratios in twin births in cattle.

1. The embryological evidence

There are two ways in mammals of deciding whether twins are monozygotic or dizygotic: 1) If there is a single chorion for both of the twins, this would usually be regarded as evidence of monozygotic origin. But a single chorion is not decisive evidence, because, though one would expect monozygotic twins to have a single chorion, yet it is theoretically possible that two separate chorions may fuse to form one. The monochorial condition may be primary or secondary. 2) The number of ova concerned in a pregnancy may be ascertained by the number of corpora lutea, which correspond accurately. If we should find two corpora lutea for all free-martin twins, the dizygotic origin would be proved, provided that the rule holds for cattle.

I have had the opportunity of examining a large number of twin pregnancies in cattle through the courtesy of Messrs. Swift and Company of the Chicago Stockyards; and I wish to express my appreciation of their generous coöperation, without which this study could not have been made. The superintendent of

the cattle-house kept watch for uteri containing twins, and when they were not of too large size notice was telephoned to the department of Zoology of the University, and our collector, Mr. Adams, went over and brought the specimens to the laboratory. The collection has been going on for two years and a half, and 55 pairs of twins have been studied. For a long time most of the uteri were received with one or both ovaries missing, but recently special pains have been taken to secure the ovaries also attached to the uterus. A large proportion of the earlier records are therefore incomplete in this respect.

It was a great surprise to find that nearly all twins of cattle are monochorial; only two complete exceptions have appeared in 55 cases. The first case (no. 40) is of great theoretical interest, and will come up for detailed consideration later on. In a very few other cases the connection between the two halves was slight (see cases 8, 9, 10, 24, in table); generally it was broad and strong. At first I had expected to decide the question of monozygotic or dizygotic condition by the monochorial or dichorial state; so that for a considerable period not much attention was paid to the question of the corpora lutea. Later attention was directed to this question and the unexpected result was reached that in all cases in which both ovaries were present each had a corpus luteum. The exact data are: 22 cases in which both ovaries were present; in all of these there was a corpus luteum in each ovary; 7 of these were ♂♂, 4 ♀♀, 10 ♂♀, and one too young for sex diagnosis; 11 cases in which only one ovary was present, 9 of which had the corpus luteum present, and 2 absent; the two latter were same-sexed, one pair of males, and one of females; they may have been monozygotic, but the missing ovary may have contained two corpora lutea in each case. In 22 cases both ovaries were missing or not recorded. If we consider only those cases, 22 in number, in which both ovaries were present, there is no exception to the rule that cattle twins are dizygotic, using the corpus luteum as evidence for a separate zygote. This is a sufficiently large number to make it certain that the occurrence of monozygotic cattle twins is at least extremely rare. The free-martin condition cannot possibly be interpreted as a result of monozygotic twinning.

It may, however, be objected that we are relying on a rule which has not been proved for cattle, viz: that in a single pregnancy only one corpus luteum is present. To settle this question examination was made of 81 uteri each containing a single calf; in 45 of these both ovaries were present, and in every case only a single corpus luteum was found; in 36 cases one ovary only was present and 18 of these contained a single corpus luteum each, the other 18 lacked a corpus luteum. There can, therefore, be no doubt that the rule holds for cattle. This conclusion necessitates the inference that the monochorial condition of cattle twins is secondary. In a later section we shall consider the question of the probable time and nature of the fusion of the two chorions.

The conclusion drawn from the embryological evidence that the heterosexual cattle twins are dizygotic is supported by two other important considerations, viz: lack of close resemblance between the bull and heifer of such pairs, and by the sex ratios of all cattle twins.

2. Concerning the degree of resemblance of such twins

A careful study of this point remains to be made; but it is noteworthy that no one has recorded resemblances similar to those of identical twins in the case of the free-martin and its bull mate. My own somewhat limited observations lead me to the conclusion that the resemblances are no closer than ordinary fraternal likeness. One striking case was recently observed by me in pure bred Holstein-Friesian cattle in which the free-martin was about half black and white in patches, and the male was almost entirely black except its forehead and legs below the knees.

3. Sex-ratio of cattle twins

On this point we have observations by Cole and myself. Cole ('16) finds in a study of records of 303 multiple births in cattle that there were 43 cases male twins, 165 cases two-sexed twins (male and female), 88 cases female twins, and 7 cases of triplets. This gives a ratio of about 1 ♂♂ : 4 ♂♀ : 2 ♀♀ for the twins, instead of the expected ratio of 1 : 2 : 1. Cole then states:

> The expectation may be brought more nearly into harmony with the facts if it is assumed that in addition to ordinary fraternal (dizygotic) twins, there are numbers of 'identical' (monozygotic) twins of both sexes, and that while in the case of females these are both normal, in the case of a dividing male zygote, to form two individuals, in one of them the sexual organs remain in the undifferentiated stage, so that the animal superficially resembles a female and ordinarily is recorded as such although it is barren. The records for monozygotic twins accordingly go to increase the homosexual female and the heterosexual classes, while the homosexual male class in which part of them really belong, does not receive any increment.

Cole thus tentatively adopts the theory, which has been worked out most elaborately by D. Berry Hart, stated also by Bateson, and implied in Spiegelberg's analysis ('61), that the sterile free-martin is really a male co-zygotic with its mate.

Cole's figures represent the only statistical evidence that we have previously had on this subject. Let us follow his suggestion and take from the two-sexed class enough cases to make the male twins equal in number to the female pairs; this will be approximately one-fourth of the class, leaving the ratio 2:3:2 instead of 1:4:2. Which one of these is the more satisfactory sex ratio I leave others to determine; I wish only to point out the fatal objection, that according to the hypothesis, the females remaining in the two-sexed class are normal; in other words, on this hypothesis the ratio of normal free-martins (females co-twin with a bull) to sterile ones is 3:1; and the ratio would not be very different on any basis of division of the two-sexed class that would help out the sex ratio. Hitherto there have been no data from which the ratio of normal to sterile free-martins could be computed, and Cole furnishes none. I have records of 24 cases statistically homogeneous, 3 of which are normal and 21 abnormal. That is, the ratio of normal to sterile free-martins is 1:7 instead of 3:1.

This ratio is not more adverse to the normals than might be anticipated, for breeders' associations will not register free-martins until they are proved capable of breeding, and some breeders hardly believe in the existence of fertile free-martins so rare are they.

My own records of 55 cases of bovine twins, all examined *in utero*, and their classification determined anatomically without the possibility of error, give 19 ♂♂: 24 ♂♀: 11 ♀♀ and 1 (no. 49) too young for determination. It will be observed that the sum of the one-sexed classes is 25 per cent greater than the two-sexed class; and the ♂♂ class is much larger than the ♀♀ class instead of being equal to it, as it should be if males and females are produced in equal numbers in cattle. The material cannot be weighted statistically because every uterus containing twins below a certain size from a certain slaughter house is sent to me for examination without being opened.[4] Cole's material shows twice as many female as male pairs, and the two-sexed class is about one-third greater than the sum of the other two classes. I strongly suspect that it is weighted statistically; the possibility of this must be admitted, for the records are assembled from a great number of breeders. But, whether this is so or not, if we add the sterile free-martin pairs of my collection to the male side in accordance with Cole's suggestion, we get the ratio 40 ♂♂: 3 ♂♀: 11 ♀♀, which is absurd. And if we take Cole's figures, divide his heterosexual class into pairs containing sterile females and pairs containing normal females according to the expectation, 7 of the former to 1 of the latter, and add the former to his male class, we get an almost equally absurd result (186 ♂♂: 20 ♂♀: 88 ♀♀). On the main question our statistical results are sufficiently alike to show that the free-martin must be interpreted as female.

Prof. Alexander Graham Bell has kindly furnished me with a catalogue of the lambs born from 1890 to 1914 in his well-known experiments on his Beinn Bhreagh Estate in Nova Scotia from which I have taken all the records of twin births, 139 in number;

[4] The great preponderance of the ♂♂ over the ♀♀ class in foetal cattle twins of the sizes dealt with in this study appears to be real, though it must be admitted that the numbers are too small to make this quite certain. Cole's data on the other hand indicate a great preponderance of the ♀♀ class over the ♂♂ class in cattle twins after birth. It may be that abortion, which is so frequent in cattle, is even more adverse to the males in the case of twins than in single births; it is conceivable that the difference is largely a question of viability, but other explanations are possible.

of these 38 were ♂♂, 67 ♂♀ and 34 ♀♀, thus an exceedingly close approximation of the expected 1:2:1 ratio. In his "Problems of Genetics" Bateson cites Bernadin (La Bergerie de Rambouillet, 1890, p. 100) as to the frequency of twin combinations in Merino Sheep, viz: 87 ♂♂ to 187 ♂♀ to 83 ♀♀, which also approximates the expected 1:2:1 ratio. These statistics, therefore, also support the interpretation of the free-martin as female, for they show that the actual ratios of the distribution of sex among twins are as a matter of fact the expected ones in ungulates.

On the other hand in man there is a very significant and interesting departure from the expected ratio: Simpson ('44) collected statistics of 788 cases of human twins, the various sex combinations being ♂♂ 229, ♂♀ 298, ♀♀ 261, thus very far removed from the 1:2:1 ratio. Nichols ('07) has made a very much larger collection of statistics with the following ratios ♂♂ 234,497, ♂♀ 264,098, ♀♀ 219,312. It is obvious that there is a very large disturbing factor here; this is almost certainly the factor of monozygotic twinning. As the two-sexed combination must be dizygotic, we may estimate the dizygotic ♂♂ and ♀♀ combinations at one-half of the ♂♀ combination, on the slightly inaccurate basis of a 1:1 sex ratio of male and female zygotes. This would give 132,049 dizygotic ♂♂ and ♀♀ twin pairs each, and the excess viz: 102,448 ♂♂ and 87,263 ♀♀ would represent the monozygotic twin couples. This is not very far from the proportion of monozygotic twin pairs among one-sexed twin couples estimated by physical resemblance. If, then monozygotic twinning is the disturbing factor in the unexpected sex-distribution ratios of human twins, we may argue from the fact that the ratios in cattle and sheep approach expectation that monozygotic twinning either does not occur, or is very rare in them, and this is confirmed by the embryological evidence. This matter is discussed more fully in Newman's book on twins ('17) to which the reader is referred.

4. Discussion

The preceding considerations constitute an argument that the free-martin is zygotically female, which may be summarized as follows: 1) The only basis on which it could be logically interpreted as male is that it is co-zygotic with its male mate, because it is impossible to suppose that the association of two males *in utero* should cause the transformation of one of them into a free-martin in a certain definite proportion of cases. But we have seen that the free-martin and its male mate arise from separate zygotes. From this point of view the free-martin must be interpreted as zygotically female. 2) The somatic resemblances between the free-martin and its mate are not of the order of identical twins. 3) The assumption that the free-martin is male leads to an absolutely incomprehensible sex-ratio, while the interpretation that it is female comes nearer fulfilling the expected sex-ratio. From this point of view also the free-martin is female.

The only argument that remains for its male nature rests on the anatomy of the internal organs of reproduction, which unquestionably are more or less of the male type. But, as the external genitals and the mammary gland are almost invariably of the female type, the argument from anatomy may be made to turn either way depending on what anatomical characters are recognized as diagnostic of sex. In a later section we shall go fully into the anatomical problems involved. Here it may suffice to say that the anatomical argument is necessarily inconclusive.

In what follows, therefore, we shall treat the free-martin as demonstrated to be zygotically female, and the question becomes how the association of a male and female *in utero* may so transform the female.

List of cattle twins examined in utero

NUMBER	SEX ♂	SEX ♀	SEX ⚥	SIZE	CHORION	MATERNAL OVARIES
1	2			35 cm. each	Single	Not observed
2	1		1	♂ 23 cm. ⚥ 21.5 cm.	Single	Not observed
3	2			25.5 cm. 26.5 cm.	Single	Not observed
4	1		1	♂ 24 cm. ⚥ 22.5 cm.	Single	Not observed
5		2		17 cm. each	Single	Not observed
6	1		1	♂ 16.8 cm. ⚥ 16.3 cm.	Single	Only one ovary present. Contains corpus luteum
7	1		1	About 20 cm.	? (See note 1)	Both ovaries present. Corpus luteum in each
8	1	1		♂ 26.5 cm. (wt. 2 lb., 6 oz.) ♀ 23.3 cm. (wt. 1 lb., 6.5 oz.)	Single, narrow connection	Ovaries absent
9	1	1		♂ 20 cm. ♀ 20 cm.	Single, narrow connection	Ovaries absent
10		2		23 cm. each	Nearly separated; united by narrow strand	Ovaries absent
11		2		17 cm. 16 cm.	Single	Ovaries absent
12	1		1	♂ 30 cm. (2 lb., 10.5 oz.) ⚥ 28 cm. (2 lbs., 13.5 oz.)	Single	Ovaries absent

NUMBER	SEX ♁	SEX ♀	SEX ☿	SIZE	CHORION	MATERNAL OVARIES
13	1		1	♂ 20 cm. (14 oz.) ☿ 20 cm. (14 oz.)	Single[2]	Ovaries absent
14	1		1	♂ 27 cm. ☿ 27 cm.	Single	Ovaries absent
15		2		17.5 cm. 18 cm.	Single	Both present. Corpus luteum in each
16	2			13 cm. 12.75 cm.	Single[3]	Ovaries absent
17	1		1	♂13.75 cm. ☿ 13.1 cm.	Single	One missing, other had 1 corpus luteum
18	2			12.5 cm. 13 cm.	Single	Ovaries absent
19	1		1	♂ 8 cm. ☿ 7.5 cm.	Single	Ovaries absent
20	2			20.5 cm. 20 cm.	Single	One ovary absent. Other contains corpus luteum
21	1		1	♂ 27.5 cm. ☿ 26.5 cm.	Single	Ovaries absent
22	1		1	♂ 24.5 cm. ☿ ?	Single[4]	Both ovaries present. Corpus luteum in each
23	1		1	♂ 18 cm. ☿ 17.5 cm.	Single	One ovary absent. Other has 1 corpus luteum
24	2			21 cm. each	Single; narrow connection	Both ovaries present. Corpus luteum in each
25	2			11.2 cm. each	? Injured	One ovary absent. Corpus luteum in other
26	1		1	♂ 12.5 cm. ☿ 12.25 cm.	Single	One ovary absent. Other has 1 corpus luteum

NUMBER	SEX ♂	SEX ♀	SEX ⚥	SIZE	CHORION	MATERNAL OVARIES
27	2			42.5 cm. each	Not examined	One ovary absent. Other has 1 corpus luteum
28	2			17 cm. each	Single	One ovary absent. Other has 1 corpus luteum
29	2			15 cm. each	Single	Both ovaries present. Corpus luteum in each
30	2			23 cm. 22 cm.	Single	One ovary absent. No corpus luteum in other
31		2		12 cm. each	Single	Both ovaries present. Corpus luteum in each
32	1		1	♂ 18 cm. ⚥ 16.75 cm.	Single	Both ovaries present. Corpus luteum in each
33	2			10.5 cm. each	Single	Both ovaries present. Corpus luteum in each
34		2		25 cm. (about)	Single	Both ovaries present. Corpus luteum in each
35	2			13 cm. each	Single	Both ovaries present. Corpus luteum in each
36	1		1	♂ 18 cm. ⚥ 17.5 cm.	Single	Both ovaries present. Corpus luteum in each
37	1		1	♂ 16 cm. ⚥ 15.5 cm.	Single	Both ovaries present. Corpus luteum in each
38	1		1	♂ 23.5 cm. ⚥ 22.5 cm.	Single	Both ovaries present. Corpus luteum in each
39	2			18 cm. each	Single	Both ovaries present. Corpus luteum in each
40	1	1		♂ 10.4 cm. ♀ 10.2 cm.	Two separate chorions	Both ovaries present. Corpus luteum in each
41	1		1	♂ 22.7 cm. ⚥ 21.8 cm.	Single	Both ovaries present. Corpus luteum in each

NUMBER	SEX ♁	SEX ♀	SEX ♂	SIZE	CHORION	MATERNAL OVARIES
42—	se	e	N	ote 5		
43	2			10.75 cm.	Single	Not observed
44—	se	e	N	ote 5		
45		2		34 cm. 31.25 cm.	Single	Both ovaries present. Corpus luteum in each
46	2			23 cm. (about)	Almost separate	Both ovaries present. Corpus luteum in each
47	1		1	♁ 22.75 ♂ 22.25 cm.	Single	Both ovaries present. Corpus luteum in each
48		2		20 cm. (about)	Single	One ovary absent. No corpus luteum in other
49	?	?		1.5 cm. each	Separate, but overlapping	Both ovaries present. Corpus luteum in each
50	1		1	♂ 21 cm. ♂ 21 cm.	Single	Both ovaries present. Corpus luteum in each
51	2			5 cm. each	Single	Both ovaries missing
52		2		18 cm. 19.5 cm.	Single	One ovary absent. Single corpus luteum in other
53		2		19 cm.	Single	Both ovaries absent
54	2			20.5 cm. 21 cm.	Single	Both ovaries present. Corpus luteum in each
55	2			13.75 cm.	Single	Both ovaries absent
56		2		12.5 cm.	Two chorions separate	Both ovaries absent
57	1		1	♂ 19.25 cm. ♂ 18 cm.	Single	Both ovaries absent

[1] Case 7 was received in my absence, and the entire uterus was placed in formalin; preservation of its contents was bad, and condition of chorion must be recorded as doubtful.

[2] Case 13 uterus injured by butcher; chorion cut in two.

[3] Case 16 uterus injured by butcher; chorion cut in two.

[4] Case 22 uterus injured by butcher; chorion cut in two.

[5] Cases 42 and 44 are not included because they were selected heterosexual pairs taken after birth.

III. THE TIME OF FUSION OF THE TWIN CHORIONS AND THE DEVELOPMENT OF THE VASCULAR ANASTOMOSES BETWEEN THE TWINS

In order to form an estimate of the probable time of fusion of the twin chorions it is necessary to present a few data concerning the development of the usual single chorion. Figure 1 shows the non-pregnant uterus of the cow partly dissected. It will be noted that the horns of the uterus open by constricted apertures into the small body. The blastodermic vesicle forms in the horn of the uterus on the same side as the ovary from which the ovum was derived, as I have observed in numerous cases. It grows out into a long strand-like sac extending both distally and centrally. The embryonic area forms near the center in the sheep (Bonnet) and presumably also in the cow. The growth of the strand-like vesicle in length is extraordinarily rapid, and it soon enters the body of the uterus centrally, and penetrates into the opposite horn. By the time that the embryo is 10 mm. long the vesicle has extended completely through the body of the uterus and far into the other horn (two cases observed); the embryo is thus excentrically placed in the very long vesicle. The allantois forms later than the blastodermic vesicle; it grows from the embryo both centrally and distally, and ultimately completely fills the blastodermic vesicle and occludes its cavity. In the case of an embryo of 19 mm. length the allantois had passed from the horn of the uterus containing the embryo well into the body of the uterus. In another case of an embryo of 21 mm. length the allantois had extended through the entire horn of the uterus opposite to that containing the embryo.

I have one case of a twin pregnancy in the cow in which the embryos were only 15 mm. long (no. 49). Unfortunately the

collector did not recognize the case as a twin pregnancy until after the uterus had been opened and one foetus removed, thus cutting the membranes; the other foetus was also removed in the same way, and when the uterus and specimens reached me for examination it was necessary to reconstruct the original condition from the parts. This was, however, successfully done, as the central end of the one chorion was found still in place in the body of the uterus and extending into both horns. In this one the chorion with contained allantois had passed the body of the uterus. In the other the end of the chorion had been drawn out of the uterus with the foetus, but measurement showed that it also with the allantois contained had passed the body of the uterus. The two chorions were thus not fused at this stage, but they overlapped and were in closest apposition in the body of the uterus. The conditions precedent to fusion were thus fully established at this early stage long before sexual differentiation begins.

Comparison of these twins with the stages of 19 and 21 mm. described above indicates some variation in the degree of development of the chorion relative to the length of the embryo. But these stages demonstrate the possibility of fusion of twin chorions a considerable time before the stage of beginning sex-differentiation, which I estimate at about 25 mm. Vascular anastomosis between the twins is possible as soon as the allantoes from the two sides meet, or even earlier, because after the allantois has once fused to the chorion the blood-vessels tend to spread out more or less in the chorion beyond the area of fusion.

Owing to the extreme difficulty of obtaining early stages of twin pregnancies in cows the next earliest stage that we have (no. 51) is a case of male twins 5 cm. long in which there is no evidence of the place of fusion of the twin chorions, and there is a perfect vascular anastomosis between the two sides. Fusion is already perfect and any overlapping parts have entirely disappeared. The next case is a two-sexed pair (no. 19) in which the male foetus was 80 mm. and the female 75 mm. long. The twin chorion was single with a broad connection provided with cotyledons between the two halves; no evidence of the place of

fusion of the twin chorions remained. The urinogenital system of the female, described in section 4, was already definitely of the sterile free-martin type. The inference is, therefore, that, fusion had taken place some time previously in order to account for the completeness of the fusion and the transformation of the reproductive system of the female.

In the case of twin pregnancies in cattle, therefore, the two vesicles starting in opposite horns of the uterus will meet in the body of the uterus before the 10 mm. stage; the allantoes of the two vesicles will not however meet until about the 15 mm. stage, and the opportunity for vascular anastomosis therefore dates from this time.

Bonnet ('89) describes a very early twin pregnancy of the sheep, which confirms in the strongest way my conclusion concerning the early time of fusion of twin chorions in ungulates. His description is of so much interest that I quote it entire, he describes a pair of sheep twins 6 mm. long, secured 18 days and 6 hours after copulation,

> deren serösen Hüllen an den sich berührenden Enden auf eine Strecke von 6 cm. in einander eingestülpt und verklebt, aber noch nicht verwachsen waren. Sie liessen sich vielmehr noch leicht auseinanderlösen. Beide Eier maassen zusammen vom freien Ende des einen bis zum freien Ende des anderen 35 cm. und waren *in maximo* 1.5 cm. weit. Die Kürze der Eier ist eine im Vergleiche zur Länge einzelner Eier in diesem Stadium auffallende; sie betrug bei einem 15, bei dem anderen 17 cm. Wahrscheinlich behindern sich die bald einander mit den Spitzen berührenden Eier einigermaassen in der sonst normalen Längenentwicklung.

It will be noted that in this case the ova met at their apices and invaginated one another, and that the stage of such union was only 6 mm. The sheep's uterus is of precisely the same type as the cow; fusion follows the union of the ova in the sheep as in the cow; but vascular anastomosis does not occur in the sheep, as I describe in detail later on, and for this reason the female of two-sexed twins remains unaffected in the sheep.

We have already referred frequently to the vascular anastomoses between twin foetuses of the cow, and it is now time to describe the matter fully. The working hypothesis with which

the investigation began was that the free-martin and its twin were monozygotic, and it was not until after 27 cases had been examined that I was convinced that they were dizygotic. The real explanation of the phenomenon then for the first time became evident. No vascular injections were therefore made during the first part of the investigation, and the evidence for vascular anastomosis among these rests upon incidental observations, the significance of which was not realized at the time. Relatively few of the twin ova received thereafter were in a fit state for complete injections. Of the 28 cases involved injections were made only in seven cases; two of which will be described in detail below. But in 21 of the 28 cases vascular anastomosis could be satisfactorily demonstrated either in the uninjected chorions or in injections. Some of the uninjected cases were just as demonstrative as though they had been injected. In four cases of the remaining eight there was no anastomosis; one too young (no. 49); the second was a case of normal male twins in which the connection between the two chorions was merely a narrow band-like connection (no. 46); the third was a case of male twins with entirely separate chorions (no. 56); the fourth was another case of completely separate chorions (no. 40) of the greatest theoretical interest because one foetus was male and the other a normal female. Finally there were three cases with inadequate records. Eliminating these three we have 25 cases, in 21 of which, including the three possible twin combinations, vascular anastomosis could be demonstrated and 4 in which it was absent (nos. 40, 46, 49, and 56).

This is not, however, the only evidence that more or less complete vascular anastomosis between the pairs is the rule in cattle twins. I can distinctly remember the continuity of the thickened chorionic band that carries the main arteries as the rule in the first 27 cases, and this was recorded in certain cases in my notes.

There cannot be the least doubt that in bovine twins fusion of the chorions usually occurs and is followed by anastomosis of the blood vessels of the two sides, and that intermixture of the blood of the two foetuses results. Nor can it be doubted that

rarely this does not occur either because the chorions fail to fuse (cases 40 and 56) or because a slender connection is not vascularized (case 46). The significance of the exceptions is very great.

The nature and extent of the vascular connections may now be illustrated by a detailed study of two cases. 1. Case no. 33 Males, 10.5 cm. long (figs. 2 and 3). The entire arterial system of both chorions was injected from one umbilical artery of one partner; the mass easily passed the constriction between the two halves of the chorion, and penetrated even into the umbilical arteries of the other; every cotyledon was injected on both sides. The venous system was also injected from one of the umbilical veins of the same specimen; the injection mass also passed the constriction far into the chorion of the opposite side, but the blood present in the veins prevented as complete an injection of the veins as of the arteries.

The two umbilical arteries of each foetus have a cross connection at the distal end of the umbilical cord, so that an injection from one artery outwards flows both centrally and distally. The two veins lack such an anastomosis.

The arterial anastomosis (fig. 3). The main artery from the right of the drawing divides in three branches 1, 2, and 3 as it approaches the center. Branch 3 need not be farther considered as it does not anastomose with the opposite side. Branch 1 can be followed directly through into communication with the arterial system of the other side, branch 2 has a strong anastomosis with the through trunk *1-1* (at *1-2*), but branches for the most part within its own venous territory. The side branches of the through trunk *1-1* are of considerable interest, inasmuch as some are oriented in the direction of the blood flow from the right, and others from the left. Thus following the trunk from the right the first branches that we meet are directed against the blood stream coming from this direction (*1a*); immediately after passing the anastomosis (*1–2*) we meet a branch *1b*, directed with the blood flow from the right; the next two branches, *1c* and *1d*, are directed similarly, but the large branch *1e* immediately beyond has the reverse orientation. If we suppose the blood

flow to come from the left *1e* is directed with the current, *1d*, *1c*, and *1b* against it, etc. The orientation of these branches seems to indicate an alternation in direction of flow in the main trunk, as is to be expected with a beating heart at each end of it. It is significant that this is the only place in the arterial system of the membranes where reversal of orientation of branches is found in the course of a single trunk.

The venous anastomoses. The venous anastomoses are two in number (*4* and *5* in figure 3). The larger one *4* was on the opposite side from which the drawing was made, and is therefore represented as a broken line; *5* comes from the same main venous stem. There is no reversal of orientation of side branches.

The circulation. It is obvious that any arterial blood that is pumped by either foetus into the capillary system situated beyond about the line *A–B* will be taken up by the venous system to the other foetus. There must therefore be a constant interchange of blood between the two foetuses, which, considering the size of the arterial intercommunications, must be very considerable. The venous anastomoses are not significant for the intermingling of the two circulations. The direction of flow along the main arterial trunk (*1–1*) will depend on the blood pressure on the two sides. If for any reason an excess of blood is received by one of the two foetuses, this will have a tendency to raise the blood pressure on that side and thus to equalize the distribution. There is of course the possibility that the beat may alternate on the two sides, but nothing is known of this, and the effect of such an arrangement would not be easily deduced.

2. Case no. 47. ♂ 22.75 cm. and ♀22.25 cm. (fig. 4). Arteries injected yellow; veins blue. The injection was made first into an umbilical artery and vein of the male. The arterial injection flowed regularly into the opposite chorion and through to the free-martin; the venous injection also flowed into the opposite chorion, but not so freely. The injection was then completed from an umbilical artery and vein of the free-martin in order to fill the vessels on this side more completely.

The arterial anastomosis is a single strong vessel, the relationships of which are shown clearly in the figure and require no further description. The stage is much more advanced than the preceding case, and the cotyledons are much more developed. Most of the arterial branches are distributed directly to the cotyledons. The venous anastomosis is much less viable than the arterial; macroscopically it consists exclusively of a connection between the two veins of one cotyledon (*2*, fig. 4) one of which returns to the male side and the other to the side of the freemartin. This is the only cotyledon that appears to be connected with the umbilical veins of both sides; therefore any other venous anastomisis must be through the capillary circulation of the extra-cotyledonary chorion if it exists.

The circulation in this case must be according to the same principles as in the preceding: whenever the arterial pressure is higher on one side than the other blood must be distributed from the side of higher pressure to that of the lower pressure; it will thus reach the veins and the foetus of the opposite side; variations in pressure on the two sides must constantly occur, if there is any difference in the time of occurrence of systole and diastole of the twin hearts. The blood of the twins must therefore intermingle intimately, and internal secretions of either must reach the other.

These cases adequately illustrate the time and nature of the vascular anastomosis; we may therefore turn to the question of duration of the intermingling of the blood during foetal life.

We have seen that the vascular anastomosis probably begins at the stage of about 19–20 mm. The two cases we have considered in detail indicate a strengthening of the arterial anastomosis, and a weakening of the venous anastomosis after a certain stage as development proceeds. This is to be expected because the arterial flow is stronger and toward the center, whereas the venous flow is slower and away from the center. The circulation itself tends therefore to strengthen any primitive arterial connection, and to diminish relatively any venous connection. Moreover as development proceeds the cotyledons increase in size, and the intercotyledonary circulation in the chorion becomes

correspondingly reduced in a relative sense with the result that the prominent arteries and veins become exclusively cotyledonary with the single exception of the artery connecting the two sides; and any intercotyledonary venous connections become insignificant.

It is an important question whether this condition persists throughout foetal life, even though completely sterilizing effects on the female reproductive system are produced by the stage of 7.5 cm., as we shall see in more detail in another section. The question therefore relates to possible influences on later stages of the female reproductive organs, and on the somatic characters of both twins. The latest stage that I have examined with reference to this question was a pair of female twins, 35.3 cm. and 31.25 cm. in length respectively. The arterial connection was even stronger than in earlier stages proportional to the more advanced stage of development. There is no reason to suppose that the connection is interrupted until birth, but the actual observations have not been made.

Thus the available records indicate a growth of the arterial anastomosis throughout foetal life and a consequent duration of action of the male hormones up to the time of birth. The possibility exists that in certain cases the connection may be interrupted at different stages of development; but so far no such cases have appeared. In any event the decisive effects on the reproductive system of the female are determined very early and they are presumably irreversible in their character.

Triplets occur rarely in cattle, and cases of even more young at a birth are on record. Unfortunately records of their breeding history appear to be very rare. The only one that I have been able to discover is given by Pearl ('12). In this case there were two females and one male. The females were kept until they were about three years old, but they never came in heat. They were then killed, and "The man that dressed them said that they never would have bred. Neither uterus nor tubes were recognized, but the vagina apparently ended at its anterior end as a blind sac." Both were apparently sterile free-martins. The male was put in service and got good calves. We have here,

therefore, in all probability a case in which the circulations of the three individuals anastomosed, and in which the male sterilized both females.

IV. THE HORMONE THEORY OF THE FREE-MARTIN

We may now proceed to a consideration of the argument for the hormone theory. In our previous considerations we have dwelt upon the separate zygotic origin of the free-martin, and the foetal vascular connections; it is obvious that these conditions suggest a hormone theory; but, before such a theory could be regarded as demonstrated an explanation of the existence of fertile free-martins would need to be offered, and the limitation of the phenomenon of sterility of the free-martin to cattle as a common occurrence would have to be explained; the possibility of the existence of sex hormones at such an early period of the foetal life would also need to be demonstrated, and reason for limitation of the sterilizing effect to the female is needed.

We shall consider first the fertile free-martin; three cases of a normal female twin to a male have been found in my 24 cases of bovine two-sexed foetal twins. These are readily explained *a priori* on the hormone hypothesis on the supposition that they represent cases in which anastomosis of the foetal blood-vessels did not occur. It is important to notice that such cases are exceedingly crucial, for if we should find a case of two-sexed bovine twins in which foetal vascular anastomosis was absent, and in which the female was nevertheless a sterile free-martin, the hormone theory would have to be abandoned.

The first two cases of fertile free-martins were nos. 8 and 9 of my series (figs. 7 and 8); they were collected before the hormone theory was formed and the records are incomplete. In my notebook I had merely recorded that the connection between the two chorions of each pair was narrow; it was probably not vascular, but this cannot be certainly known, and these cases must be left out of consideration. Fortunately the third case, no 40, is a veritable *experimentum crucis*. In this case organic connection of the two chorions was entirely lacking. The central ends of the two chorions merely overlapped in the body of the uterus, and fell

apart when removed; injection of the chorion of the male showed its circulation to be entirely closed. Dissection of the female showed its reproductive system to be perfectly normal (fig. 6); sections of the gonad showed it to be an ovary (Chapin, '17); each maternal ovary had a corpus luteum in it. Even though this case stands alone, it is obvious that it fulfills all the conditions of a radical experiment; so that we can say that foetal vascular anastomosis of two-sexed twins involves the sterile condition of the female, and absence of such anastomosis its fertile condition.

The sheep and other normally uniparous ruminants should furnish another test of the theory; for though twin births are fairly common in sheep the female of two-sexed pairs is usually normal. This is a matter of common experience among breeders, and is strikingly demonstrated by Prof. Alexander Graham Bell's well-known experiments (Bell '12) on the production of a multi-nippled race of sheep; 36 per cent of the lambs born on Professor Bell's farm were twins; and in 1912, 60 per cent of the lambs born from three year old ewes were twins; the records show that the twin ewes are used commonly for breeding purpose, which would not be the case if any considerable percentage were sterile. The fact that there is no reference in this very careful series of experiments to sterility of ewes from two-sexed twins would also show that such a phenomenon must be at least very uncommon. On the other hand Bateson states that it sometimes occurs among sheep; though, on what authority, I do not know.

In response to a letter of inquiry Wm. John G. Davidson who has had charge of the breeding operations at Dr. Bell's estate for a great many years writes:

> I may say that in all my experience in sheep breeding I have yet to find a case where lambs born twin to males have turned out sterile. In fact when lambs are born twin male and female if they have the desired qualifications required in the flock both lambs would be retained in the flock and I have not had the slightest trouble with either male or female being unfruitful. I know there is nothing in the free-martin theory in sheep breeding.

It was therefore very interesting to examine twin pregnancies of sheep with reference to the relations of their membranes. I

found in the four cases, that I examined, that the twins were dizygotic (in one case both corpora lutea were in one ovary) and that the membranes were fused in the body of the uterus as in cattle. But when injections were made, as was done in all four cases, it was found that the circulation of each individual was entirely closed; the injection mass could not be forced from one side to the other, either through the arteries, or through the veins. Figure 5 gives a faithful representation of one case; it will be observed that the arteries and veins of each side end in a central neutral zone that they do not cross; this zone is no doubt occupied by capillaries, and it is possible that these anastomose from the two sides, though it is uncertain. The other cases were similar, though in one of them a single centrally placed cotyledon received an artery from each side; each artery was accompanied by its own strong vein returning to the same side, which indicated that there was little, if any, intermixture of blood in the cotyledon; the starch injection masses, yellow on one side and red on the other were not forced through.

In the sheep we have, then, all the necessary conditions for the production of sterile free-martins except the actual vascular anastomosis. If the vascular anastomosis should also occur exceptionally, such a condition should be accompanied by sterility of the female in the case of heterosexual pairs. This lends probability to the assertion that this condition actually occurs occasionally in sheep.

The hormone theory thus gives a satisfactory explanation of the occurrence of occasional fertile free-martins in cattle as well as of the usual condition of sterility of the free-martin; and it fits the case of the sheep equally well. As regards other ruminants we have unfortunately almost no information. But I have been much interested to find that the famous discoverer of the circulation of the blood, William Harvey, in his "*Exercitationes de Generatione Animalium*" 1651 has some statements on the subject of twin pregnancies in ruminants: thus in Ex. 69, p. 487 (Sydenham Society edition, translated by Willis), he says of the deer, "if the conception be double, one in either horn (of the uterus), each sends its umbilical vessels to its own

horn alone; the embryo in the right horn deriving nourishment from the right part of the conception, that in the left from the left portion of the same." He made similar observations on the sheep, goat, and "other bisulcated animals" and notes that "in the dog, rabbit, hog, and other animals that produce a considerable number of young at a litter, the thing is otherwise. In these each foetus has two humors, these being severally surrounded with their proper membranes." So far as I know there are no other published observations on the foetal membranes of twins in ungulates from Harvey's time to the present with the exception of Bonnet's single case already referred to. Harvey's observations show that fusion of chorions is wide spread in twin pregnancies in ungulates; but he states definitely that in the deer the umbilical vessels of each foetus are distributed to its own side only, in which it resembles the sheep. A more careful examination of the female of two-sexed twin pairs in these animals would be of interest in order to determine the possible sporadic occurrence of sterility.

The theory requires that if the same condition of common circulation of the foetal blood were to occur in other mammals as in twins of cattle the sterile free-martin condition should occur there also. Now in multiparous mammals such conditions certainly do not occur commonly; for, if they did, the very numerous researches on their embryology would have brought them to light. In the pig one can find occasional, but rare, fusions of adjacent chorions, but I have never found any vascular connection. A number of mammalian groups could be at once excluded from consideration because the conditions of placentation are such as to prohibit chorionic fusion; in mammalian groups such as primates and many rodents in which the ovum becomes embedded in the uterine mucosa, there is of course an insuperable bar to early chorionic fusion. And in those mammalian groups in which the placenta is a highly localized organ as in the remainder of the rodents, the insectivores, carnivores, and edentates the circulation in the chorion outside of the placental area is so restricted that, even if chorionic fusion did occur, it is difficult to believe that the circulation of separate foetuses would intermingle to any great extent.

Fernandez ('15) has described fusion of dizygotic chorions in one of the armadilloes (Dasypus villosus). This form has usually two young at a birth, which may be one-sexed or two-sexed. The two chorionic vesicles are separate at first, but they gradually fuse and by the time that the embryos are 3.5 cm. long the fusion of the two vesicles is so intimate that they appear as a single one. As this is the usual condition it cannot be supposed that it is accompanied by genital abnormalities. The author does not consider the problem of vascular anastomosis; but it can hardly be supposed to occur.

Although the hormone theory invokes a cause of the utmost generality in mammals, it is obvious that the conditions leading to its intervention must be restricted to forms with a relatively diffuse placentation, i.e., ungulates for the most part, and among these to forms in which quite special conditions obtain. Such conditions are found only in normally uniparous ungulates in which the ovum grows to an extreme length very rapidly, so that the associated ova meet at an early stage which favors their organic union. Even then vascular anastomosis is not likely to occur to any considerable extent unless the development of the foetal cotyledons is relatively late, so as to be preceded by a condition of general vascularization of the chorion, before the highly specialized circulation of the cotyledons becomes dominant. Such is the condition in cattle. In sheep the development of the cotyledons appears to be more precocious; I would at least venture this suggestion, although based on relatively few observations, to explain the difference between cattle and sheep in this respect.

Although these considerations give a color of great probability to the hormone theory we have still to deal with two difficulties:— 1) it is evident that we must be dealing with specific sex hormones, for their influence is limited to sex characters so far as our present evidence goes; is there any other evidence for such early production of specific sex hormones available? 2) It is certain that the intermixture of blood of the twins must be reciprocal, but the effect is exclusively on the female; in what way can this be explained?

Both of these difficulties receive a satisfactory explanation in certain previously known facts not hitherto correlated with the phenomenona in question, and in certain new facts which are described by Miss Chapin ('17). They constitute concurrent evidence which appears to me to render the entire evidence perfectly conclusive.

The previously known facts to which I refer are (1) the early development of the interstitial tissue of the mammalian testes, from the very beginning of sex-differentiation, and (2) the fact that the differentiation of the ovary is later than that of the testis, inasmuch as in the female of mammals the first generation of ingrowths from the germinal epithelium, a complete homologue of the seminiferous tubules of the male, forms only the medulla of the ovary, and the ovarian cortex is formed from a distinct second generation of ingrowths. Thus (1) interstitial tissue of the testis is present at the time for which male hormones are postulated, and (2) the testis has a start over the ovary in this respect which results in the suppression of specific ovarian tissue from the beginning as shown by Miss Chapin's study.

On the question of the embryonic origin of interstitial cells in the testes we have the excellent studies of Whitehead ('04) and Allen ('04) on the pig; Allen also deals to a certain extent with the interstitial cells of the ovary. Allen finds that sex differentiation is strikingly shown in the structure of the testis and ovary in embryos of 2.5 cm. length. The sexes cannot be sharply distinguished at 1.8 cm. length so that the initial stages of sex-differentiation lie in between. Interstitial cells are present in both testis and ovary at the stage of 2.5 cm., but while they are very numerous in the testis at this stage they are very rare in the ovary. Whitehead finds that they appear in the testis of the pig embryo at 2.4 cm. Although these authors do not note it, it is unquestionably significant that these cells appear, and exhibit the usual evidences of active secretion at the time of the onset of sex-differentiation. It is also significant that they appear first in the testis and more abundantly than in the ovary; this is of course correlated with the fact that the cortex of the ovary in which they appear is a later formation than the seminiferous tubules or their homologue in the female, the medullary cords.

As regards the origin of the interstitial cells of the ovary we have relatively few observations. Allen notes, as we have seen, that they are very rare in the ovary of the 2.5 cm. pig as compared with the testis; they are also very short-lived, disappearing at the stage of 4 cm. "No interstitial cells are found in the rabbit ovary until the stage of 45 days after birth." They would thus appear to form a very inconspicuous and transitory feature of the embryonic ovary. It is not clear from Allen's account whether they occur in the medulla or cortex of the embryonic ovary; a point which is of some significance in connection with the following discussion.

As regards the testis Whitehead notes

> Leydig's cells (the interstitial cells of the testes) pass through two phases of growth, between which a phase of atrophy intervenes. Growth is very rapid from their appearance in the embryo 2.4 cm. long until the length of 3.5 cm. is reached. This is followed by the phase of atrophy, during which the cells return almost to their first state of nearly naked nuclei (figs. 4 and 5). This process reaches its acme in the embryo 14 cm. long. Synchronous with it there is extensive growth of the seminal tubules, particularly in length, so that they are much convoluted, and the intertubular spaces are correspondingly narrowed (fig. 6).

The atrophy proceeds slowly from 3.5 to 14 cm., and is not at all marked at 5.5 cm.

> In the embryo 20 cm. long the cells enter upon the second phase of growth, which attains its maximum in the pig of 28 cm., very near to term. Here the cells are enormously increased in number and size, so that they constitute the predominating feature of the microscopic picture.

It seems to me that these facts are of great significance, viz: the intensive growth of the interstitial cells of the testis at the time of most rapid sex differentiation, between 2.4 and 3.5 cm., the subsidence of their activity after embryonic sex differentiation is once attained, and the second phase of activity which probably leads on to the juvenile sex differentiation. It would thus appear that both phases of sex differentiation are covered by periods of intensive activity on the part of the interstitial cells of the testis.

These data relate for the most part to the pig, but there is no doubt that we are justified in assuming that the processes in cattle would not differ essentially; and hence that at the time of sex-differentiation in cattle there is an active secretion of male sex hormones which pass into the blood, and thus in the case of twins by the vascular anastomosis reach the circulation of the other twin. At this time, if the other twin is a female, the cords of Pflüger have hardly begun to form; no interstitial cells can therefore be present; hence there can be no conflict of hormones. Now Miss Chapin's study of the embryonic gonad of the sterile free-martin shows that the cortex of the ovary does not develop in these animals. Hence no conflict of sex hormones arises; hence also, there can be no question of the male of two-sexed twins being influenced in its sexual development by its mate.

There is at least no escape from the conclusion that it is the circulation of the blood of the male twin in the female that accounts for the results. The probability of the presence of interstitial secretion of the testis in the blood of the male at the time of beginning sex differentiation and the limitation of the action of the male blood to the reproductive system of the female are the reasons for attributing the effect to sex hormones of the male. The possibility of course exists that the blood of the male in such foetal stages differs from that of the female in this specific respect owing to other causes than secretion of interstitial cells of the testis; but there seems to be no reason for making such an assumption.

In my preliminary paper I left open the question whether the invariable result of sterilization of the female at the expense of the male was due to more precocious development of the male hormones, or to a certain natural dominance of male over female hormones. It now appears from the results of more detailed investigation that the latter alternative probably does not arise.

The main assumptions that are involved up to the present point are (1) that cattle resemble pigs with reference to the early origin of interstitial tissue in the testis; (2) that such tissue has in foetal life properties similar to those that have been demonstrated in post foetal life by a considerable number of investi-

gators. While it will no doubt be desirable to clear up these assumptions by farther observations and experiments, it is nevertheless true that the facts that we have presented form a solid basis for both assumptions, which are in no sense unsupported or forced.

The material presents the problem whether the intermixture of other internal secretions of the twin individuals modifies any of their characters? It is obvious that modifications could be expected only in the case of differentiating characters, of which the most fundamental are those of sex; all other main features of organization are common to the sexes, and there is no evidence of individual or sexual differences in the hormones of a species except in the sexual hormones themselves. A negative answer would therefore be expected to this question in general. Individual variations of course exist, and it is an interesting question whether the blood community of foetal life tends to reduce such variations and to approximate their resemblances towards the correlation of variability of identical twins? We have seen reason to believe that this is not the case in regard to color, but the problem remains for future investigation.

According to the conceptions involved in this discussion, the deviations of the sterile free-martin from the female type are due to the action of the male sex-hormones. In order to appreciate the full extent of this action, it is necessary to understand minutely the anatomy of the sterile free-martin in comparison with normal individuals. This comparison is made in a detailed way for numerous cases in section V. The results there given will enable us to correlate the whole series of phenomena and to discuss in a very general way the phenomena of sex-differentiation in mammals.

In terminating the present section then we may repeat our main steps up to this point:—(1) We have demonstrated the separate zygotic origin of the free-martin and its male twin, (2) We have studied the foetal vascular connections in twin pregnancies of cattle. (3) We have explained the existence of occasional fertile free-martins. (4) We have explained the reason for the usual limitation of sterility of the female of two-sexed

twins to cattle. (5) We have shown that there is a firm basis for the hormone theory in the known data concerning the time of origin of interstitial tissue in the testis and in the ovary. (6) We have also shown why the effect is limited to the female.

V. THE EMBRYONIC AND ADULT ANATOMY OF THE FREE-MARTIN

We can now proceed to a discussion of the anatomy of various stages of the free-martin with a firm basis for interpretation of the conditions. This will be considered under two heads, A. the gross anatomy, B. the microscopic anatomy. The second section has been worked up by Miss Catherine L. Chapin from my material, and is published as a separate paper immediately following this.

The gross anatomy

The mammalian embryo passes through a stage of complete sexual indifference anatomically, in which all the organs of both sexes are represented by embryonic rudiments in each individual; the anatomical sex differentiation that follows is due to progressive development of certain parts and degeneration of others in different ways in the two sexes. This is such a commonplace in embryology that it seems unnecessary to describe the anatomy of the indifferent stage.

For comparison with the anatomy of the free-martins I have introduced figures of the normal female reproductive system of embryos 10.2 cm. long (fig. 6), 17 cm. long (fig. 9), 20 cm. long (fig. 7) and 23.3 cm. long (fig. 8), and of the normal male reproductive system of embryos 10.4 cm. long (fig. 10), 15.8 cm. long (fig. 11), and 26 cm. long (fig. 12). The free-martins will be described in the order from the youngest to the oldest. The written descriptions are given very briefly, and the reader should refer to the figures for details. The exact interpretation of the ducts is often difficult or impossible on the gross anatomical evidence alone; Miss Chapin's descriptions should be compared.

1. Case 19. ♂ 8 cm.; ♀ 7.5 cm. in length; figure 13, urinogenital Septem of Female. × 4. (Cf. Miss Chapin's description of the histology of this specimen in the paper following.)

This is the youngest free-martin found; its reproductive system is already definitely of the sterile free-martin type. The gonads are much smaller than in the normal female (cf. description by Miss Chapin '17, p. 455). Miss Chapin's observations described in the following paper demonstrate a complete absence of cortical tissue in the ovary; the medullary component is hypertrophied as compared with the normal, and an albuginea is present. The ducts also show a sifting in the male direction (Chapin, p. 459), the Müllerian duct being in process of degeneration. Thus already at this stage the characters of the sterile free-martin are definitely established, and it is proved that the primary effect of the male sex hormones is to prevent the formation of the ingrowths that normally form the cortex of the ovary. This is a matter of great significance, which is repeated in all the other cases.

History. Collected October 20, 1915. Maternal ovaries absent. These twins were contained in a single chorion with broad connection occupied by cotyledons between the two halves.

2. The next stage (♀ 12.5 cm., case 26) was used for histological work; it is described by Miss Chapin. No study of the gross anatomy.

3. Case 17. ♂ 13.75 cm.; ♀ 13.1 cm. in length; figure 14, urinogenital system of ♀. × 4.

In this preparation the very minute size of the gonad strikes the eye at once; (compare fig. 6, normal female 10.2 cm. long, and 9 normal female 17 cm. long). The Wolffian body has entirely degenerated. Phallus typically female. About midway between the gonad and the genital cord the inguinal fold arching over the umbilical arteries unites the ducts (urinogenital fold) to the lateral body-wall; this fold is the foundation for the round ligament in the female and is the site of formation of the gubernaculum in the male. The ducts are interpreted as Wolffian ducts, though they appear to be united in the posterior third of the genital cord; Müllerian ducts are not visible macroscopically.

History. Bovine twins ♂ and ♀. Collected October 9, 1915. One maternal ovary missing; the other had a single corpus

luteum in it. The chorion was single with a broad connection occupied by cotyledons between the two halves.

4. Case 37. ♂ 16 cm.; ☿ 15.5 cm., figure 15, urinogenital system of ☿. × 2.

This figure may be compared with figure 11 (normal male of 16.8 cm.) and figure 9 (normal female of 17 cm.). The exceedingly rudimentary condition of the gonads is at once evident, about two-fifths of the length and one-third of the breadth of the normal ovary. The ducts appear to be Wolffian ducts. An extraordinary feature of the anatomy which recurs in every ☿ specimen of this or greater size is the development of a pair of gubernacula from the inguinal fold, which penetrate the musculature precisely as in the male (fig. 11) and reach the integument, extending well down to the groin. In the normal female the round ligament is definitely formed (fig. 9 A and B) in place of the gubernaculum, the horns of the uterus are definitely differentiated from the Fallopian tube, the vagina is enlarged.

History. Bovine twins ♂ and ☿. Collected January 25, 1916. Both maternal ovaries present, each with a single corpus luteum. The uterus was damaged by a cut above the cervix and the two halves of the chorion severed. Examination showed that the chorion had been single with both arterial and venous anastomosis between the two halves. Cotyledons continuous from side to side.

5. Case 6. ♂ 16.8 cm., ☿ 16.3 cm., figure 16, urinogenital system of female. (cf. Miss Chapin's description of the histology of this specimen and interpretation of the ducts.)

For comparison normal female of 17 cm. (fig. 9) and normal male of 16.8 cm., twin to this specimen (fig. 11).

The very minute size of the gonads is again apparent. Gubernacula again take the place of the round ligament. The Müllerian ducts are undeveloped. Seminal vesicles, however, have not begun to form (cf. normal male, fig. 11); the contrast between the genital cord of the ☿ and normal ♀ is very striking.

History. Bovine twins. Collected March 4, 1915. Only one of the maternal ovaries was present; corpus luteum in it. Single chorion for the twins somewhat constricted. No record of vascular conditions.

6, 7, and 8. Cases 32, 23, and 36. 16.75, 17.5, and 17.5 cm. respectively may be mentioned together (figs. 17, 18, and 19). The drawings of these three specimens show essentially the same anatomical characteristics as the preceding stage; the gonads are very small in all, but somewhat larger in 32 (fig. 17) than in the others. They all exhibit gubernacula and the ducts resemble the male ducts much more than the female; the urinogenital sinus is intermediate in length between the female (fig. 9) and the male (fig. 10).

Histories. Case 32. Figure 17. Bovine twins. Male 18 cm., female 16.75 cm. Collected January 1, 1916. Both maternal ovaries present; corpus luteum in each. The uterus was injured and the single chorion cut in two; there was an arterial connection between the halves, and cotyledons continued through.

Case 23. Figure 18. Bovine twins. Male 18 cm., female 17.5 cm. Collected October 29, 1915. Only one maternal ovary present, containing single corpus luteum. The chorion was single; broad connection between the two halves; cotyledons in connecting part.

Case 36. Figure 19. Bovine twins. Male 18 cm., female 17.5 cm. Collected January 21, 1916. Both maternal ovaries present with a single corpus luteum in each. The chorion was single with broad connection occupied by cotyledons between the two halves. Arterial anastomosis present; veins interdigitate, and may communicate.

Summary of the preceding cases. The gross anatomical findings for the stages 7.5 to 17.5 cm. thus show that the effects (either direct or indirect) of the male hormones on the reproductive system of the female are (1) to inhibit the growth of the gonad, (2) to cause the formation of typical gubernacula in place of the round ligament, (3) to favor the development of the Wolffian ducts and inhibit the development of the Müllerian ducts. The urinogenital sinus is intermediate between the male and female condition and the phallus develops similarly to that of the female; moreover the disposition of the teats is female, differing definitely from that of the male (figs. 4, 12, 25 A) and no

trace of the scrotum which is well developed in males of corresponding size is to be found. In other words the derivatives of the urinogenital ridge (gonads, Wolffian and Müllerian ducts and ligaments) tend to develop in the male direction, but other parts of the urinogenital system of such affected females are less involved.

In later stages the conditions established at the beginning undergo farther development. Drawings of various stages from 21.5 to 28 cm. are shown in figures 20 to 27, and these will hardly require detailed description after what has gone before. A rather common anomaly is the evagination of the right gubernaculum into the body cavity in place of growing into the body-wall (figs. 20, 22, 25, and 27); it is difficult to assign a cause for this; inasmuch as it rarely happens on the left side (fig. 27) it is presumably associated with the asymmetry of the urinogenital complex which is particularly striking in the case of the kidneys (see all figures of dissections). The gonad remains exceedingly small in these stages, nor is there much change in other directions. Seminal vesicles, which occur frequently at least in the sterile free-martins after birth are not yet found though they are very evident in the male from 16.8 cm. on. In the normal male the testes are drawn into the saccus vaginalis at the 26 cm. stage (fig. 11); in the sterile free-martin this may happen (fig. 25 A and B), or the gonads may remain in the peritoneal cavity (fig. 26).

History of cases:

Figures 20A and 20B. Case 2. Bovine twins. Male 23 cm., female 21.5 cm. Collected October 30, 1914. Chorion single with constricted connection occupied by cotyledons between the two sides. This being one of the earliest cases no record was made of vascular conditions in the chorion. No record of maternal ovaries.

Figure 21. Case 41. Bovine twins ♂ and ♀. Collected February 16, 1916. Male 22.7 cm., female 21.8 cm. Both maternal ovaries present, corpus luteum in each. The uterus had been cut above the cervix and membranes injured; the fusion of the two chorions was broad, and the cut ends of the arteries connecting the two were readily discovered.

Figure 22. Case 4. Bovine twins ♂ and ♀. Collected January 30, 1915. Male 24 cm., female 22.5 cm. Single chorion, constricted between the two foetuses. No other records.

Figure 23. Case 38. Bovine twins ♂ and ♀. Collected January 26, 1916. Male 23.5 cm., female 22.5 cm. Both maternal ovaries present, corpus luteum in each. The uterus was received cut off above the cervix severing the connecting part of the chorion. The large vessels run to cut ends; extensive vascular anastomosis between the two foetuses involved.

Figure 24. Case 22. Bovine twins ♂ and ♀. Collected October 26, 1915. Male 24.5 cm., female not measured. Both maternal ovaries present, corpus luteum in each. Uterus cut into destroying connection of the two halves of the chorion; the chorion was single and the maternal cotyledons occurred throughout the corpus uteri. Vascular conditions of chorion not observed.

Figure 25, A and B. Case 21. Bovine twins ♂ and ♀. Collected October 20, 1915. Male 27.5 cm., female 26.5 cm. Maternal ovaries absent. The two halves of the chorion were broadly connected, and cotyledons and blood vessels cross over from side to side.

Figure 26. Case 14. Bovine twins ♂ and ♀. Collected July 15, 1915. Male 27 cm. long; female 27 cm. long (measurements by assistant). Maternal ovaries absent; the two halves of the chorion were broadly connected with cotyledons and arteries crossing over.

Figure 27. Case 12. Bovine twins ♂ and ♀. Collected October 2, 1915. Male 30 cm., female 28 cm. long. Maternal ovaries absent. The chorion was single, not much constricted between foetuses. Cotyledons form continuous series from side to side. No observations on vascular conditions of chorion.

The gross anatomy after birth

The anatomy of the reproductive organs of a seven weeks old free-martin (case 44) born on my farm is shown in figure 28. Incidentally it may be noted that the twins were pure bred Holsteins, and that while the male was mostly black, the female

was about half white. This is an exceedingly interesting case, as it represents the most extreme transformation of the internal organs towards the male type which I have seen. When the body cavity was opened gonads appeared to be entirely absent; however the ducts were found to perforate the body wall; and farther dissection revealed the gonads lying in peritoneal sacs situated in the groin on each side between the skin and abdominal muscles. There is no difficulty in interpreting the parts (fig. 28): the vulva is typical; it leads into a short urinogenital sinus beyond which there is no trace of vagina, uterus, or tubes. Opening into the dorsal wall of the urinogenital sinus at its anterior end are two Wolffian ducts (vasa deferentia); laterally to them seminal vesicles. The Wolffian duct of the left side is much larger than that of the right. They run in a broad membranous septum corresponding to the broad ligament of the uterus and enter peritoneal evaginations, open to the body cavity, which perforate the abdominal muscles in the manner already described. When the peritoneal sacs are opened, as shown in the insert figures, figure 28, B and C, they are found to contain testis-like gonads, with an epididymys-like organ associated. It is an interesting fact that the smaller right gonad is associated with a smaller Wolffian duct and a smaller seminal vesicle; this is the side on which disturbances in relation of parts is found in foetal stages. Sections of the testis show a superficial dense albuginea and entire absence of cortical ovarian tissue. The interior is occupied by exceedingly wide branching tubes lined by a one-layered epithelium. Connective tissue is abundant between the tubes.

History. Figure 28. Case 44. Holstein-Friesian twins ♂ and ♀ born March 12, 1916. The female (free-martin) was killed and dissected April 29, 1916.

In the foetal stages of the free-martin, which we have considered, the gonad presents a rudimentary aspect and seminal vesicles are not formed, although they appear in the males at much earlier stages (fig. 1). On the other hand, the gonads may attain a very considerable size after birth, and seminal vesicles appear usually to be present (cf. literature). It therefore fol-

lows that there must be a belated growth of the gonads and formation of seminal vesicles in the later foetal stages which I have not had an opportunity to examine, in some cases at least.

The literature contains descriptions more or less complete of the anatomy of the reproductive system of about 30 free-martins. These have been tabulated by D. Berry Hart ('09–'10). All agree in one fundamental respect, viz: that the gonads never show the least evidence of possessing the structure of an ovary; Graafian follicles have never been described. In some cases the gonad is represented merely by clumps of fat, or is absent; in others it is rudimentary and situated in the body cavity, or may be of considerable size in the same situation, or finally may be found in the groin. Whenever present it presents more or less superficial resemblance to a testis, sometimes an exceedingly close resemblance. Histologically also it resembles a testis. However it is probable that spermatozoa are never formed, and even that the earliest stages of spermatozoa are lacking in the tubules. All agree likewise in the very great reduction or complete absence of vagina, uterus, and tubes, and the presence of Wolffian ducts in a greater or lesser degree of development. However, it would be merely tedious to review the variations in detail.

The very interesting question presents itself how far the modification of the female reproductive organs towards the male side may be carried. I have already described the most extreme case that I have met (no. 44, fig. 28); but one of Numan's cases indicates that the transformation may proceed considerably farther. This case seems to be well authenticated in all respects, and on account of the rarity of Numan's publication I include a full description of it: a pair of calf twins was delivered by Caesarian section in 1832 at Maarssen in Holland, one was male and the other was supposed to be a free-martin. Numan bought the latter the following year; on examination a small opening was found in the perineum a short distance below the anus through which urine dropped, the urethra passed over the pubic symphysis and opened externally about two handsbreaths below the anus through an opening surrounded by an

apparent female vulva, within which appeared an imperforate glans penis; the vulva was provided ventrally with a tuft of hair as normally. The animal was slaughtered in 1835, and the anatomy of the reproductive system studied. Numan's figure is here reproduced (fig. 29). It will be seen that the internal anatomy is quite similar to my case 44 (fig. 28), but the external parts are also modified in this case to a very considerable extent in the male direction.

If this case is well authenticated, as it appears to be, we would have to conclude, contrary to the evidence from my cases that the external organs of reproduction of the female are also susceptible of modification in the male direction by the male hormones; it may be that this occurs in cases of exceptionally early action of the male hormones. The embryological history of the external male and female organs shows that the sexual type is fixed very early.

It is of course by no means certain that the extreme possible modification of the female reproductive system by male hormones will be found in free-martins, indeed when we consider the various exigencies of the admission of the male hormones to the female circulation under the conditions of production of the free-martin, it seems improbable that the optimum conditions for modification of the female reproductive system are ever realized in such cases: the onset of the action can not be much before morphological sex differentiation has begun in any case, and its intensity must certainly be of a low order quantitatively at first not only on account of the minuteness of the first vascular inter-connections, but also on account of the rudimentary character of the interstitial gland of the testis at first, if we suppose that the effective hormone is derived entirely from this source. Nevertheless search should certainly be made for more pronounced cases of transformation of the free-martin towards the male condition. But it is obvious that definitely controlled experimental investigation will be the only means of deciding where the real limit of action lies, whether or not the complete transformation of a female zygote of mammals into a male individual by hormone action is possible.

VI. GENERAL DISCUSSION

The precision and definiteness of the transformation of the specific organ system, and the undoubted character of the primary cause of the transformation appears to offer a more definite basis than we have hitherto had for the analysis of the origin and differentiation of some fundamental sex characteristics of mammals. At the present time there is a general recognition of the primary zygotic determination of sex in mammals; we have some scattering data on sex limited inheritance, a great deal of miscellaneous information on the effects of castration, and a little on the effects of cross transplantation of gonads. There is also a large literature on hermaphroditism and pseudohermaphroditism of mammals. But these data are by no means capable of arrangement in any general scheme. They are on the whole exceedingly confusing in spite of the recent advances concerning the zygotic character of the initial sex impulse, and the analyses of sex hormone action in post-foetal life.

The free-martin gives us additional evidence of considerable value concerning the problem of sex-determination and sex-differentiation in mammals, especially in its suggestion that the course of embryonic sex-differentiation is largely determined by sex-hormones circulating in the blood. The evidential value of this case is, however, limited, in the first place by the fact that only the female is affected,—we have no information on the reverse situation—and in the second place by the fact that we cannot study separately the effect of early embryonic castration of the female, but only as it is modified by the simultaneous presence of male hormones, On the male side there is complete absence of information as to the effects of early embryonic castration and the possible effect of the presence of female hormones[5]

[5] A curious problem however presents itself in this connection viz: how it happens that the sex-hormones of the mother do not affect the reproductive system of the unborn sons. Steinach's results on feminization of infantile male rats by castration and implantation of ovaries demonstrates the far reaching effects of female sex hormones in the male system; and we can hardly doubt that even greater effects would result in embryonic life. It would appear probable, therefore, that the embryo is in some way protected from the sex hormones circulating in the mother's blood. Either there is cessation of production of sex

in the absence of male hormones. These facts must be kept clearly in mind as definitely fixing the very provisional character of such speculations as we may make.

The present standpoint for the analysis of sex-characters of mammals would have to include (1) primary zygotic determination of the male and female sex and (2) secondary differentiation of the sex characters, in which internal secretions play a very specific and fundamental rôle. The production of intermediate zygotic conditions is theoretically possible, since Goldschmidt has demonstrated all grades of intersexuality in the gypsy moth depending on variable conditions of the gametes. But it is obvious that such conditions are not involved in the present case and that we have to consider only the secondary factors.

It follows from the data that the female zygote must contain factors for both sexes; the primary determination of the female sex must therefore be due to dominance of the female factors over the male. If we think of this as a simple quantitative relation, as Goldschmidt ('16) has done, we can explain the intersexual condition of the free-martin as due to an acceleration or intensification of the male factors of the female zygote by the male hormones. The degree of the effect which is quite variable, as we have seen, would of course be subject to all quantitative variations of the hormone. Thus the case of the free-martin could come under the same general point of view as that of the intersexes of Lymantria according to Goldschmidt with the one exception that the quantitative differences between the male and female factors of the female zygote necessary for the differentiation of female characters, are reduced in the free-martin by internal secretions instead of by variations of potency of the

hormones during foetal life, or they are neutralized in some way, or the placenta is impervious to them. The first possibility does not seem consistent with our knowledge of the physiology of the mammalian ovary, or with the cytology of the organ during pregnancy; the second one offers no point of attack at present; the third seems the most probable *a priori*, and is no doubt susceptible of experimental analysis. But whatever explanation may prove to be correct it seems probable that disturbance of the equilibrium that protects the male from the sex hormones of the mother would result in malformations of the male sex characters to a degree commensurate with the extent of the disturbances. There is, therefore, here a possible explanation of the greater mortality among male foetuses, and of certain types of intersexes (pseudo-hermaphroditism).

male factors in different varieties as in the intersexual hybrids of Lymantria.

The case of the free-martin shows that a gonad with a primary female determination may form a structure which is morphologically a testis (Chapin '17), through suppression of the cortex and overdevelopment of the medullary cords and urinogenital union under the influence of male sex-hormones. Lesser degrees of transformation are of course possible, so that it is certain that the gonad of a mammalian female zygote is capable of most, at least, of the series of transformations that may exist between an ovary and a testis. Whether the transformation in the male direction may proceed under such conditions to the production of true spermatocytes and spermatozoa is at least doubtful. Such elements have not hitherto been described for free-martins, if we except D. Berry Hart's statement concerning the gonads of Hunter free-martins, that "in only one are spermatozoa present." More than six words seem necessary to establish so important an exception.

Pick's investigation of 'true hermaphroditism' in man and mammals shows that in all bilateral cases the gonad is part ovary and part testis; but that whereas normal germ cells may arise in the ovarian part, there is no trace of spermatocytes or spermatozoa in the testicular part. Four such cases are described for the hog, and it would seem that the theoretical possibility exists of explaining them as due to embryonic foetal anastomosis of blood-vessels between opposite sexes, by making the farther assumption that such anastomosis was only temporary and ceased after the transformation of part of the genital ridge. The interpretation of 'hermaphroditism'[6] in mammals is in any

[6] The term 'hermaphroditism' has so many and various connotations that it seems better to drop it from our vocabulary, so far as mammals are concerned, and to describe the conditions hitherto gathered under this head simply as intersexual. Some conception of the confusion that results from present methods of classification of these cases may be felt by stating that our case 44 (fig. 28), would be classified as Hermaphroditismus spurius masculinus externus (Neugebauer '08): 'spurius' because both kinds of gonads are not present; 'masculinus' because the gonads are apparently testes; 'externus' because the external organs are female. But the animal is female, not male, and it represents merely a certain stage in a series. The animal is not a sex-mosaic, e.g., male in front of a certain transverse level and female behind ('transverse hermaphroditism'); it is a step in a series of sex intergrades.

event a very difficult problem, and it seems worth while to indicate the hitherto unconsidered possibility of action of heterologic sex hormones, however brought about, in this connection.

Regarding other parts of the internal reproductive system we have seen that the free-martins exhibit a graded series of inhibition of the female ducts, and of development of the male ducts which may obviously correspond to variable time of onset, intensity, and perhaps duration of action, of the male sex-hormones. The series extends nearly to the normal male limit in exceptional cases (no. 44, fig. 28). There is indicated a rough parallelism at least between the grade of transformation of the gonad and that of the remainder of the internal reproductive system. The external organs of reproduction are the least liable to modification, but they do not escape in all cases, and may even exhibit considerable transformation in the male direction if we can accept Numan's case described on p. 413 and illustrated in figure 29.

The fundamental determining factor in these events is undoubtedly the male sex hormones as has been argued previously (p. 396); but the entire causal nexus is by no means clear. We do not know what the results of embryonic castration of the female might be in itself, and hence we are unable to assert definitely in just what positive ways the male hormones act on the female zygote, because the earliest determinable result of such action is the suppression of the ovarian cortex, which must be regarded as practically equivalent to castration. This action at least is due to the male hormones; how much of the subsequent events is due to mere absence of ovarian tissue, and how much to positive action of male sex-hormones is more or less problematical. It is well-known that spayed females of certain birds and mammals tend to develop male characters; heifers with cystic degeneration of ovary also develop certain male characteristics (Pearl and Surface, '15), so that we must admit in principle the possibility that much of the male development in the free-martin is due to the lack of inhibitions normally furnished by the ovary.

It is also probable that the various parts of the reproductive system have other means of correlation, and act and react on one another in various ways. Certain indications of this are

seen in lateral variations, as for instance in figure 28 where a large gonad on one side is associated with a large Wolffian duct, and seminal vesicle, and a much smaller one on the other side with a correspondingly smaller duct and vesicle.

When, therefore, we attribute the free-martin condition to the male hormones we only mean to assert that they are the primary cause, and not that they are the decisive factors in each member of the series of events.

It follows from this discussion that sex in mammals cannot be diagnosed by the character of the gonads alone because a testis-bearing individual may develop from a female zygote. The unexpected result is reached that the external genitalia and the mammary gland are more reliable criteria of the female sex than the internal parts.

We have no comparable data on the male side, and we can only speculate as to the transformation that would be produced in the male reproductive system by the action of female hormones beginning before sexual differentiation. Theoretically we would have to assume that the male zygote contains female as well as male factors, but the male zygote may not be capable of such extensive transformation as the female, owing to the embryological fact that the male gonad never forms normally any homologue of the cords of Pflüger in the female, i.e. of the ovarian cortex, whereas the female does form the homologue of the seminiferous tubules before the cords of Pflüger begin to arise. In the case of the free-martin we do not find that male hormones cause the development of any structure which is not represented embryologically in the normal female; the hormones act in this case merely by inhibition or stimulation of normal embryonic rudiments. If this should hold as a principle also on the male side we could not expect that the transformation of a testis into an ovary should ever occur, although suppression of complete testicular development would probably happen. But apart from this admittedly uncertain principle there is no reason for assuming that the possibility of the male zygote for acquisition of female characters may be any less than the reverse case.

For their proper evaluation the results concerning the free-martin should be associated with the other studies indicating that

zygotic determination of sex is not irreversible predestination, but a quantitative overbalance in the direction of one sex on the other. I refer more especially to the studies of Richard Hertwig ('06), Kuschakewitsch ('14), of Whitman-Riddle on pigeons (Riddle '16), and especially the very convincing demonstration of reversibility of sex in the gypsy moth by Goldschmidt ('16). These studies demonstrate that sex differentiation is controlable within variable limits in certain groups, to which mammals must be added as a result of the present study. To determine the means, limits and subsequent results of such control is now one of the most important tasks of biology.

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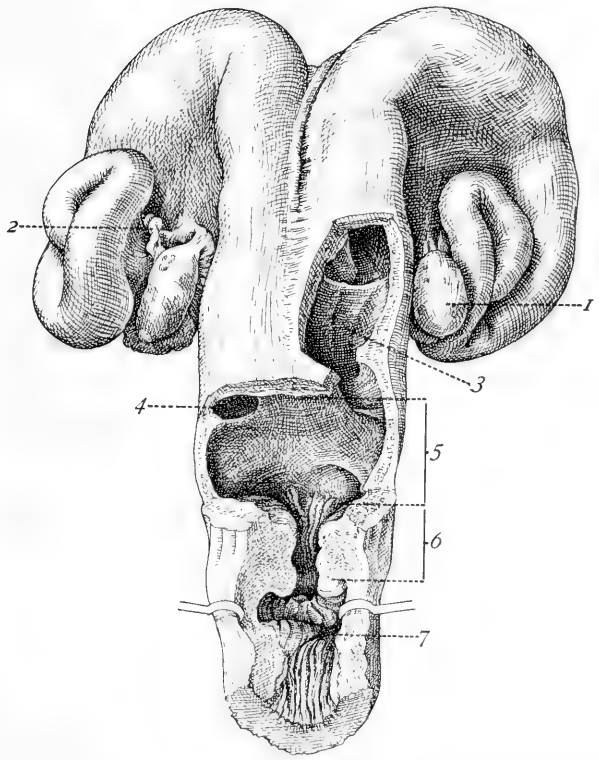


Fig. 1[7] Cow's uterus strongly distended with formalin; ventral view; parts cut out. × ⅔. *1*, left ovary; *2*, right fallopian tube; *3*, left horn of uterus cut open; *4*, opening from right horn into body of the uterus; *5*, body of the uterus; *6*, cervix; *7*, vagina.

[7] Drawings made by Kenji Toda.

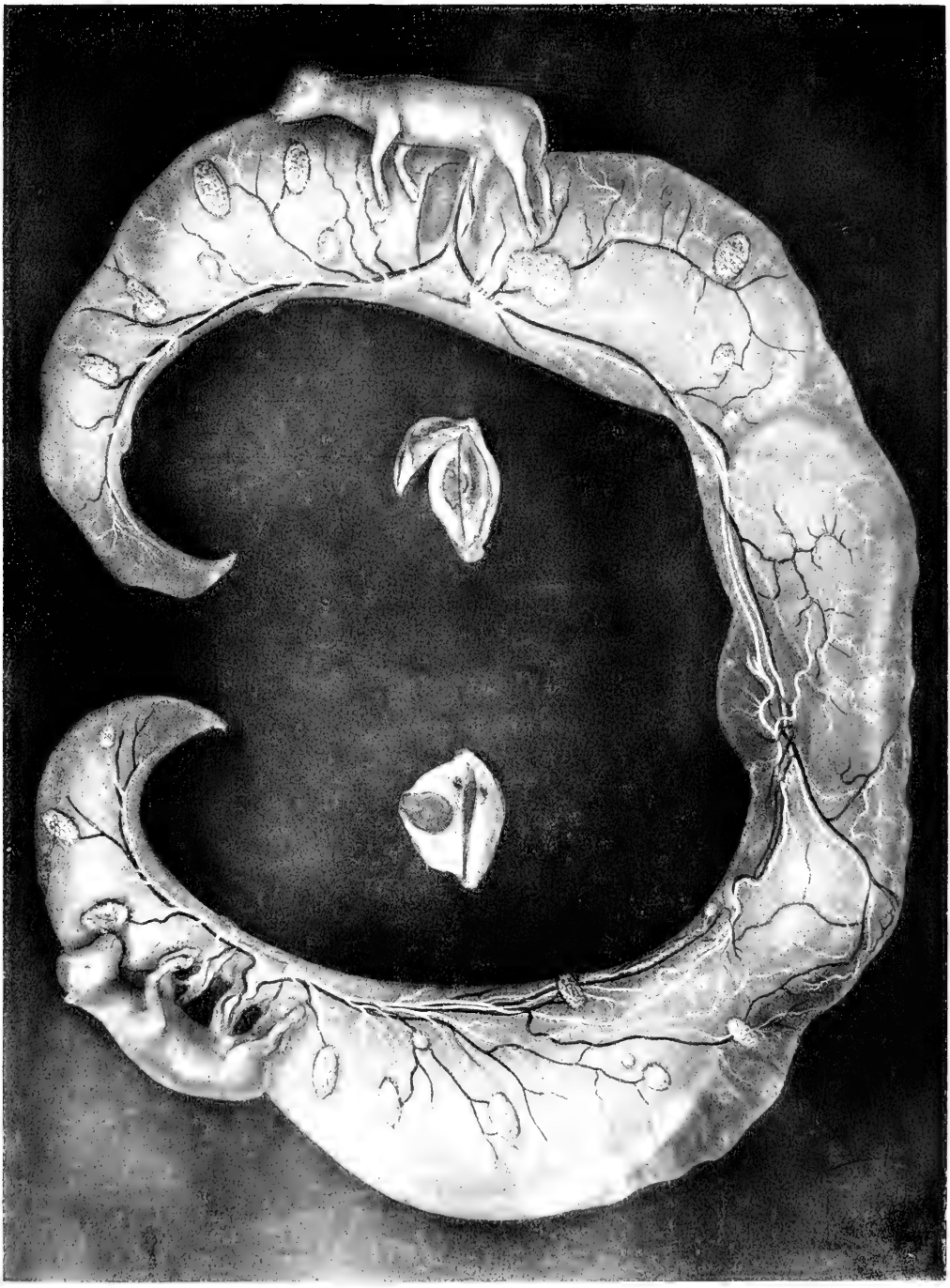


Fig. 2 Twin chorionic vesicle of cow; double injection; case no. 33 ♂♂, 10.5 cm. each. × ⅓. The figure also shows the two ovaries of this pair of twins cut so as to exhibit the corpus luteum in each. Amnions entirely removed. The arteries are represented in white, the veins in black. (See text for further description.)

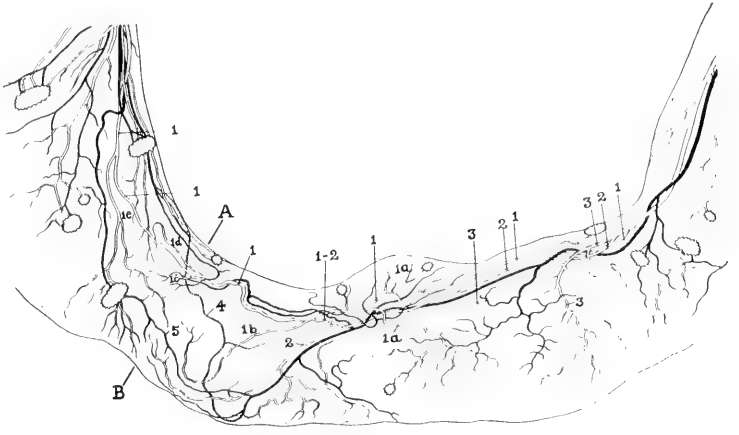


Fig. 3 The central part of the twin chorionic vesicle shown in figure 2 slightly schematized. See text p. 393 for description.

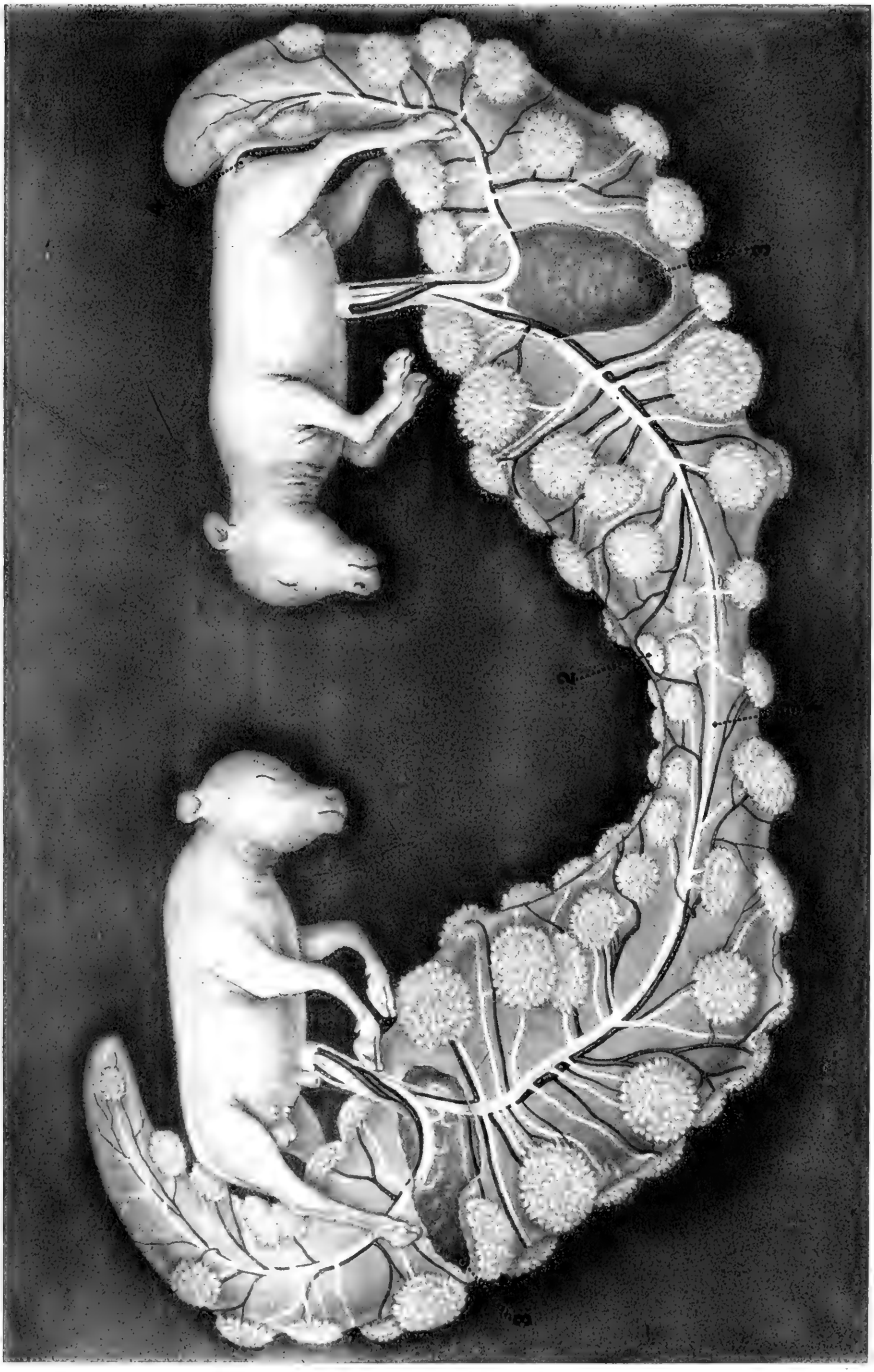


Fig. 4 Twin chorionic vesicle of cow; double injection; case no. 47. ♂ 22.75 cm. ♀ 22.25 cm. × $\frac{1}{4}$. *1*, arterial through trunk; *2*, cotyledon with venous connection with both sides; *3*, amniotic sacs opened; *4*, clitoris of free-martin; note female arrangement of teats; cf. with male. (See text p. 394 for further description.)

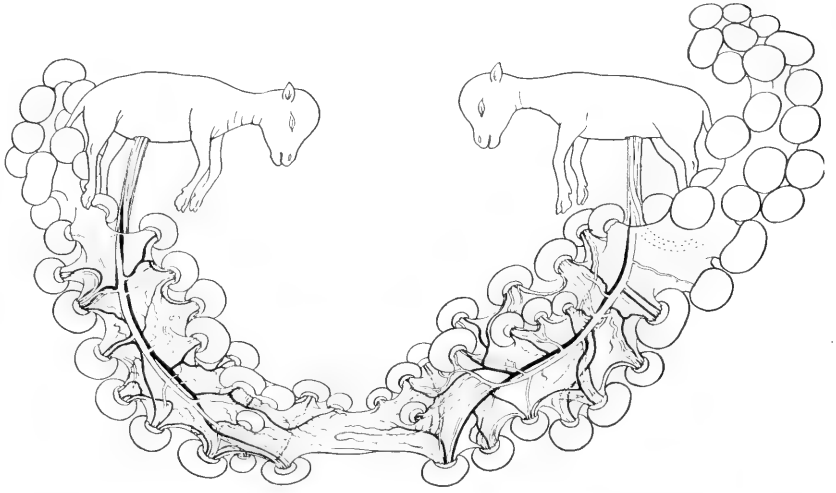


Fig. 5 Twin chorionic vesicle of sheep. × $\frac{3}{5}$. Double injection toward the center. ♀ ♀ 13 cm. each. The maternal ovaries had a corpus luteum in each. The chorionic vesicles have fused, but there is no anastomosis of blood-vessels from the two sides.

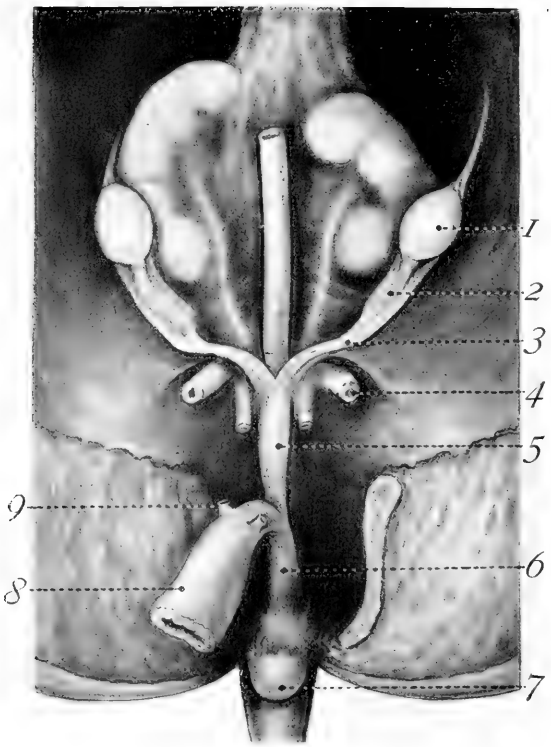


Fig. 6 Case 40. Reproductive organs of fertile free-martin; 10.2 cm. long. × $\frac{8}{3}$. In this case there was no fusion between the two chorions, and the reproductive system is normal. See figure 10 for male twin. *1*, ovary; *2*, remains of Wolffian body; *3*, Müllerian duct; *4*, umbilical artery; *5*, vagina; *6*, urino-genital sinus; *7*, clitoris; *8*, neck of allantois; *9*, ureter.

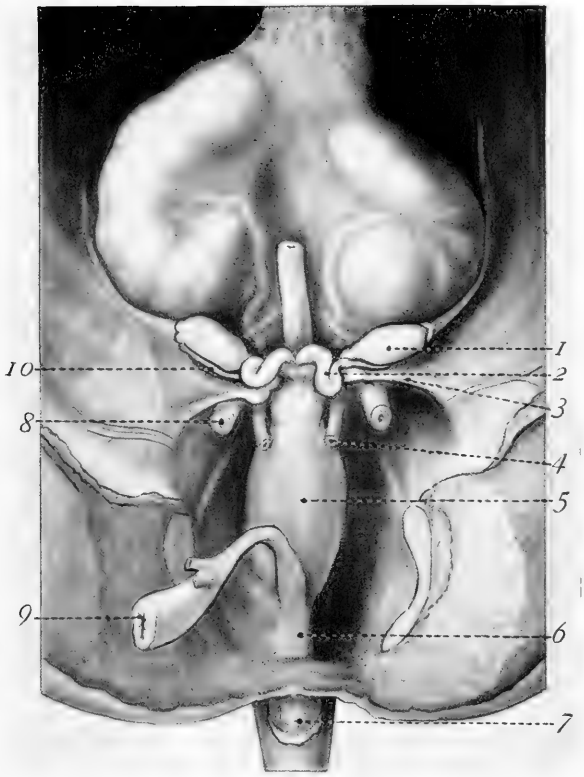


Fig. 7 Case 9. Reproductive organs of fertile free-martin 20 cm. long. × ⅔. The chorion of the twins was single with a narrow connecting part between the two halves devoid of cotyledons for a space of about three inches. No record of vascular connections, which were presumably lacking. *1*, ovary; *2*, left horn of uterus; *3*, round ligament; *4*, ureter; *5*, vagina; *6*, urinogenital sinus; *7*, clitoris; *8*, umbilical artery; *9*, neck of allantois; *10*, fallopian tube.

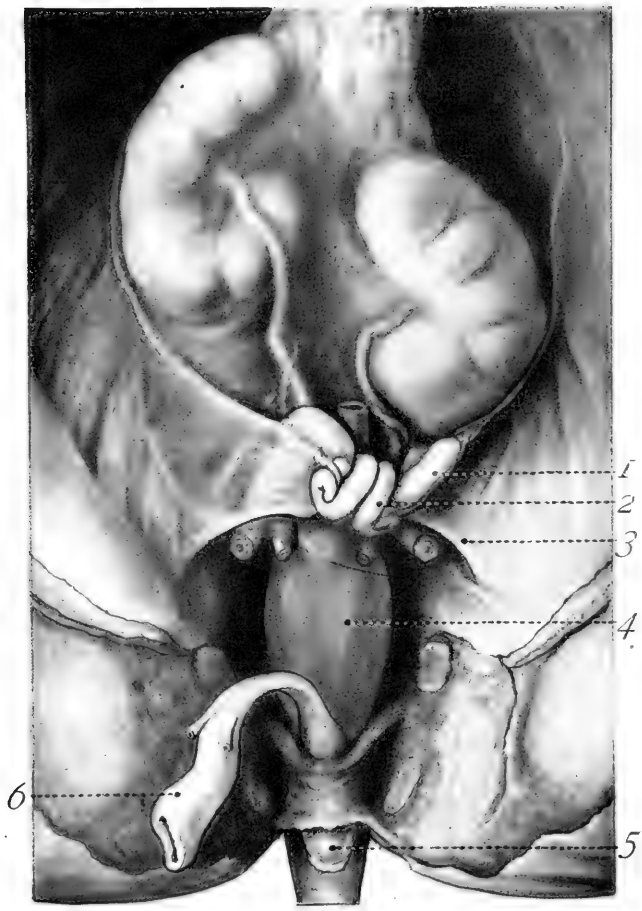


Fig. 8 Case 8. Reproductive organs of fertile free-martin 23.3 cm. long. × 1. The chorion of the twins was single with a narrow connection between the two halves. Presumably no vascular anastomosis. The male was 26.5 cm. long, nearly 80 per cent heavier than the female, and its skin was unpigmented, whereas the female was darkly pigmented. *1*, ovary; *2*, left horn of uterus; *3*, round ligament; *4*, vagina; *5*, clitoris; 6, neck of allantois.

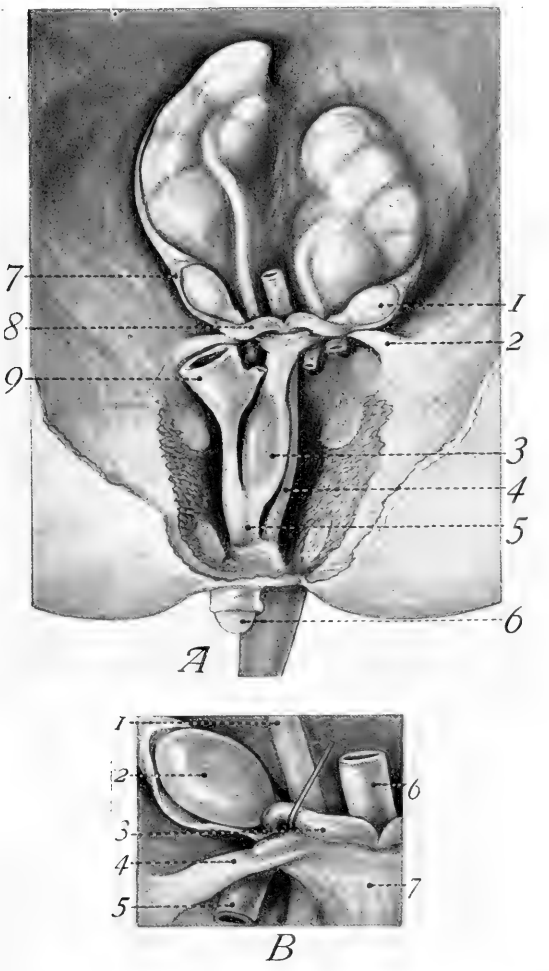


Fig. 9 Urinogenital system of normal female from twin females, pair 5. 17 cm. long. Collected February 11, 1915. Chorion was single constricted between the two foetuses. Figure B shows part of the right side $\times \frac{8}{3}$ with the horn of the uterus lifted to show the insertion of the round ligament. Contrast the round ligament of the female with the gubernaculum of the male (fig. 11) to which it corresponds exactly in position. The horns of the uterus begin to show the spiral coil; body of the uterus small; vagina distended; the urinogenital sinus much shorter than in male (fig. 11).

A. $\times \frac{4}{3}$. *1*, ovary; *2*, round ligament of uterus; *3*, vagina; *4*, rectum; *5*, urinogenital sinus; *6*, clitoris; *7*, fallopian tube; *8*, right horn of uterus; *9*, allantois.

B. $\times \frac{8}{3}$. Part of same specimen with horn of uterus raised to show insertion of the round ligament. *1*, ureter; *2*, ovary; *3*, right horn of uterus; *4*, round ligament; *5*, umbilical artery; *6*, rectum; *7*, body of uterus.

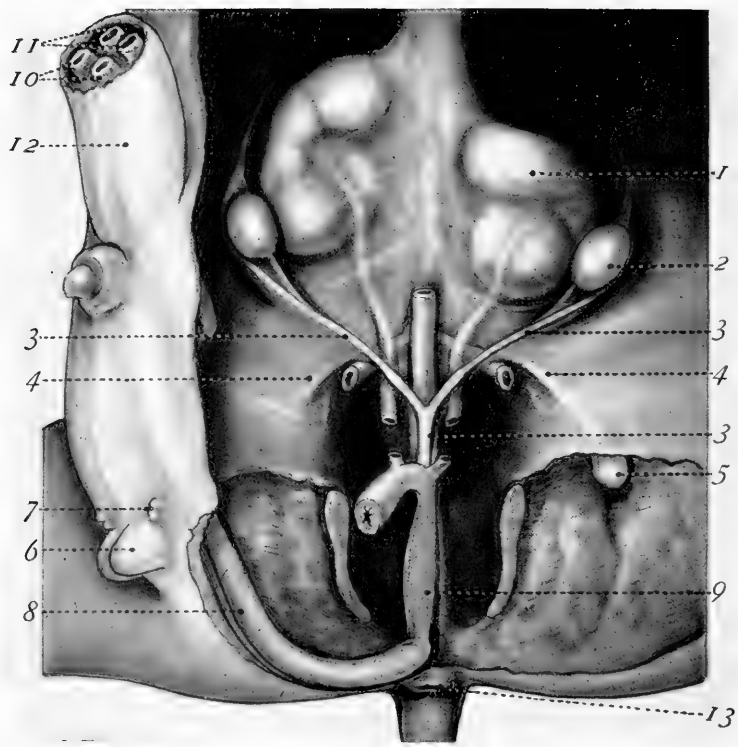


Fig. 10 Normal male 10.4 cm. long, from two-sexed pair 40. × $\frac{8}{3}$. Note that the gubernaculum is well formed on the left side, but not yet on the right. *1*, kidney; *2*, testis; *3*, Wolffian ducts; *4*, inguinal fold; *5*, left gubernaculum; *6*, scrotal sacs; *7*, teats; *8*, penial tube; *9*, urinogenital sinus; *10*, umbilical arteries; *11*, umbilical veins; *12*, umbilical cord; *13*, anus.

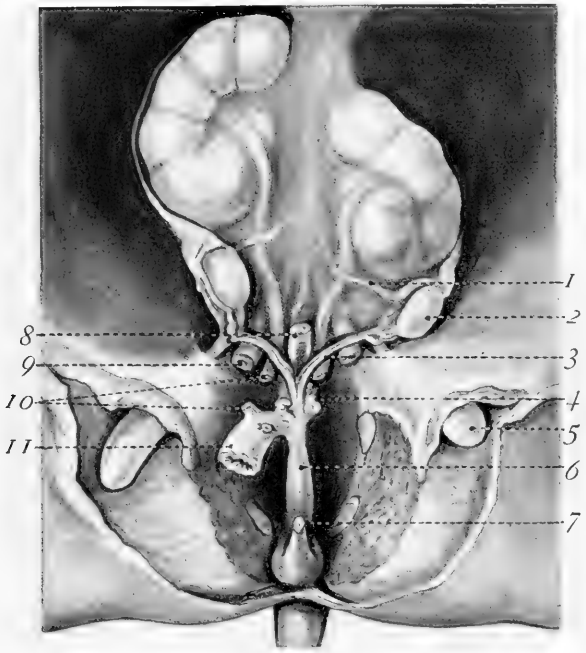


Fig. 11 Normal male 15.8 cm. long. × $\frac{4}{5}$. From two-sexed pair 6 collected March 4, 1915. Chorion was single, constricted between the two foetuses. The long gubernacula have grown into the groin but have not yet entered the scrotal sacs. The testes are still in the body cavity, though close to the entrance to the saccus vaginalis. The left testis, posterior in position to the right, corresponding to the more posterior location of the left kidney. The seminal vesicles are well formed with distal buds. The urinogenital system of the free-martin twin is shown in figure 16. *1*, spermatic artery; *2*, testis; *3*, Wolffian duct (vas deferens); *4*, vesiculae seminales; *5*, gubernaculum; *6*, urinogenital sinus; *7*, root of penis; *8*, rectum; *9*, umbilical artery; *10*, ureter; *11*, allantois.

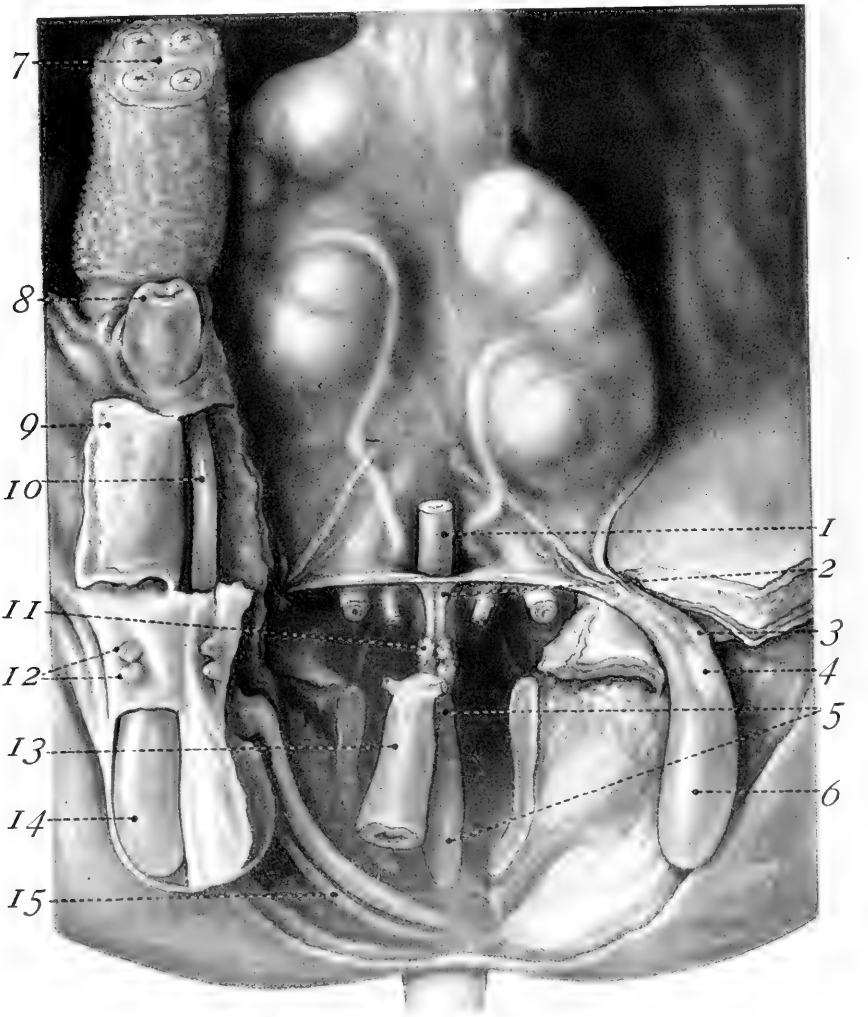


Fig. 12 Normal male 26 cm. long; from twin one-sexed male pair 3; collected January 28, 1915. × $\frac{1}{3}$. Single chorion, constricted between the two foetuses. This figure shows the entire male urinogenital system; the gubernacula have entered the scrotal sacs. The testes are drawn into the vaginal sacs. The disposition of the teats for the normal male should be noted. *1*, rectum; *2*, vasa deferentia; *3*, epididymis; *4*, testis; *5*, urinogenital sinus; *6*. gubernaculum withdrawn from scrotal sac; *7*, cut end of umbilical cord; *8*, prepuce; *9*, wall of penial sheath; *10*, penis; *11*, vesiculae seminales; *12*, teats; *13*, allantois; *14*, gubernaculum in scrotal sac; *15*, retractor muscle of penis.

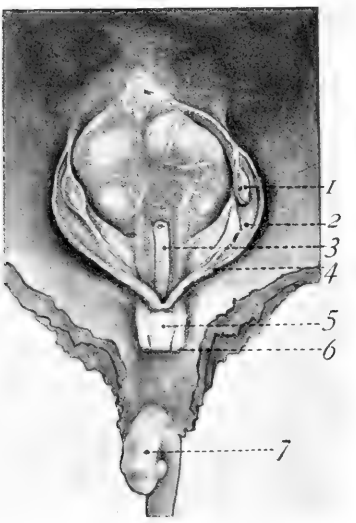


Fig. 13 Urinogenital system of sterile free-martin 7.5 cm. long. × $\frac{8}{3}$; case 19. Specimen fixed in Flemming's fluid; not so fully dissected as the following cases, as it was preserved for microscopical study. (Other drawings from the same specimen are figures 4 and 5, Chapin '17.) *1*, gonad; *2*, Wolffian body; *3*, rectum; *4*, genital duct; *5*, allantois; *6*, umbilical artery; *7*, clitoris.

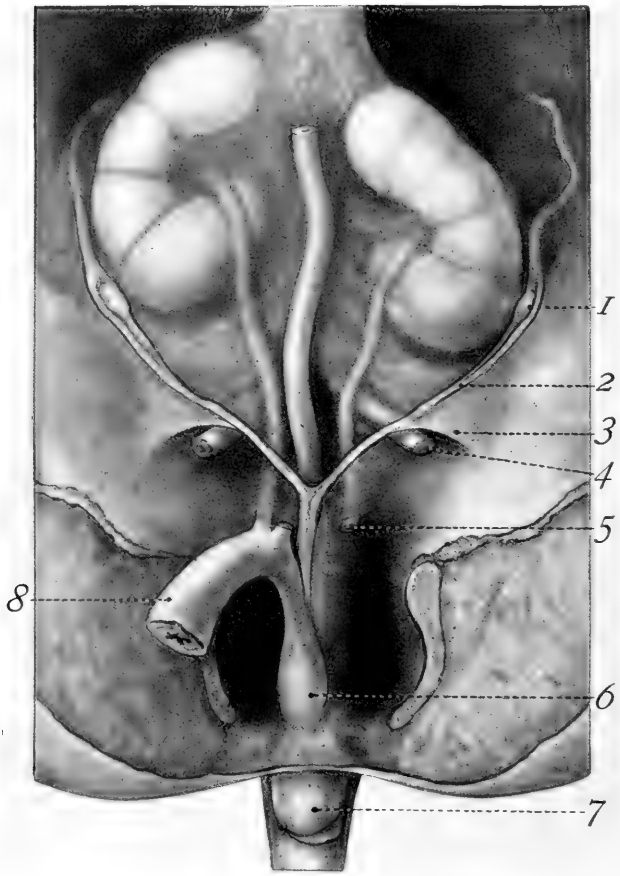


Fig. 14 Sterile free-martin 13.1 cm. long. × ⅔. From two-sexed pair 17. Collected October 9, 1915. Gubernacula are not developed; cf. male of earlier stage (fig. 10). *1*, gonad; *2*, Wolffian duct; *3*, inguinal fold; *4*, umbilical artery; *5*, ureter; *6*, urinogenital sinus; *7*, clitoris; *8*, allantois.

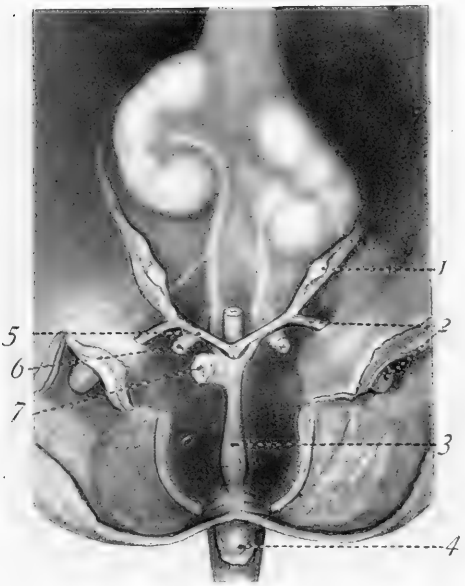


Fig. 15 Sterile free-martin 15.5 cm. long. × ¾. From two-sexed pair 37. Collected January 25, 1916. Gubernacula are developed as typically as in a male (figs. 10 and 11). Gonads small. Ducts also appear as in male. *1*, gonad; *2*, gubernaculum; *3*, urinogenital sinus; *4*, clitoris; *5*, Wolffian ducts; *6*, umbilical artery; *7*, allantois.

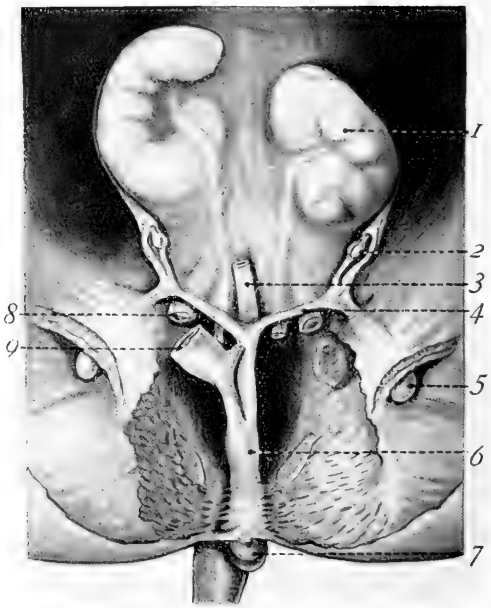


Fig. 16 Sterile free-martin 6, 16.3 cm. long. × $\frac{4}{3}$. (Cf. fig. 11 for male twin.) Gubernacula are somewhat smaller than in the male (fig. 11). Small gonads (cf. figs. 9 for normal size, and fig. 7 of Chapin for histological structure). The urinogenital sinus is intermediate in length between male and female (fig. 9); no seminal vesicles. *1*, kidney; *2*, gonad; *3*, rectum; *4*, Wolffian duct; *5*, gubernaculum; *6*, urinogenital sinus; *7*, clitoris; *8*, umbilical artery; *9*, allantois.

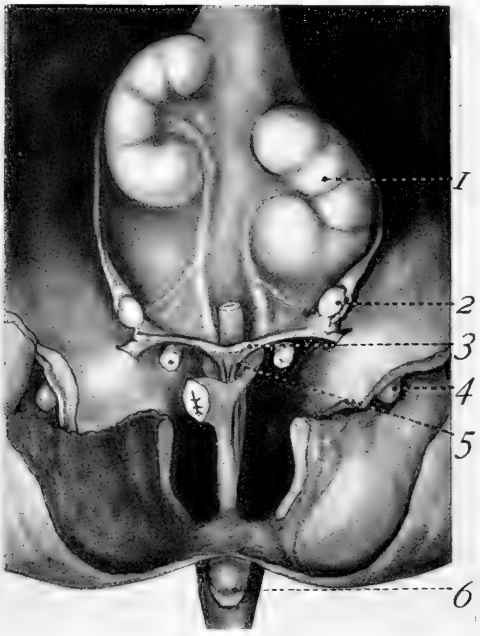
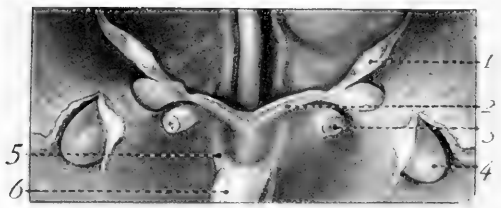


Fig. 17 Sterile free-martin 16.75 cm. long. × ⅓. From two-sexed pair 32. Similar in all essential respects to figure 16 except that gonads are slightly larger, and possible rudiments of Müllerian ducts occur in the broad ligament. *1*, kidney; *2*, gonad; *3*, rudiments of horns of uterus; *4*, gubernaculum; *5*, Wolffian ducts; *6*, clitoris.



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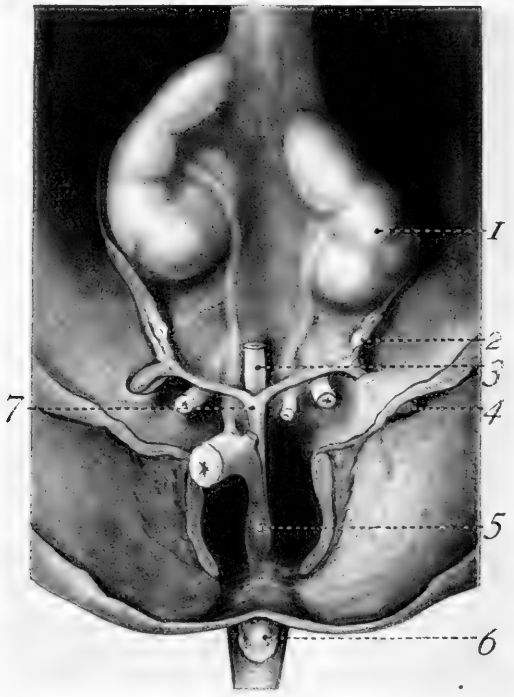

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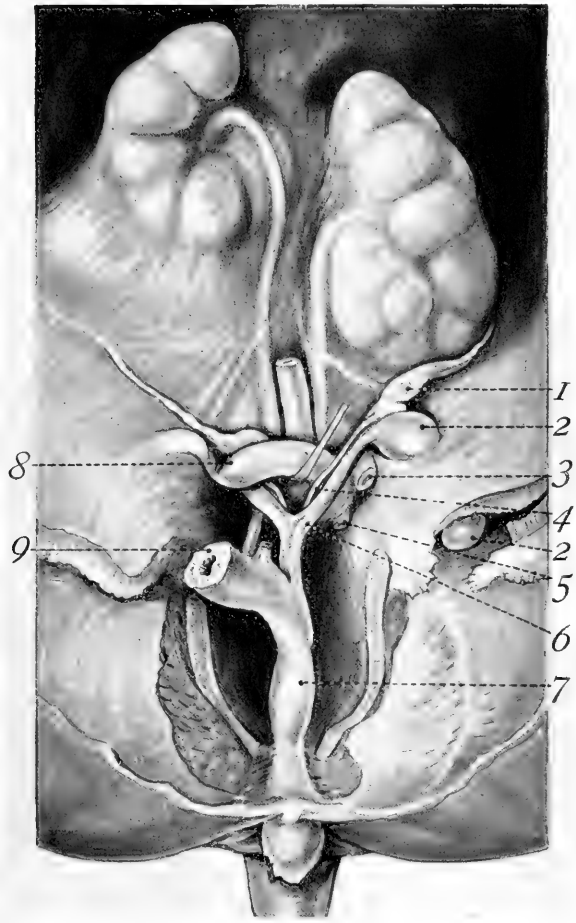
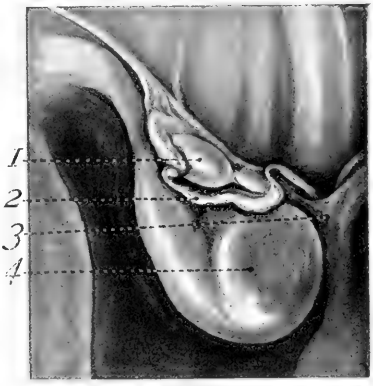
Fig. 18 Part of urinogenital system of sterile free-martin 17.5 cm. long. $\times \frac{4}{3}$. From two-sexed pair 23. The gonads are unusually rudimentary. *1*, gonad; *2*, urinogenital fold with ducts; difficult to interpret; *3*, umbilical artery; *4*, gubernaculum; *5*, ureter; *6*, allantois turned back.

Fig. 19 Urinogenital system of sterile free-martin 17.5 cm. long; $\times \frac{4}{3}$. From two-sexed twin pair 36. The gonads are exceedingly rudimentary in this specimen; the gubernacula are present, but not as well developed as in figure 18. *1*, kidney; *2*, gonad; *3*, rectum; *4*, gubernaculum; *5*, urinogenital sinus; *6*, clitoris; *7*, Wolffian ducts.



20A



20B

Figs. 20A and 20B Urinogenital system of sterile free-martin 21.5 cm. long. From two-sexed pair 2.

20A. × $\frac{1}{3}$. On the right side the gubernaculum, instead of growing into the body wall has evaginated into the body cavity and lies partly in the utero-rectal recess. Both Wolffian and Müllerian ducts appear to be present; latter very rudimentary. Gonads very small. *1*, gonad; *2*, gubernaculum, left side; *3*, umbilical artery; *4*, Wolffian duct; *5*, ureter; *6*, Müllerian duct; *7*, urinogenital sinus; *8*, gubernaculum of right side evaginated into body cavity; *9*, allantois.

20B. × $\frac{5}{3}$. Part of 20A with the right gubernaculum withdrawn from the utero-rectal recess and turned over to expose the gonad and Wolffian duct. *1*, gonad; *2*, Wolffian duct; *3*, Müllerian duct; *4*, gubernaculum.

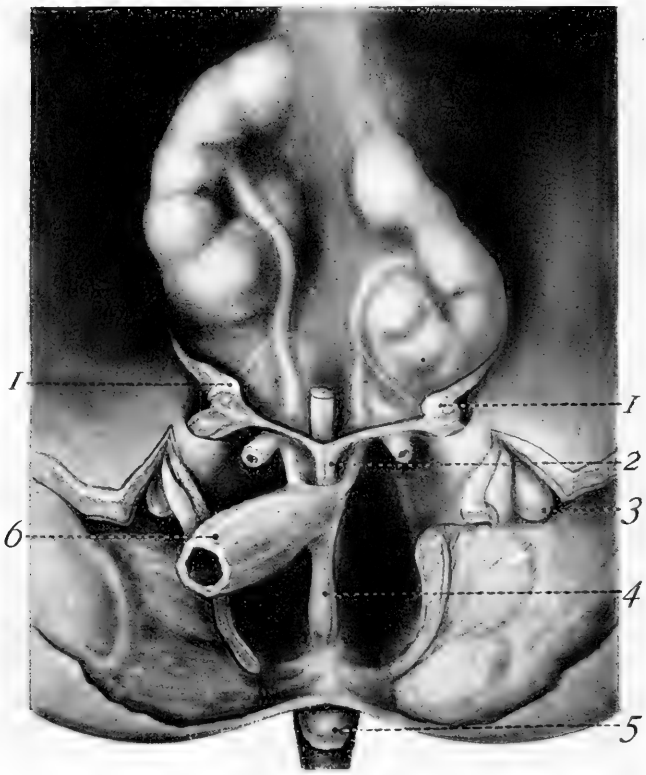
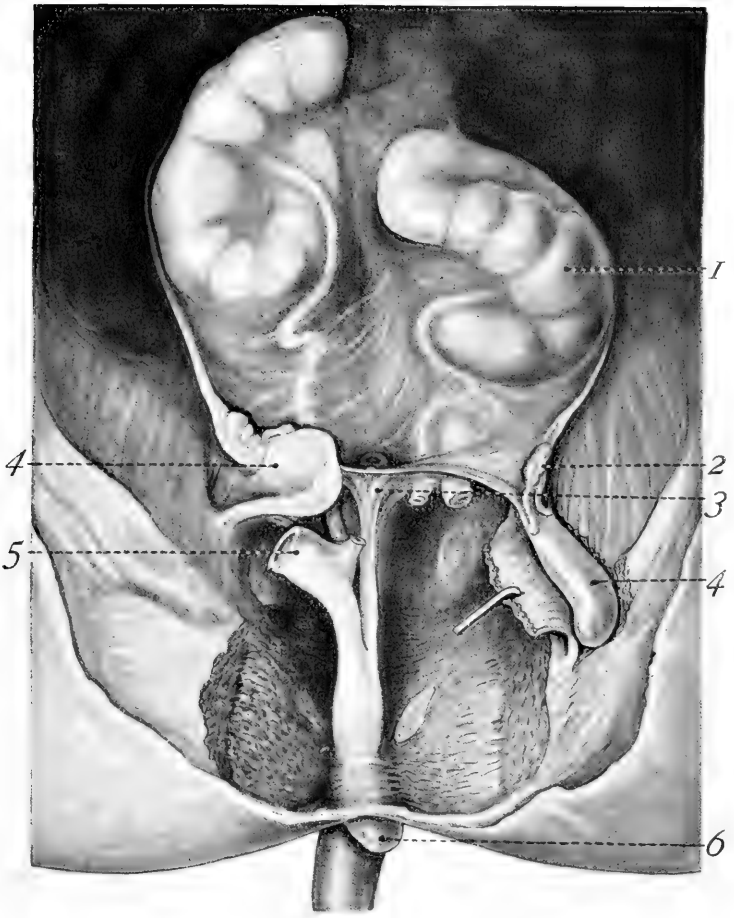


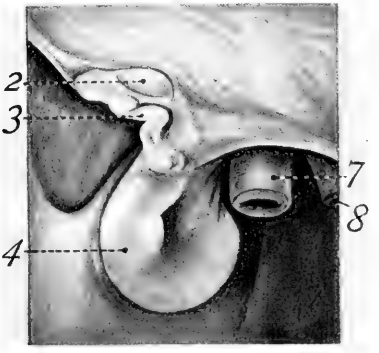
Fig. 21 Urinogenital system of sterile free-martin 21.8 cm. long. × $\frac{4}{3}$. From two-sexed pair 41. Exceedingly small gonads drawn close to entrance of the saccus vaginalis. *1*, gonad; *2*, Wolffian duct; *3*, gubernaculum; *4*, urinogenital sinus; *5*, clitoris; *6*, allantois.

Fig. 22A Urinogenital system of sterile free-martin 22.5 cm. long. × $\frac{4}{3}$. From two-sexed pair 4. The right gubernaculum is evaginated into the body cavity.

22B. Part of 22A. × $\frac{8}{3}$ with the right gubernaculum turned over to show the gonad and Wolffian duct. *1*, kidney; *2*, gonad; *3*, Wolffian ducts; *4*, gubernaculum; *5*, allantois; *6*, clitoris; *7*, umbilical artery; *8*, ureter.



22A



22B

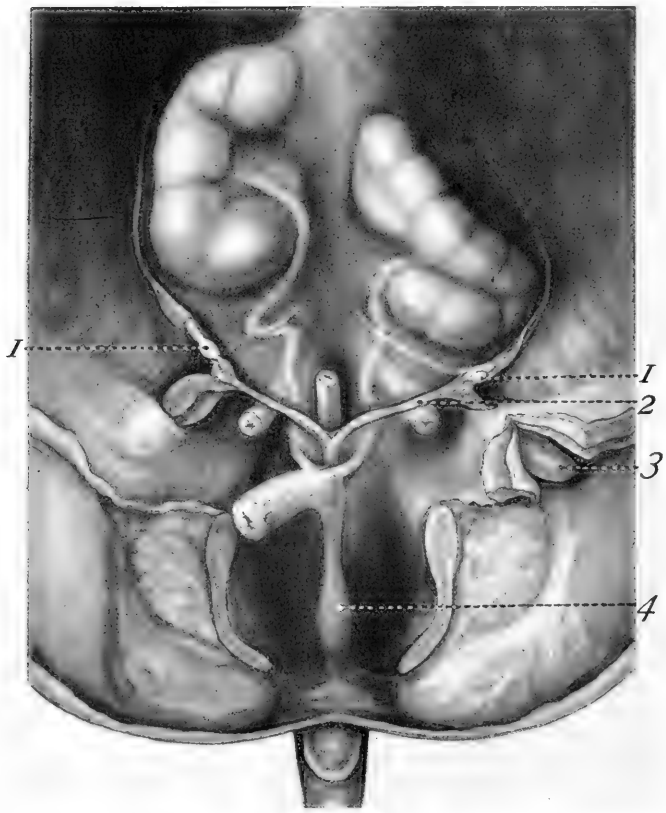


Fig. 23 Urinogenital system of sterile free-martin 22.5 cm. long. $\times \frac{4}{3}$. From two-sexed pair 38. The typical features recur here; the gubernaculum of the right side has grown only partly into the body wall; compare the left side. *1*, gonad; *2*, Wolffian duct; *3*, gubernaculum; *4*, urinogenital sinus.

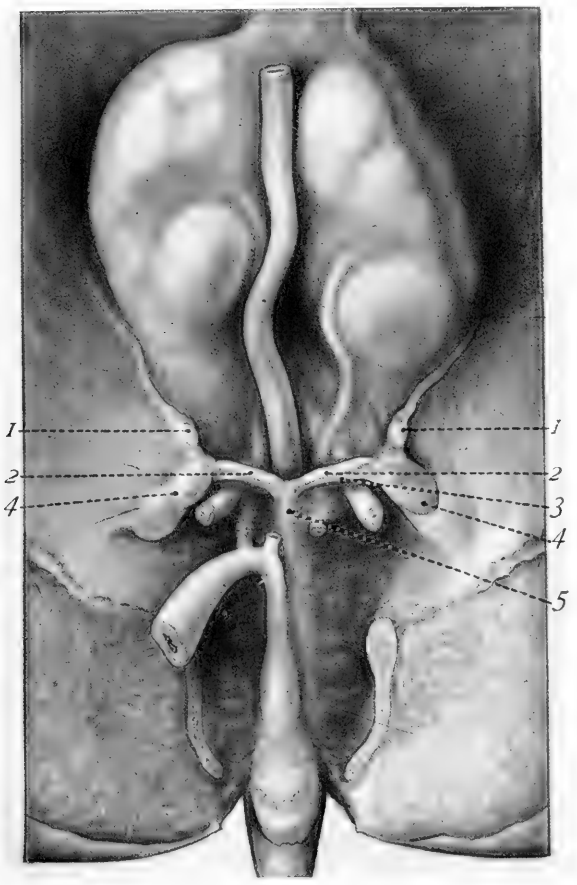
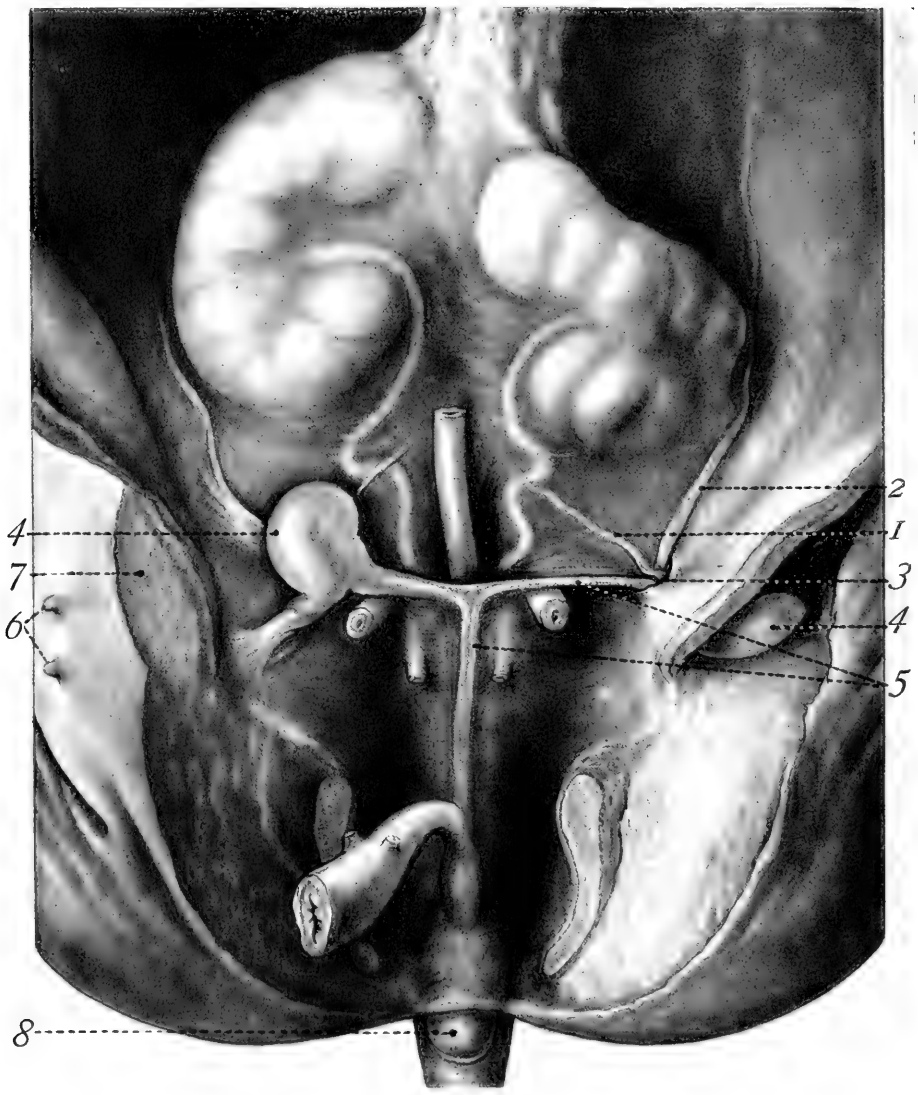
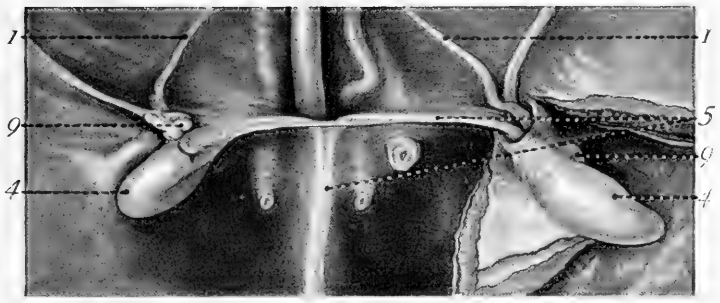


Fig. 24 Urinogenital system of sterile free-martin about 24 cm. long. × ⅓. From two-sexed pair 22. The typical features recur here again; the right gubernaculum lies in the body-cavity and is relatively undeveloped; compare the left side. Parts of the cornua uteri seem to be developed in this case. *1*, gonad; *2*, Müllerian ducts (cornua uteri); *3*, Wolffian duct; *4*, gubernaculum; *5*, rudiment of corpus uteri, cervix and vagina.



25A



25B

Fig. 25A Urinogenital system of sterile free-martin 26.5 cm. long. × ⅓. From two-sexed pair 21. In this case the gonad of the left side has entered the saccus vaginalis; compare figure 12 for normal condition of the male. The right gubernaculum has grown into the body-cavity. Observe the female disposition of the teats and development of the glandular tissue of the mammary gland, and compare male (fig. 12).

Fig. 25B Part of 25A further dissected to show the left saccus vaginalis containing the gonad on the left side; on the right side the gubernaculum is turned over. Designations for 25A and 25B. *1*, ovarian artery; *2*, remains of urinogenital ridge; *3*, entrance to saccus vaginalis; *4*, gubernaculum; *5*, Wolffian duct; *6*, teats; *7*, mammary gland tissue; *8*, clitoris; *9*, gonad.

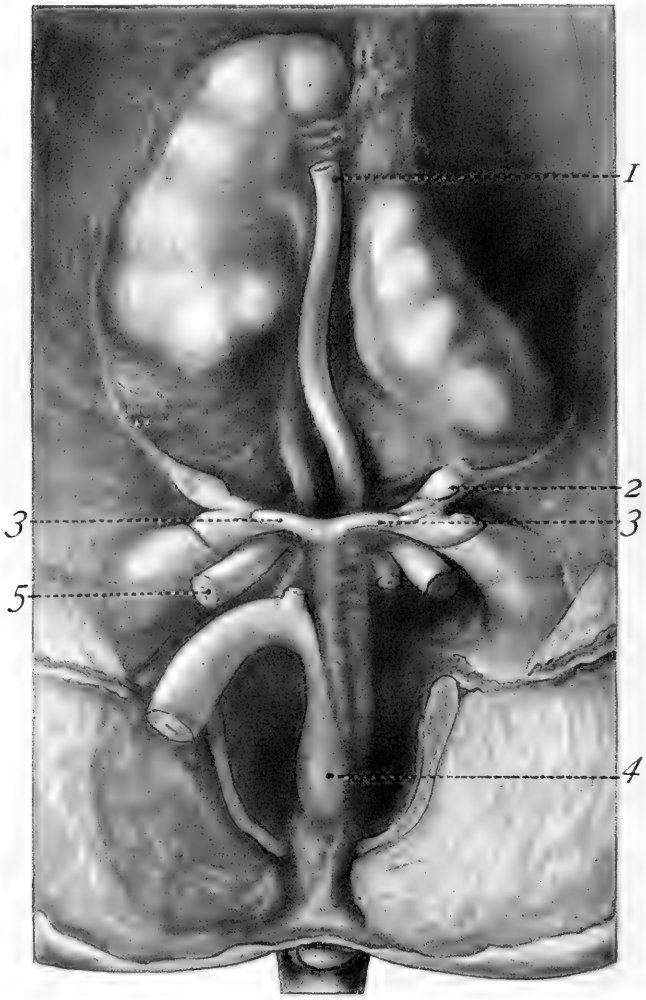
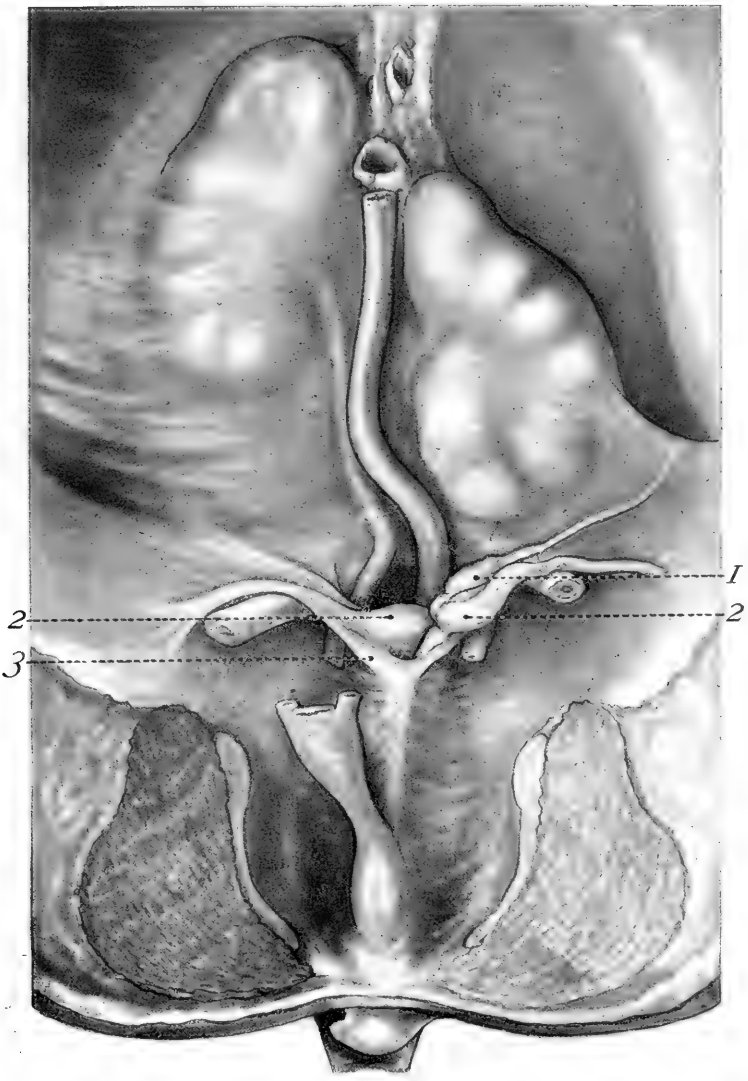
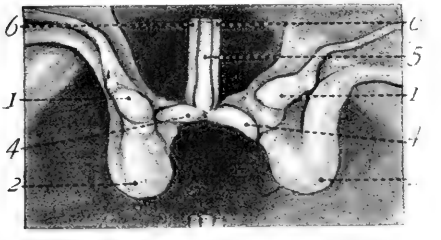


Fig. 26 Urinogenital system of sterile free-martin 27 cm. long. × ⅓. From two-sexed pair 14. Although this case is beyond the stage in which the testes of the male normally enter the saccus vaginalis, the rudimentary ovaries are here in the body cavity. In this case there appear to be rudiments of the cornua uteri. *1*, rectum; *2*, gonad; *3*, cornua uteri; *4*, urinogenital sinus; *5*, umbilical artery.

Fig. 27 Urinogenital system of sterile free-martin 28 cm. long. × ⅓. From two-sexed pair 12. In this case both gubernacula have grown into the body cavity; the right gubernaculum lies in the utero-rectal recess of the body-cavity. In figure B the gubernacula are rearranged, the right one being drawn out of the recess, and the genital cord is cut across and turned forward. Rudiments of both Müllerian and Wolffian ducts present. *1*, gonads; *2*, gubernaculum; *3*, sex-ducts; *4*, cornua uteri; *5*, corpus uteri, cervix and vagina; *6*, Wolffian ducts.



27A



27B

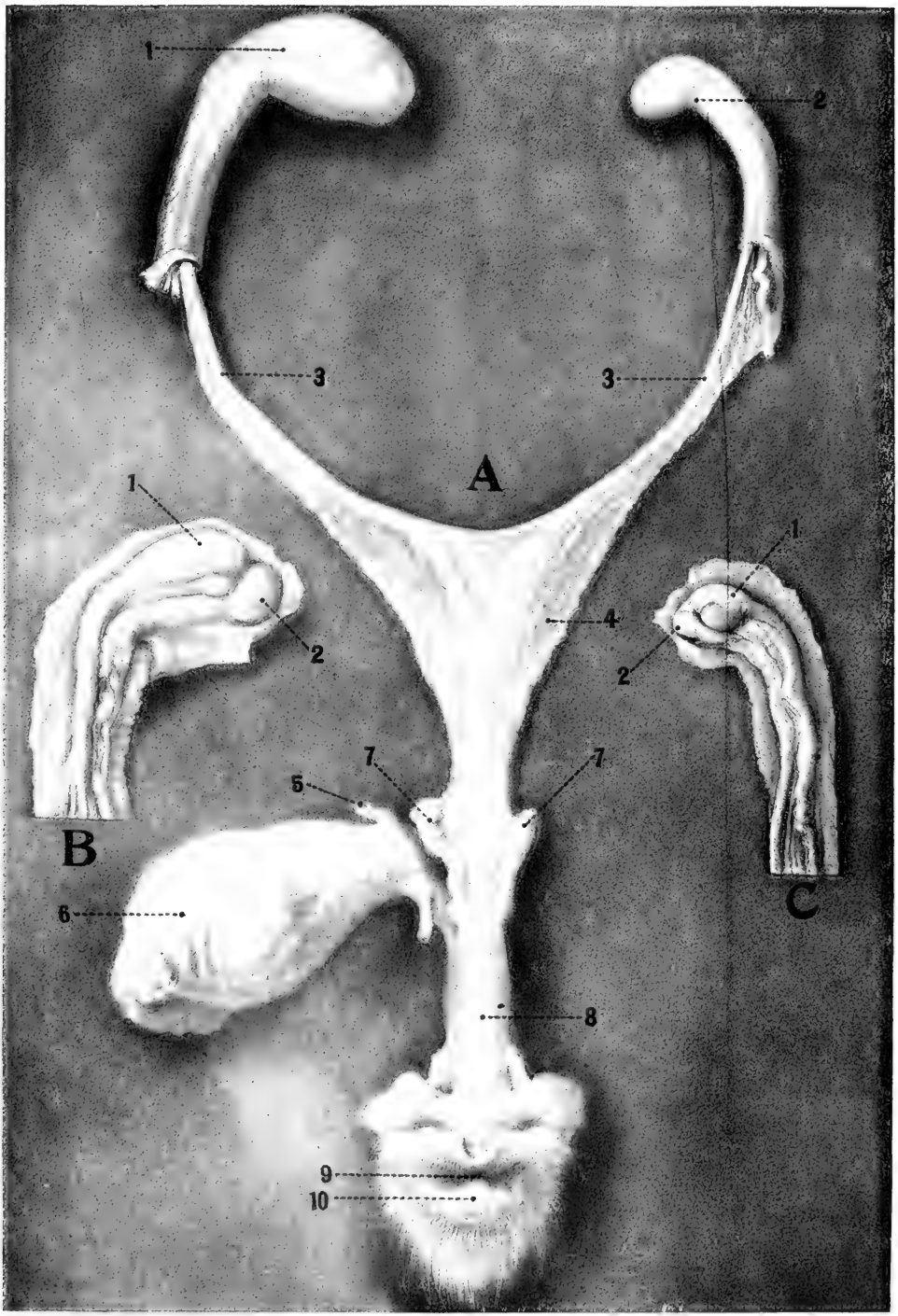

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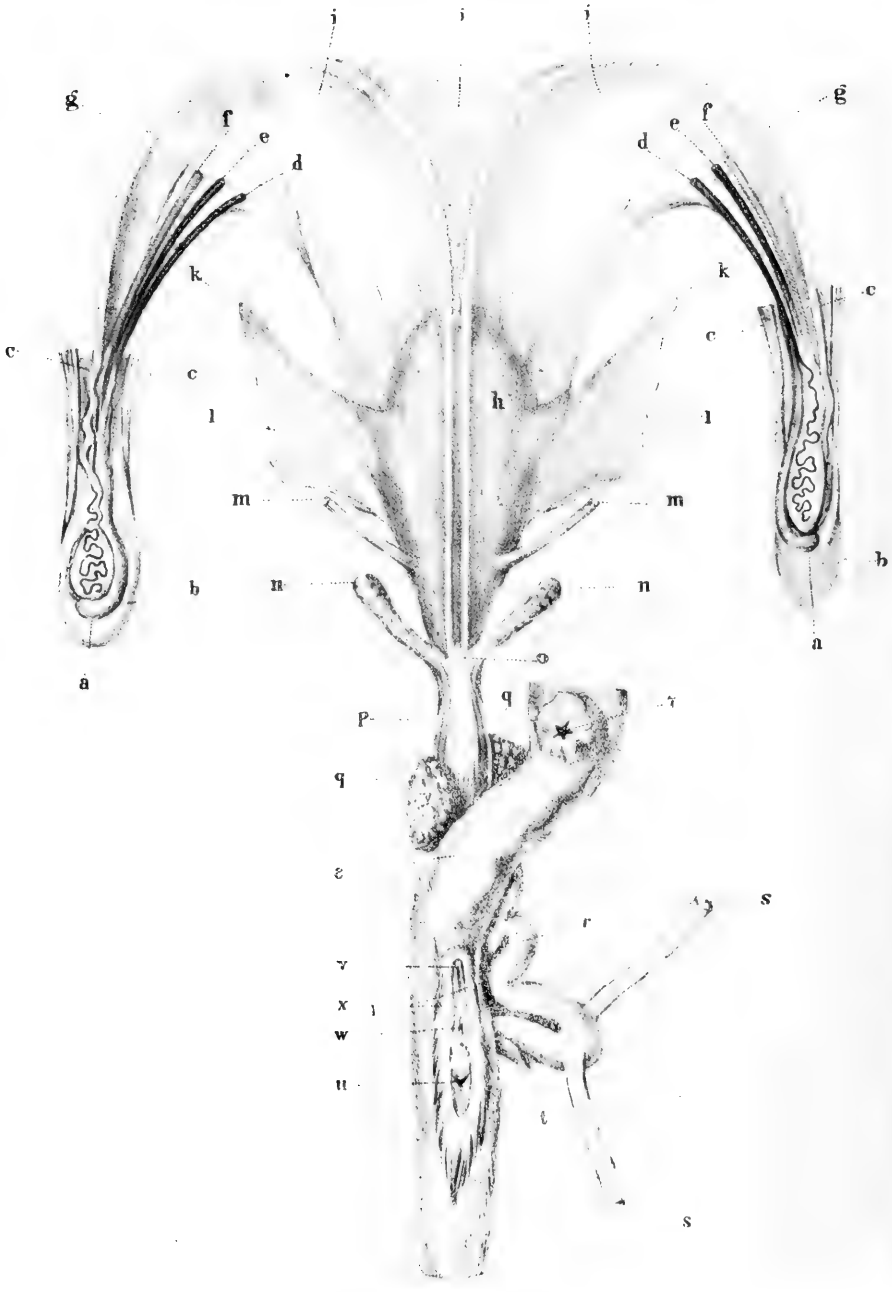
Fig. 28 Reproductive organs of a seven weeks old free-martin. $\times \frac{2}{1}\frac{3}{8}$. Born twin to a male, case 44. The dissection shows a dorsal view. Description in text.

Fig. A *1*, left saccus vaginalis containing gonad; *2*, right saccus vaginalis containing gonad; *3*, Vas deferens (Wolffian duct); *4*, broad ligament; *5*, ureter; *6*, bladder; *7*, seminal vesicles; *8*, urogenital sinus; *9*, vulva; *10*, clitoris.

Figs. B and C Left and right sacci vaginales opened. *1*, testis; *2*, epididymis.

Fig. 29 Reproductive organs of a free-martin described by Numan ('43) from his plate XI. Description in the text (p. 413). The description of this plate was missing. The explanation of the letters is therefore my own interpretation. *a*, epididymis with testis above; *b*, Saccus vaginalis; *c.c*, cut wall of saccus vaginalis; *d.e.f*, spermatic artery, vein and nerve (?); *g*, Vasa deferentia (Wolffian ducts); *h*, bladder; *i*, broad ligament; *k.l*, ligaments of bladder; (?); *m*, ureters; *n*, seminal vesicles; *o*, entrance of vasa deferentia into the urinogenital sinus; *p*, urinogenital sinus; *q*, prostate; *r*, penis; *s*, retractor muscles of penis; *t*, ?; *u*, external opening of urinogenital sinus (urethra) beneath the glans penis; *v.w*, accessory openings in the urethra; *x*, vulva; *7*, anus; *8*, perineum.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A MICROSCOPIC STUDY OF THE REPRODUCTIVE SYSTEM OF FOETAL FREE-MARTINS

CATHARINE LINES CHAPIN

Hull Zoölogical Laboratory, University of Chicago

SIXTEEN FIGURES

The following study of one phase of the free-martin problem was suggested to me by Prof. F. R. Lillie and has been pursued under his direction. To him my thanks are due for his kindly advice and constructive criticism.

The foetal free-martins used in this investigation are those described by Professor Lillie in the preceding paper (this Journal, p. 371 to 452). Some of these specimens were preserved in toto in 5 per cent formalin. Other specimens were dissected as soon as possible after being brought to the laboratory and parts to be used for histological study were preserved in Zenker's fluid or in strong Flemming solution. The gonads and related organs, including in most cases the Wolffian body and the Wolffian and Müllerian ducts, of the foetal free-martins were sectioned. These organs were studied also in normal males and females of approximately the same size as the free-martins.

Two series of records of specimens were kept; one, of the series of twins described in the preceding paper and one, of the series of normal embryos collected to study in comparison with the free-martins found in the twin series. Individuals of the twin series, which includes twins of both the one sexed and the two sexed types are designated by the letter T and their serial number; individuals of the normal series are designated by N and their serial number. Histological preparations were made from some of the normal males and females for comparison with the free-martin. There is no indication in the early stages studied that, at a given degree of development, there is any marked difference in size between a single embryo and a twin. Thus a

20 cm. free-martin may justly be compared with a 20 cm. normal embryo of either sex, as well as with its twin, the normal male.

In the description of the reproductive glands and related organs which follows, most of the terms are employed in their usual sense; Wolffian body, epididymis, epoöphoron, Wolffian or mesonephrotic duct and Müllerian duct, germinal epithelium and others. A few terms, which have not been used in their ordinary sense may best be explained at this point. Rete is used to describe the network of tubules of the rete testis, the rete ovarii which for the most part degenerates, and the modified rete of the free-martin which persists. The term sex cords is used to designate the proliferation of the germinal epithelium in the two sexes. In the male, there is one set of sex cords, the seminiferous tubules. In the female there are two sets of sex cords; first, the medullary cords which degenerate during foetal life and second, the cords of Pflüger, at the inner ends of which are formed nests of cells including the primordial ova, the primary ovarian follicles.

The term albuginea is used to designate the tissues lying just beneath the peritoneum and surrounding the sex cords, including not only the tunica albuginea, but also the tunica vasculosa which, in the male embryo, merges into the tunica albuginea. According to Allen[1] this term is equally applicable to the similar structure found in the adult female, between the cords of Pflüger and the germinal epithelium, but which has not yet developed to any great extent in a 29.5 cm. female (N20). For the sake of clearness this structure is designated definitive albuginea, to distinguish it from the primary albuginea which separates the medullary cords from the cords of Pflüger.

The term interstitial cells is used to refer to those cells described by R. H. Whitehead[2] as the cells of the interstitial gland; interstitial material refers to all the material between the seminiferous tubules, including not only the interstitial cells, but also the connective tissue stroma.

[1] B. M. Allen, Am. Jour. Anat., vol. 3.
[2] R. H. Whitehead, Am. Jour. Anat., vol. 3.

The following pages comprise detailed descriptions and comparisons with normal males and females of eight free-martins, viz.:—(1) f.m. T19, (2) f.m. T26, (3) f.m. T6, (4) f.ms. 13, 2, and 4, (5) f.m. 12, and (6) f.m. 42.

1. FREE-MARTIN, 7.5 CM. T 19

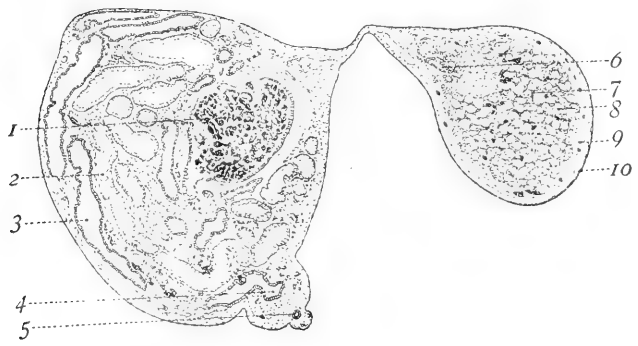
The smallest free-martin which has been accessible for study, T19, measures 7.5 cm. in total length. Lillie, figure 13. The gonads are very small, being only 2.07 mm. long, whereas a testis of its twin brother is 3.5 mm. long, and an ovary of an 8.5 cm. female (N4) measures 3 mm. The gonads of this free-martin are lemon-shaped structures which lie close to the Wolffian body, near its anterior end. The area of the central cross section is about one-fourth of that of the testes of normal males measuring 7 cm. (N10) and 8 cm. (T19) and of the ovary of a normal female, 7.3 cm. long (N8), (figs. 2, 3, and 5).

a. Normal conditions at this stage

In the normal male T19, twin with free-martin T19, and measuring 8 cm., the sex cords, seminiferous tubules, are distinct, much branched, and separated from each other by connective tissue and the cells of the interstitial gland. (Unfortunately, the preservation of ♂ T19 was not made with a view to histological study and the central portion of the gonad is indistinct.) In ♂ N10, 7 cm. long and preserved in strong Flemming (figs. 1 and 2), the seminiferous tubules make up the larger part of the testis. The outer layer, in both cases (T19 and N10) is the typical albugineal layer of connective tissue with the long axis of the cells parallel to the peritoneum. The rete enters the testis near the anterior end and runs posteriorly, forming the core about which the seminiferous tubules radiate, figures 1 and 2. Most of the germ cells of the seminiferous tubules are still indifferent but a very few are found in the early stages of the growth period.[3]

In a normal female 8.5 cm. long (N4), the medulla is relatively large in cross section. The medullary cords have not yet begun

[3] Schoenfeld, Arch. de Biol., T18, 1901–02.



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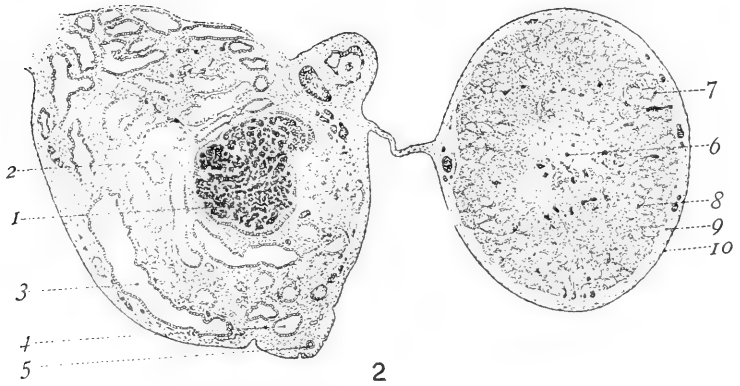

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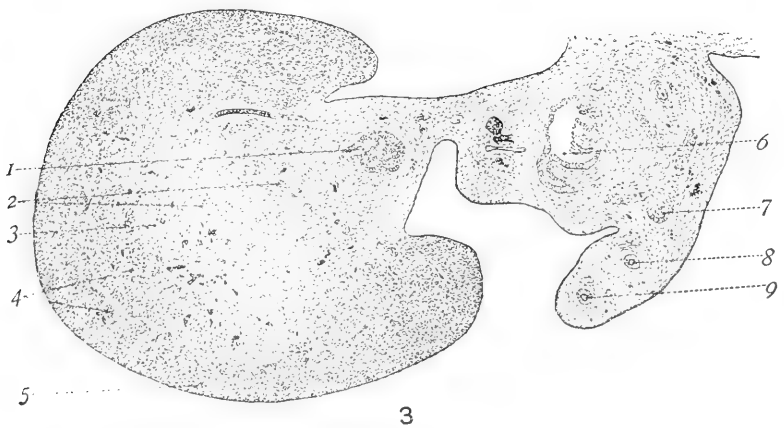

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to degenerate. In an 8.3 cm. ♀ N7 (fig. 3) and in a 7.3 cm. ♀ N8, primary follicles are found in the medulla. The inclosed ovum is usually indifferent but may have begun to degenerate. Separating the medullary cords from the outer layer of sex cords, the cords of Pflüger, is a layer of connective tissue, the primary albuginea. Strands of connective tissue extend outwards from this layer, toward the periphery of the gland, separating the cords of Pflüger from each other and forming the stroma of the cortex. This cortex is narrow. The cords of Pflüger are still in an early stage of development, the cells not yet being arranged in 'nests' surrounding growing ova. The germ cells are still in the indifferent stage. No interstitial cells can be distinguished. The germinal epithelium is a single layer of cuboidal epithelial cells. The cords of Pflüger have not yet been separated from it by the development of the definitive albuginea.

The normal ovary has a different shape from that of the testis. The rete, entering near the anterior end, lies close to the mesentery which connects the ovary with the Wolffian body. The rete ovarii does not grow far posteriorly at as early a stage as does the rete testis. During the further development of the foetal ovary, the cortex grows more rapidly than the medullary region, extends toward the Wolffian body on each side of the mesentery, and the two sides approximate each other secondarily, making the rete appear to be in the center of a round gland as it is primarily in the testis.

Fig. 1 Cross section of testis and Wolffian body of 7 cm. ♂ Bos embryo, N10, near anterior end, showing rete entering the testis. *1*, glomerulus; *2*, secretory tubule; *3*, collecting tubule; *4*, Wolffian duct; *5*, Müllerian duct; *6*, rete cord, (with lumen); *7*, interstitial material; *8*, seminiferous tubule; *9*, albuginea; *10*, superficial epithelium (peritoneum). × 20.

Fig. 2 Cross section of testis and Wolffian body at 7 cm. ♂ Bos embryo, N10 near middle of gonad. Designations as in figure 1. × 20.

Fig. 3 Cross section of ovary and Wolffian body of 8.3 cm. ♀ Bos embryo, N7, near middle of ovary, showing position of rete in ♀ as compared with that in ♂, figure 2. *1*, rete; *2*, medullary cords; *3*, primary albuginea; *4*, cords of Pflüger; *5*, germinal epithelium; *6*, Bowman's capsule (Malpighian body); *7*, Wolffian tubule; *8*, Wolffian duct; *9*, Müllerian duct. × 42.

The Wolffian bodies of a normal 7 cm. ♂ N10, and of a normal 8.3 cm. ♀ N7, are shown in figures 1, 2 and 3. These contain the Malpighian bodies and Wolffian tubules of the young embryo. The tubules in all the specimens of this stage which were examined, showed the typical form of wall, low epithelium in collecting portions and tall, columnar epithelium in secretory portions.[4] The tubules are a little less numerous in the 8 cm. ♂ T19, but N10 was a better preparation from which to make a drawing.

The relations of the Wolffian duct, Wolffian body and rete are still in the indifferent stage. In both ♂ and ♀ the rete connects sex cords with the Malpighian bodies, from which the Wolffian tubules lead into the Wolffian duct.

In the normal male T19 and female N8, measuring 8.0 and 7.3 cm. respectively, not only the Wolffian, but also the Müllerian ducts are present. In the female the Müllerian duct, at its anterior end, opens into the body cavity by the ostium abdominale, a funnel-shaped opening lined with the large ciliated type of epithelial cell of which consists the inner layer of the whole duct. Degeneration of the Müllerian duct in the male starts at an earlier stage than that reached by an 8 cm. male. The ostia abdominalia are present in a 4.8 cm. male N13. In N10, 7 cm. long, there is a suggestion of ostia abdominalia, but the preservation is poor in the critical region. In the 8 cm. male 19, which is twin with a free-martin, no ostium abdominale is present. Slightly anterior to the testis there begins a rod of connective tissue which runs posteriorly, parallel to the Wolffian duct. For a short distance the anterior end consists simply of a few concentric layers of connective tissue, but throughout the greater part of its course this structure encloses the epithelial Müllerian duct. The wall of the duct is made up of epithelial cells but these are not ciliated, as they are in the female. In a few sections (not consecutive) the lumen is obliterated, but this may be due to preservation and staining rather than to an abnormality in the duct. (This specimen

[4] J. B. MacCallum, Am. Jour. Anat., vol. 1.

had not been preserved carefully for microscopic study.) The whole duct is smaller in diameter than the duct of a normal female of the same size.

b. Condition in the free-martin

The gonad of the free-martin 19, on microscopic examination, is found to consist of a central, rather dense mass of deeply staining cells, surrounded by a layer of cells less dense than the central mass, figures 4 and 5. The outer structure is albuginea, similar to that of the testis, but it is not arranged in definite layers as in the normal ♂ T19. In the anterior part, the central mass shows two divisions. The smaller one, round in cross section, is the rete. This is easily traced from its connection with the glomeruli in the Wolffian body, into the gonad which it penetrates almost at the anterior apex, figure 4. A short distance posterior to its entrance into the central mass of the gonad it becomes scarcely distinguishable from the rest of the mass, figure 5. The rete is not differentiated into cords. The remaining portion of the central mass of the gonad partly surrounds the rete. It is an undifferentiated mass of cells, but represents the medullary cords. A very few germ cells are found. These are in Schoenfeld's growth stages c, d and e. Interstitial cells cannot be distinguished. A single layer of peritoneum covers the whole gonad, as in the testis. Although the gonad is much smaller than those of the normal ♂ and ♀ studied in comparison with free-martin 19, the Wolffian body is of normal size and structure. The Wolffian tubules and Malpighian bodies in 7.5 cm. free-martin 19 are as numerous as they are in the 7 cm. ♂ N10 and 7.3 cm. ♀ N8, and more numerous than in its twin, the 8 cm. ♂ T19 and in the 8.3 cm. ♀ N7.

The Wolffian duct of the free-martin is normal. The Müllerian duct, on the other hand, is abnormal. At its anterior end it is like the Müllerian duct of the normal male twin 19. The connective tissue rod lies in the normal position of the Müllerian duct, but the epithelial duct which it encloses is discontinuous. The lumen is present only at irregular intervals and even the

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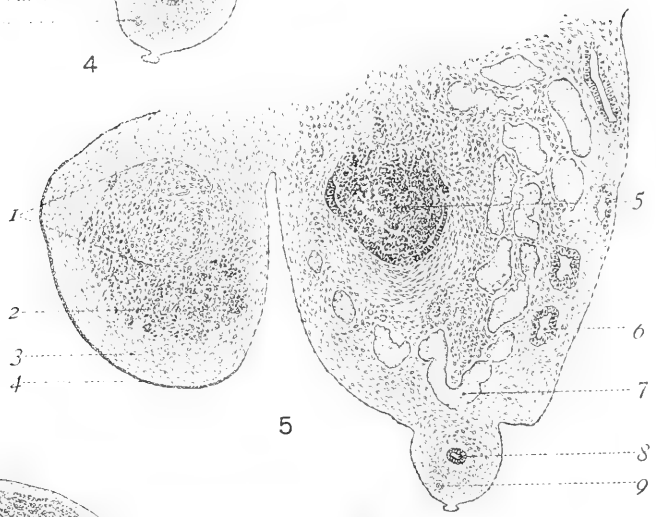

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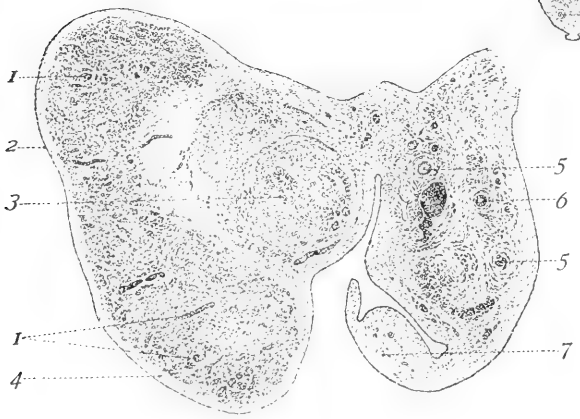

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epithelial cells which form the duct are not present through its entire length.

Summary. The gonad of this 7.5 cm. free-martin resembles a reduced testis in the presence of albuginea, thin covering of peritoneum, and absence of cords of Pflüger, and differs from the ovary in the same respects. It is like the ovary in the position of the rete. The ducts resembles those of the male in that the Wolffian duct is complete and degeneration of the Müllerian duct has commenced. The Wolffian body is like that of normal embryos of the same size.

2. FREE-MARTIN, 12.5 CM. T26

The next size of free-martin which was examined microscopically, T26, measured 12.5 cm. This may be compared with the 12.75 cm. male T16, and the 14 cm. female N26.

a. Normal conditions at this stage

The testis of the male is 4.45 mm. long. The seminiferous tubules are already branched, contorted, and very numerous, but have not yet developed lumina. They are separated by a small amount of interstitial material; most of this material is connective tissue, but some of the cells resemble interstitial cells as described by B. M. Allen[1] and R. H. Whitehead.[2] Throughout the seminiferous tubules are found primitive sex cells, some of which have started upon the growth period.[3] A very thin layer of peritoneum covers the surface of the testis. Beneath this lies the albuginea which is wide and compact.

Fig. 4 Cross section of Wolffian body of 7.5 cm. free-martin T19, at point where rete is pushing out to enter anterior end of gonad. *1*, rete; *2*, Wolffian tubule; *3*, Wolffian duct; *4*, Müllerian duct; *5*, Glomerulus. × 42.

Fig. 5 Cross section of Wolffian body and gonad of 7.5 cm. free-martin embryo, T19 through middle of gonad. *1*, rete; *2*, sex cord region; *3*, albuginea; *4*, germinal epithelium; *5*, glomerulus; *6*, collecting Wolffian tubule; *7*, secretory Wolffian tubule; *8*, Wolffian duct; *9*, rudimentary Müllerian duct. × 42.

Fig. 6 Cross section through Wolffian body and middle of gonad of 12.5 cm. free-martin, T26. *1*, medullary cords; *2*, superficial or germinal epithelium; *3*, rete; *4*, albuginea; *5*, degenerating Wolffian tubules; *6*, Wolffian tubules; *7*, urogenital fold in which ducts normally lie. × 42.

The rete tubules branch and anastomose. The lumina are wide. The epididymis is formed from the anterior end of the Wolffian body, but as yet it is very small, consisting only of a few tubules, the vasa efferentia, which connect the rete with the Wolffian duct. Lateral and posterior to the testis the Wolffian body is degenerating. No glomeruli are to be found. Tubules are fewer than in the Wolffian body of younger males and cells filling their lumina indicate degeneration. The Wolffian duct is fully developed. The Müllerian duct has undergone complete atrophy in the region of the gonad. The entire length of the ducts was not sectioned so the vestiges of Müllerian ducts —uterus masculinus—were not observed.

The ovary of the 14 cm. ♀ N26 measures 3.4 mm. Compared with an 8.3 cm. ♀ the medulla is small and the cortex large. The cortical region is less dense and the cells stain less deeply. The cords of Pflüger are separated by connective tissue stroma. No 'nests' of cells are as yet formed at the inner ends of the cords of Pflüger. The interstitial material consists mainly of connective tissue but a few large cells are found which may be the interstitial cells of Leydig.[1 and 2] The germ cells which are distinguishable in the cords of Pflüger are mainly in the indifferent stage. In the deeper part of the cortex are found primary oöcytes in the early stages of growth.[3] The germinal epithelium is a layer of cuboidal cells on the surface of the ovary, continuous with the cords of Pflüger; no definitive albuginea has as yet developed. Some of the follicles which were formed in the medullary cords at a much earlier stage, have degenerated in the 14 cm. ♀ leaving only round clusters of cells, sometimes arranged in concentric layers. Others contain germ cells, but the stage which these ova had reached, could not be determined.

The rete is smaller in cross section than that of the testis and does not extend as far into the ovary. The rete cords are less numerous and less branched than those of the male, but they also have lumina. The rete can be traced into the Wolffian body. Only a few glomeruli persist in the Wolffian body. Those are in the posterior part. There remain none of the large

Wolffian tubules which were such a noticeable feature of the Wolffian body of younger females (N8, etc.). Opposite the anterior end of the ovary, a few tubules form the epoöphoron, which is the homologue of the epididymis in the male. Throughout the Wolffian body are signs of degeneration such as a large amount of connective tissue, and cells in the lumina of tubules.[4] The Wolffian duct has atrophied, although it is present in a 17 cm. female sectioned. The Müllerian duct is complete.

b. Conditions in the free-martin

The gonad of the 12.5 cm. free-martin is 1.735 mm. long. The area of the central cross section is one-fifth that of the ovary of the 14 cm. female and one-eighth the size of the testis of the 12.75 cm. male. The shape of the gonad resembles that of an ovary of an 8 cm. ♀ (fig. 6). The rete enters the gonad at one side, near the anterior end, but runs posteriorly near the mesentery. It is more clearly defined than the rete of the 14 cm. ♀ N26. The cords are distinct and have lumina. Connective tissue surrounds the rete. On the distal side the connective tissue forms the fan-shaped stroma of the medullary region. In the anterior part of the gland, this region is large but the sex cords are with difficulty distinguished from each other. In the posterior part of the gland are found some degenerating medullary cords which look like those of the female. The few germ cells present are in Schoenfeld's growth stage d. Outside the sex cords and just beneath the peritoneum lies a layer of albuginea, less compact than that of the testis of a 12.75 cm. ♂. Both the peritoneum and the albuginea are strikingly like the male of this stage. There seems to be but one set of sex cords present—the homologue of medullary cords of the ♀ and seminiferous tubules of the ♂. There is no structure present to correspond to the cortex of the normal ovary.

Neither the Wolfian nor the Müllerian duct is complete. Anterior to the gonad, a groove runs along the genital fold at about the place where Müller's duct is normally found. In cross section this groove is suggestive of the ostium abdominale.

On a level with the anterior end of the gonad there is a portion of the Müllerian duct, 0.080 mm in length, surrounded by the connective tissue structure which normally encloses it (cf. f-m. 13, page 474). The Wolffian duct begins anterior to the gonad. At intervals, other ducts enter it from the Wolffian body. It is irregular, in some places large and clearly defined, with a wide lumen, at other points small with the lumen indistinct.

Summary. The gonad of the 12.5 cm. free-martin resembles a testis in the appearance of albuginea and peritoneum, and in the fact that only one set of sex cords is present. In the position of the rete, it resembles an ovary. The Wolffian duct is incomplete as in the female and the Müllerian duct is degenerating, as in the male.

3. FREE-MARTIN, 16.3 CM. T6

(See fig. 16, Lillie.) Both the free-martin and the normal male twin were studied. The free-martin measured 16.3 cm. and the male measured 16.8 cm. The gonad and Wolffian body were sectioned in both embryos; in the free-martin, the ducts posterior to the Wolffian body were also sectioned.

a. Normal conditions at this stage

The male, as is usual in this type of twins, is normal. The testis is 4.86 mm. long. The rete enters the gonad almost at its extreme anterior end. The seminiferous tubules which radiate from the rete are numerous and more branched than those of younger males (cf. ♂ T16 or T25). They are separated from each other by connective tissue and interstitial cells. Germ cells in the seminiferous tubules are found in the indifferent stage and early stages of the growth period. A wide, compact albuginea is present. The anterior part of the Wolffian body has become the epididymis, the tubules of which are more contorted than those of the epididymis in smaller males. Degeneration is going on throughout the rest of the Wolffian body. The Wolffian duct is complete and, through part of its course, lies in the fold of peritoneum overhanging the testis The tubular part of the Müllerian duct has atrophied. (Posterior part of ducts not sectioned.)

The 17 cm. female N23 may also be described for comparison with the 16.3 cm. free-martin T6. The ovary measures 4.39 mm. In cross section its area is about half that of the testis of male T6. The medulla still makes up a large part of the ovary. Medullary cords are found containing many primary follicles and surrounded by abundant connective tissue. The cords of Pflüger are in the same condition in which they were found in the 14 cm. ♀ N26, with the exception that at their inner ends some of them have formed 'nests' of cells, primary follicles enclosing primitive ova. A few germ cells are found in early growth stages. There is no albuginea formed; cords of Pflüger are still attached to the germinal epithelium. The stroma separating the cords of Pflüger from each other is wider than that of a 14 cm. ♀, but no interstitial cells are distinguishable. The rete ovarii is much smaller in cross section and extends a shorter distance into the gonad than does the rete testis. Lumina are very few but have clearly defined epithelial walls.

Degeneration of the Wolffian body has gone farther in the 17 cm. ♀ than in the 16.8 cm. ♂. The Wolffian body of the former is smaller in cross section than that of the latter. The tubules of the epoöphoron remain in the anterior part. In the posterior end are found glomeruli, fewer in number than were found in the 14 cm. stage. The Wolffian duct, although lacking in the 14 cm. ♀ studied, is present in this 17 cm. ♀ and complete as far as it was sectioned. It is also present, though small in diameter, in a 20 cm. ♀ studied, showing some irregularity in time of atrophy of vestigeal structures in normal embryos. The Müllerian duct is complete. The ostium abdominale is relatively larger than that of smaller females and its walls are becoming convoluted. The uterine horns open into the uterus, which by this stage is large and has a muscular wall. (The uterus was studied by dissection.)

b. Conditions in the free-martin

The gonad of the free-martin is approximately 2 mm. long, as compared with the 4.86 mm. testis and 4.39 mm. ovary which have been described for comparison. The area of the cross

section through its widest region is one-fourth the area of the central cross section of the aforesaid ovary and one-eighth that of the testis.

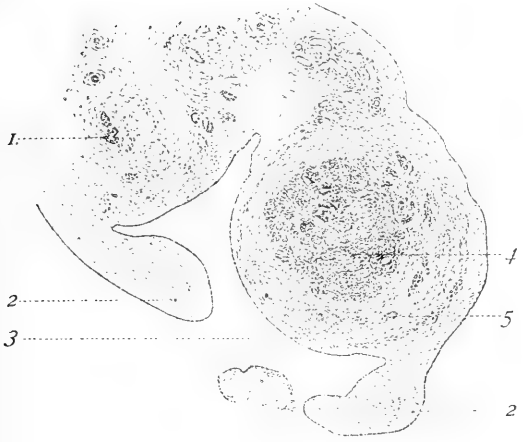
The medulla of this 16.3 cm. free-martin is relatively small, being little wider than the rete (fig. 8). It is about the size of the medulla in the 12.5 cm. free-martin T26, previously described. It shows little sign of differentiation, but occasional primary follicles are found. In some of these the vacuolated condition of the contained germ cell suggests degeneration, in this point, resembling the medullary cords of the female. The rete enters the gonad at the anterior end. It is prominent and well developed. The more anterior rete cords have lumina. The albuginea can hardly be distinguished from the undifferentiated sex cord region. Especially is this the case in the anterior part, where the medullary region is little specialized (fig. 7). The albuginea looks like a compact layer of connective tissue, whereas the medullary region looks like a loose mass of connective tissue. Farther posterior, the medulla has a more dense structure, though still homogeneous and undifferentiated, and the albuginea is quite distinct, a relatively wide and compact layer. It should be noted that, as is true of the other free-martins investigated, only one set of sex cords is found. No second set, corresponding to the cords of Pflüger, the cortex of the ovary, is ever developed.

Anterior to the gonad, the Wolffian body of the free-martin seems to have the relations found normally in the male. The Wolffian tubules connect the rete with the Wolffian duct, as in the epididymis. These tubules, however, are smaller than those

Fig. 7 Cross section through Wolffian body and anterior end of gonad of 16.3 cm. free-martin, T6. *1*, degenerating Wolffian tubule; *2*, fold of peritoneum overhanging gonad and in which Wolffian duct normally lies; *3*, albuginea; *4*, rete; *5*, sex cord (medullary cord). × 42.

Fig. 8 Cross section through Wolffian body and middle of gonad of 16.3 cm. free-martin, T6. *1*, degenerating Wolffian tubule; *2*, Wolffian tubule not yet degenerated; *3*, urogenital fold; *4*, rete; *5*, sex cord region, showing little differentiation into cords; *6*, albuginea. × 42.

Fig. 9 Cross section through epididymis of 24 cm. ♂ Bos embryo, T4. *1*, vas epididymis; *2*, rete; *3*, vasa efferentia; *4*, spermatic artery. × 20.



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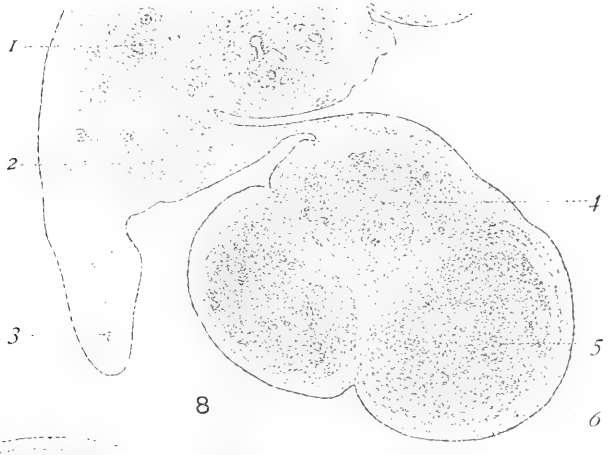

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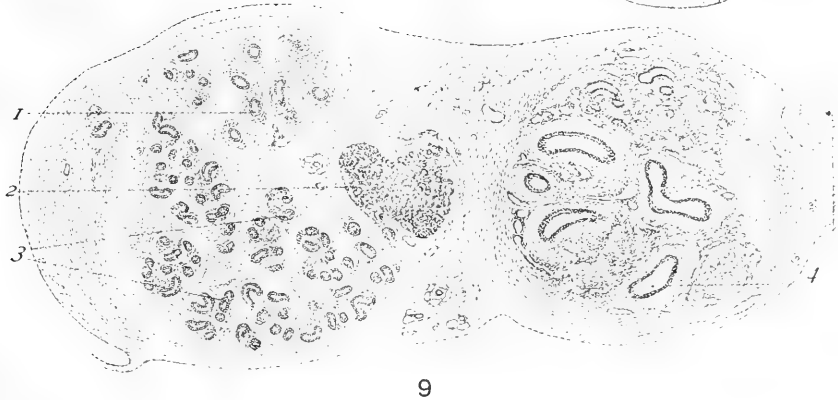

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in the normal male and, in some cases, cells are found in the lumen indicating degeneration. The Wolffian body of free-martin T6 is as large in cross section as that of ♂ T6, and is larger than the Wolffian body of the ♀ N23, examined for comparison.

Both sets of ducts of the free-martin are irregular. The Wolffian duct is almost entirely atrophied. Anterior to the gonad its relations are normal. In the region near the gonad it appears only at irregular intervals. Posterior to that it is lacking for some distance. It reappears near the caudal end of its normal course where it seems to be regular. As for the Müllerian duct, the tube is entirely absent. The enlarged posterior part of the Müllerian duct is present and seems to be complete. In a normal 14 cm. female, the horns of the uterus are much larger in diameter and unite to form the body of the uterus. In this 16.3 cm. free-martin the uterine horns do unite (of the free-martins examined, this is the only case in which any union of the cornua was found), but only for a distance of 1.905 mm. Anterior to the union, one duct lies ventral to the other. When they separate, posterior to their junction, they are once more lateral to each other.

Summary. The gonad of the 16.3 cm. free-martin resembles that of the male in the absence of cortex, the appearance of the albuginea, and peritoneum, and the large development of rete. It resembles the female gland in the structure of the sex cords and in the position of the rete. The atrophy of the oviduct and retention of the reduced uterine horns is characteristic of the male, whereas their partial union and the degeneration of the Wolffian body suggest the condition of these organs in the female.

4. SEVERAL FREE-MARTINS STUDIED MEASURED 20 CM. OR THEREABOUTS

Of these, T13 measured 20 cm., T2 measured 21.5 cm. (Lillie, fig. 20) and T4 22.5 cm. (Lillie, figs. 22A and 22B). These embryos were presumably of about the same age, but, as might be expected from the possible variations in the cause of their abnormalities, they differ among themselves, in development of

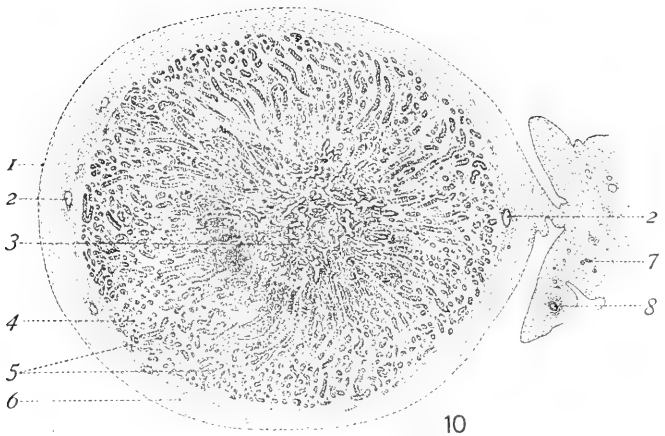
reproductive organs, much more than do normal embryos which vary even more in size.

For comparison the normal ♂ and ♀ of the same size will first be described.

a. Conditions in the normal

The males sectioned for comparative study are N21, measuring 20 cm. and T4, the twin of free-martin 4, which measures 24 cm. The testis of the 20 cm. male, which had not yet begun its descent, is 4.042 mm. long. The left testis of the 24 cm. ♂ measures 8.67 mm. and had begun its descent into the saccus vaginalis. The right testis in the same specimen had almost reached the distal end of the scrotal sac. Otherwise, the testes of these two specimens are approximately the same. Their condition is shown in figures 9 and 10. The seminiferous tubules are still solid cords, longer and more branched than in testes of younger animals. They contain germ cells, some of which are as yet in the indifferent stage, while others are in the growth stages described by Schoenfeld.[3] In the 20 cm. ♂ one finds primary spermatocytes in stages a to e. In the 24 cm. specimen they are in stages a, e and f. In neither case were any mitoses found. The spaces between the sex cords are filled with a large amount of interstitial material made up chiefly of typical embryonic connective tissue, but some of the cells have a wider layer of cytoplasm around the nucleus and are polyhedral in shape. These are young interstitial cells. The interstitial space is wider in T4, the interstitial cells more numerous. The characteristic male albuginea, consisting of layers of connective tissue pressed compactly together, lies just beneath the superficial peritoneum, surrounding the sex cord region. Blood vessels lie in the inner deeper layer (tunica vasculosa). The rete cords can be seen connected with the seminiferous tubules, but distinguished from the latter by their lumina[1] (fig. 10).

The Wolffian body lateral to the gonad is almost completely degenerated. From the anterior part of the Wolffian body is formed the epididymis, much larger in these males than it was



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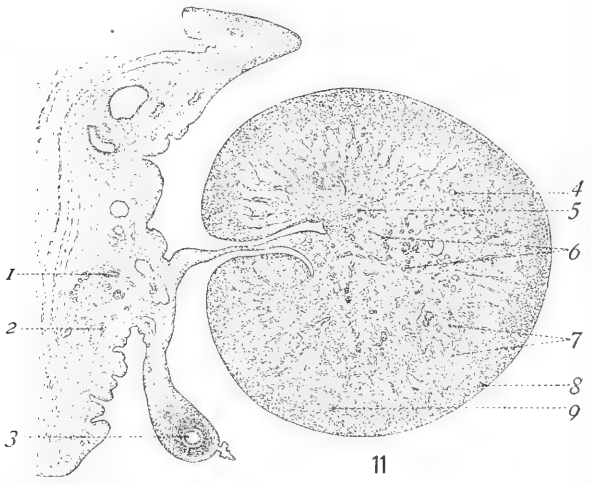

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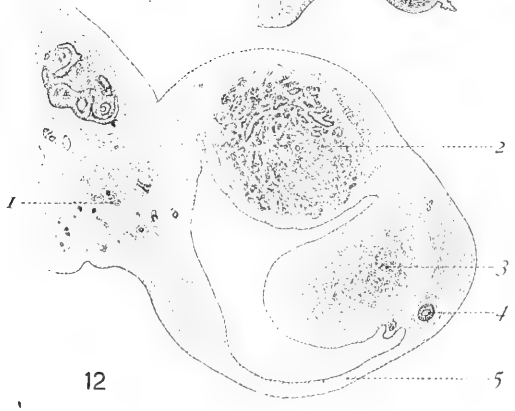

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in the 16.75 cm. male. Numerous, much convoluted vasa efferentia lead from the rete into the coiled vas epididymis which in turn opens into the Wolffian duct. The Müllerian duct has long since atrophied.

In the case of the females, there were sectioned the left ovary and ducts of a 20 cm. ♀ N24 and the left ovary and ducts of a 23 cm. ♀ N25. A dissection was made of the reproductive system of a third female, measuring about 20 cm. The sectioned ovary of the 20 cm. ♀ was 4.440 mm. long. That of the 23 cm. ♀ measured 6.830 mm. The condition of the ovaries is almost the same in these two cases. The medulla is relatively larger in the younger specimen. In N25, figure 11, it is composed mainly of fibrous stroma and contains fewer medullary cords than does the medulla of the smaller female. 'Nests' of cells, primary follicles, first found in the cortex in the 17 cm. ♀ N23, are more numerous in each succeeding stage. The individual cords of Pflüger are surrounded by wide sheaths of stroma, but in this large amount of interstitial material, no interstitial cells have been identified with certainty. Most of the cells are the long slender spindle shaped cells typical of embryonic connective tissue. In the smaller female, most of the germ cells which are found in the growth period are in Schoenfeld's early stages, though a few have reached as advanced a stage as i. In the 23 cm. ♀ they are found in these same stages, the majority of

Fig. 10 Cross section through Wolffian body and middle of testis of 24 cm. ♂ bos embryo, T4. *1*, superficial epithelium; *2*, blood vessels in tunica vasculosa; *3*, rete; *4*, interstitial material, including connective tissue stroma and interstitial cells; *5*, seminiferous tubules; *6*, albuginea; *7*, degenerating Wolffian tubule; *8*, Wolffian duct. × 15.

Fig. 11 Cross section through Wolffian body and middle of ovary of 23 cm. ♀ Bos embryo, N25. Note that the rete does not extend as far as the middle of the ovary. *1*, tubule of epoöphoron; *2*, degenerating Wolffian tubule; *3*, Müllerian duct; *4*, primary follicle formed at inner end of cord of Pflüger; *5*, primary albuginea; *6*, medullary cords; *7*, cords of Pflüger; *8*, germinal epithelium (scarcely distinguishable from cord of Pflüger); *9*, beginning of ovarian albuginea. × 22.

Fig. 12 Cross section of gonad and Wolffian body of 20 cm. free-martin T13, and entrance of rete into gonad. *1*, degenerating Wolffian tubule; *2*, rete; *3*, sex cord region (no differentiation), *4*, rudiment of Müllerian duct; *5*, fold of peritoneum overhanging gonad. × 28.

them having advanced at least as far as stage e. No mitoses are in progress. The germinal epithelium is thick and scarcely distinguishable from the outer ends of the cords of Pflüger except that in places, the strands of stroma on either side of a cord of Pflüger are uniting at their distal ends to form the beginning of the definitive albuginea. The rete ovarii, small in cross section compared with the rete testis, extends only a short distance into the ovary.

The Wolffian body, in both females, is represented only by the epoöphoron and paroöphoron. In N25 the Wolffian duct has completely atrophied, but in N24 it still persists, though it is exceedingly small in diameter. The Müllerian duct is large and lined with ciliated epithelium. The mouth of the ostium abdominale is becoming convoluted. A macroscopic study shows the uterus large, with a thick, muscular wall.

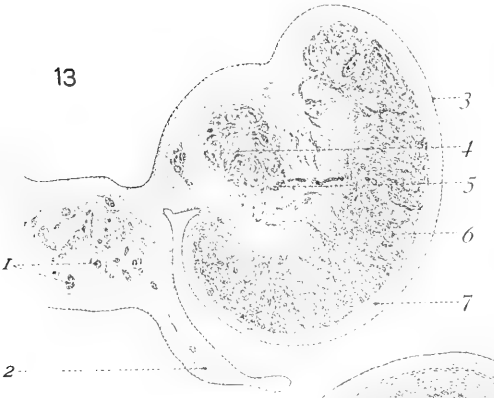
b. Condition in the free-martin

The three free-martins, 13, 2 and 4 (figs. 12 to 16) resemble each other in regard to some structures. All have well developed rete with distinct lumina in the cords. All have distinguishable medullary cords, although in case 4 they are relatively fewer in number and more widely separated by the connective tissue stroma. In every case the albuginea is a wide, compact layer, and the superficial peritoneum is a thin epithelial layer as in the male; the cortex, cords of Pflüger, is absolutely lacking.

Fig. 13 Cross section through middle of gonad and Wolffian body of 20 cm. free-martin T13. *1*, degenerating Wolffian tubules; *2*, urogenital fold; *3*, superficial epithelium like that of ♂; *4*, rete; *5*, medullary cord connecting with rete (as seminiferous tubule connects with rete in ♂); *6*, medullary cord; *7*, albuginea. × 20.

Fig. 14 Cross section through middle of gonad of 21.5 cm. free-martin, T2. *1*, sex cords resembling medullary cord; *2*, rete; *3*, sex cords resembling seminiferous tubules; *4*, Wolffian duct in fold of peritoneum overhanging gonad; *5*, rudiment of Wolffian body. × 20.

Figs. 15 and 16 Cross sections through gonad and Wolffian body of 22.5 cm. free-martin, T4. *1*, Wolffian tubules (epoöphoron); *2*, rete; *3*, sex cord; *4*, Wolffian duct in fold of peritoneum overhanging gonad; *5*, sex cord connected with rete. × 20.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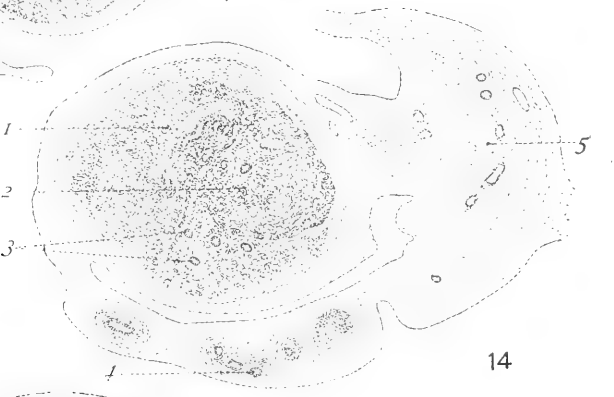
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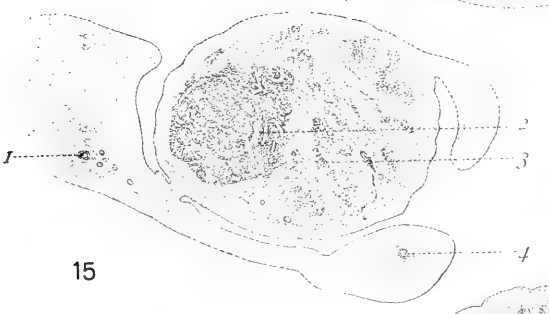
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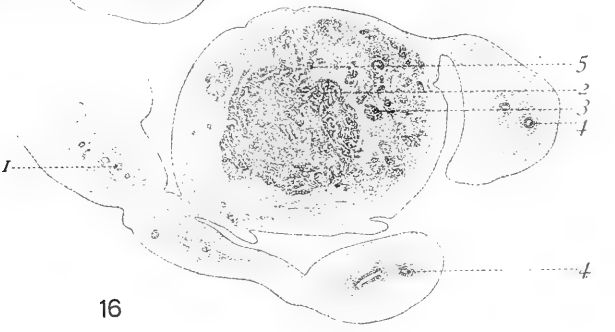
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In the 20 cm. free-martin T13, the medulla, in cross section, is shaped like the cortex and medulla of a much smaller, normal female, 7.3 cm., N8 (fig. 13). In size, the gonad is like the ovary of an 8.3 cm. female N7 (fig. 3). Very rarely one finds a germ cell enclosed in a primary follicle. In case 2, a few of the medullary cords (fig. 14), have a very definite arrangement of cells, like that of the seminiferous tubules. Such an arrangement, in a larger number of cords (in an older individual) is doubtless what D. B. Hart[5] found and illustrated. It was partly because of this that he interpreted the free-martin as an abnormal male. Occasional germ cells are found. In case 4 connections between the rete and the medullary cords can be seen (fig. 16). It should be noted that such connection is not found in the normal female, but that in the male the rete is connected with the seminiferous tubules, which are homologues of the medullary cords. No germ cells were distinguished.

The Wolffian body in all three cases has degenerated, with the exception of the part which forms the epididymis. In case 13 there are several connecting tubules between the rete and an enlargement of the Wolffian duct. The Wolffian duct extends straight anteriorly for some distance. Posterior to the vasa efferentia which are straighter than those of the epididymis of a normal ♂, the Wolffian duct ends in a posterior enlargement. In the region of the uterus, the Wolffian duct again appears. Here, the duct is not continuous. In places it is represented only by a cord of cells; in other places there is a lumen through this cord. In no place does it have the ciliated lining which is characteristic in the normal ♂.

The tube of the Müllerian duct in T13 is lacking, except for a very small portion, the lumen of which is not more than 0.025 mm. long, figure 12 (cf. f-m. 26, page 464). The anterior part of the uterine horns is present, the middle part is lacking and the most posterior part is present but very small and not like the typical Müllerian duct.

In case 2, the epididymis consists of several vasa efferentia. The Wolffian duct is complete. The more convoluted part lies in

[5] D. B. Hart, Proc. Roy. Soc. Edinb., vol. 30, 1909–10.

the fold of peritoneum which overhangs the anterior part of the gonad (fig. 14). This position of the Wolffian duct is characteristic of the male. As for the Müllerian ducts, the tube is entirely atrophied. The horns of the uterus are present and like those of a normal female, in histological structure, but much smaller in diameter. However, in the preparation studied, they do not unite, although they were sectioned posterior to the point at which they normally unite in the female.

In the 22.5 cm. free-martin T4, the epididymis and anterior part of the Wolffian duct resemble those structures in free-martin 2. The convoluted portion of the Wolffian duct (figs. 15 and 16). lies in the fold of peritoneum overhanging the gonad. Posterior to the gonad, the Wolffian duct ends in an enlargement. The caudal part of the Wolffian duct is present and, as in case 13, the lumen is discontinuous. The entire tubular portion of the Müllerian duct is atrophied. In the location where the anterior part of the horns of the uterus are found, there is a large structure which may be a rudiment of one of them. It has a typical, epithelial wall and is about the diameter of the normal uterine horn. It is closed at both ends, having no connection with any other structures. Lateral to the posterior ends of the Wolffian ducts are coiled tubules, one on each side. These are presumably the anlage of seminal vesicles which develop in the male from the Müllerian ducts at their posterior end.

Summary. The gonads of the free-martins measuring 20 cm., 21.5 cm. and 22.5 cm. resemble the testis in the entire absence of cortex, the appearance of the albuginea, peritoneum and rete, and the connection of the rete with the Wolffian duct through the Wolffian tubules. They resemble the ovary in the position of the rete and in the structure of the medullary cords, with the exception of the few sex cords in T2 which have the appearance of seminiferous tubules. T2 resembles the male in the condition of both ducts. T13 and T4 resemble the female in the partial atrophy of the Wolffian duct and resemble the male in the atrophy of the oviduct, and non-union of uterine horns.

5. FREE-MARTIN 28 CM. T12

The largest free-martin embryo studied measured 28 cm. (Lillie, figs. 27A and 27B). The male (N19) and female (N28) sectioned for comparison measured respectively 31 cm. and 29.5 cm.

a. Conditions in the normals of this stage

These specimens differ so little from the smaller normal embryos that they will be described but briefly and illustrations seem unnecessary. In the male, the seminiferous tubules are even more contorted than those of T4, and slightly larger. The nuclei, in general, are arranged along the periphery of the sex cords but no lumina are as yet formed. The interstitial material is about equal in volume to the sex cords and contains many interstitial cells. The germ cells which are found in the growth period are even more numerous in N19 than in T4. Most of them are in the later stages of the growth period (i). Otherwise the structures in the 31 cm. male are like those of the 24 cm. ♂.

In the female also some advance may be noted. The medulla is made up more largely of connective tissue stroma, and many more of the medullary cords are degenerating. At the inner ends of the cords of Pflüger are found many 'nests' of cells some of which are enclosed by more than one follicular layer. Many germ cells are in the growth period, those in the follicles being more advanced than the others. The germ cells in the follicles are in Schoenfeld's stage i while most of the other germ cells are in stages d, e, etc. No maturation or oögonial mitoses are found. The interstitial, connective tissue stroma is voluminous. A few cells in it look like young interstitial cells, but have not been identified with certainty. The albuginea is a little more advanced than it was in the 23 cm. ♀. The rete extends less than half way through the ovary and has no connections with the medullary cords. The Wolffian body, and Wolffian and Müllerian ducts are in the same condition as those of the 23 cm. ♀ N25.

b. Condition in the free-martin

The gonad of the 28 cm. free-martin T12 measures 3 mm., in comparison with the 8.260 mm. testis of N19 and the 7 mm. ovary of N28. The rete is the most prominent feature of the gonad. It is much longer than the gland, extending posterior to the sex cord region as well as anterior to it. The diameter of the rete is about one-half the diameter of the entire gonad. The rete lies at one side of the gonad, as in the female, surrounded on three sides by sex cord region. The rete cords are very numerous and have large lumina. The sex cord region shows no evidence of organization. It seems to be a homogeneous mass of connective tissue, with no differentiation into cords. A few cells are seen which have large, round nuclei resembling those of germ cells (indifferent stage or Schoenfeld's stage spc. a.) but they do not have the definitely limited cytoplasm characteristic of germ cells and probably are not germ cells. No interstitial cells are distinguishable. The thin epithelial covering of the gland and the compact albuginea lying directly beneath it are typical of the male.

The Wolffian body is represented only by a few tubules, the epoöphoron, etc. The Wolffian duct is absent. The anterior portion of the Müllerian duct, the oviduct, is absent but the horns of the uterus are present.

Summary. The 28 cm. free-martin resembles the male in the absence of cortex, condition of the albuginea and superficial epithelium, extent of the rete, atrophy of the oviduct and presence but non-union of the uterine horns. It is like the female in the position of the rete, degeneration of the Wolffian body, and atrophy of the Wolffian duct.

6. FREE-MARTIN TWENTY-ONE DAYS AFTER BIRTH

Besides the free-martin embryos studied, the gonad of a free-martin killed twenty-one days after birth was sectioned (T42). The gonad measured about 13.5 mm. in length and 5 by 3 mm. in cross section at its largest point. Data concerning the size of glands of normal calves is not at hand.

The medullary region in this free-martin is relatively larger than in the other free-martins studied, and more organized. Almost the entire region is differentiated into sex cords, separated by connective tissue stroma. Some of these sex cords resemble the seminiferous tubules, as do a few sex cords in free-martin T2; others resemble the medullary cords. Degenerating follicles are found but no germ cells. Interstitial cells have not been identified in the stroma. The superficial peritoneum and albuginea are like those structures in the male. Again there is complete absence of cortex. The rete resembles the rete testis in structure, relative size and distance of penetration into the gonad, but, lying at one side of the sex cord region, near the mesentery, it resembles the rete ovarii in respect to its location. There is a large amount of rete anterior to the gonad in the position which, in the male, is occupied by the epididymis. This resemblance to the epididymis is still more marked because in the region where the vas epididymis leads from the vasa efferentia into the vas deferens, are rudiments of three ducts. These ducts are lined with ciliated epithelium but are closed at both ends and, although the walls of tubules from the rete come in contact with the walls of these ducts, in no place do they actually open into each other. However, this mass of rete, which resembles epididymis in position does not resemble it in structure, being, in that respect like the typical rete testis.

The Wolffian body has degenerated, but lateral to the gonad are many tubules. Among other structures are found large blood vessels like those of male T4 (fig. 9).

Summary. The free-martin T42 resembles the male in absence of cortex, presence of albuginea, structure of the superficial peritoneum, and structure of the rete. Some of the sex cords are like medullary cords; others are like the seminiferous tubules. The position of the rete in the free-martin is that of the rete ovarii.

TABULAR SUMMARY OF CONDITIONS OF SEX ORGANS OF BOS EMBRYOS, INCLUDING NORMAL MALES, NORMAL FEMALES AND FREE-MARTIN

SPECIMEN				GONAD							MULLERIAN DUCT		
Centimetres	Sex	Number	Length	Area cross section	Sections, lst set	Cords of Pflüger	Interstitial material	Germ cells	Germinal epithelium	Albuginea	Rete	Wolffian body	Wolffian duct	Tube	Horns of uterus	Body of uterus
			mm													
7·0	♂	N16	3·05	4 × 1·6 m	Distinct, little branching No lumen Figure 2		Scanty connective tissue stroma	Indifferent	Absent	Thin, compact	Enters at anterior end of testis, figure 1; does not extend far posteriorly	Larger than testis in cross section and much longer. No degeneration. No formation of epididymis	Complete mesonephrotic duct. No connection with rete	Complete, except for ostium abdominale	Present	Not formed in ♂
8·0	♂	T19	3·5	6 × 1·6 m	Distinct, branching no lumen		More than in N19	(Preservation poor)	Absent	Compact	Extends further into testis. Cords more distinct. Connected with glomeruli of W. b	Cross section = central cross section of testis. Degeneration has begun; also formation of epididymis	Complete	Complete, except for ostium abdominale	Present	No union of horns of uterus
8·3	♀	N7		5 × 1·3 m	Medullary cords, not yet degenerating Figure 3	Present, short, little differentiated from one another or from germinal epithelium	Connective tissue stroma only	Degenerating or indifferent in medulla, indifferent in cortex	Wide Cord of Pflüger still attached	Primary albuginea present	Enters anterior end of gonad. Figure 4. Position like that in ♀. Cords with lumina not formed. Figure 5	As large as ovary in cross section. Degeneration has begun but has not gone far. Glomeruli fewer than in ♂ or free-martin	Complete	Complete, including ostium abdominale	Present	Horns united to form body of uterus
7·5	1-m	T19	2·07	1·4 7 m ♂ 1·4 8 3 m ♀	Medullary region small Not differentiated into cords Figure 5	Absent	Absent	Present, but very few. Growth stages a, or indifferent	Absent	Wide	Enters anterior end of gonad. Figure 4. Position like that in ♀. Cords with lumina not formed. Figure 5	Larger than gonad in cross section and in length. No degeneration as yet. Glomeruli numerous	Complete. Lillie, figure 13	Irregular, discontinuous	Not sectioned	
12·75	♂	T16	4·45	6 × 1·6 m	Branching tubules No lumina Cells beginning to be with nuclei near periphery of cords		Thin connective tissue stroma	Many indifferent. Some in early growth stages a-d few.	Absent	Compact	Runs almost entire length of testis. Lumina distinct. Cords continuous with seminiferous tubules	Much smaller than testis in cross section. Degeneration has gone far. Glomeruli reduced in size. Cells in lumina of tubules. Epididymis formed at anterior end. W. tubules, con, with rete, contorted	Continuous with Wolffian tubules which form epididymis	Absent	Not sectioned	
14·0	♀	N28	3·4	4 × 1·6 m	Medulla relatively smaller than in S 3 ♀. Cords contain primary follicles	Cortex relatively broader than in S 3 cm, ♀. Cords distinctly separated from each other by connective tissue stroma	Connective tissue stroma. A very few cells are suggestive of young interstitial cells	Majority indifferent. In deeper layer of cortex, some primary spermatogonies in early growth stages. Occasional mitoses	Present Indistinguishable from cords of Pflüger	Primary albuginea present. Suggestive of definitive albuginea	Lumina in cords distinct	Glomeruli still present in posterior part. No large W. tubules like those of 7·3 cm. ♀, N8 and 8·3 cm. ♀ N7. Signs of degeneration-cells in lumina of tubules	Atrophied	Complete. Less close to ovary	Complete. Large in diameter	Formed by union of horns. Wall thick and muscular
12·5	1-m	T26	1·735	1·6 ♂ 1·4 ♀	Anterior part of medulla little differentiated Medullary cords found in posterior part. Figure 6	Absent	Absent	Present but	Absent	Present, narrow	In position of rete ovarii. Rete cords connected with some medullary cords as with seminiferous tubules in the ♂.	Smaller than W. b. of 11·2 cm. and 12·75 cm. ♂ s. Much degenerated, as in ♀	Anterior part cords anterior to gonad. (Posterior part not sectioned)	Absent, except for small portion homologous with hydatid morgagni	Not sectioned	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Branching		Wide, composed of connective tissue stroma and some interstitial cells	Still mostly indifferent. Some spermatocytes in early growth stages	Absent	Wide and compact	Large in diameter. Cords much branched and process lumina. Connect with seminiferous tubules	Epididymis large. Posterior part of W. b. much degenerated	Complete	Atrophied Lillie, figure 14		
12·0	♀	N21	4·0	4 × 1·6 m	Medullary cords prominent Connective tissue stroma voluminous	Still attached to germinal epithelium A few tests of cells formed at inner ends of cords	Connective tissue. No interstitial cells	Majority indifferent. Some primary oögonia in early stages	Present	Primary albuginea wide	Present—less extensive than in ♂	Degeneration has gone farther than in ♂. Fewer glomeruli than in 14 cm. ♀	Present continuous as far as sectioned	Complete. Ostium large, wall becoming convoluted	Present	Present, wall thick
9·0	1-m	T1	2	1·5 × 1·4	Anterior part of medulla small, posterior enlarged Cross section of rete. Few cords distinct	Absent. Figures 7 and 8	Absent	In primary follicles in medullary cords. Some degenerating	Absent	Compact, relatively as wide as that of ♂	Relatively large. Lumina of cords distinguishable in anterior part. Position of rete ovarii. Figure 7	Degeneration has gone far	Present anterior to gonad. Present in region of horns of uterus, but discontinuous	Atrophied; Lillie, figure 16	Present; small in diameter	Horns of uterus united for 1.905 mm.
30·0	♂	N34	4·95	[illegible] T4	Seminiferous tubules many and loosely coiled		Much interstitial material, including many interstitial cells	Primary spermatocytes in growth stages a to	Absent	Wide, compact	Extends almost to posterior end of testis. Smaller than in T4	Degeneration except for large epididymis.	Complete	Atrophied	Not sectioned	
24·0	♂	T4	3·6		Seminiferous tubules longer and more branched than in N21 and N28. No lumina. Cells arranged with nuclei near periphery of cords	Figure 10	As in N21, or more voluminous. Area in any section = area seminiferous tubules in that section	Primary spermatocytes in growth stages a to c. No mitoses	Absent	Wide, compact	Large in cross section. Tubules much branched. Continuous with seminiferous tubules	Epididymis very voluminous. W. tubules composing it, much contorted. Figure 9. See 26 cm. ♂, Lillie, figure 12	Complete	Atrophied	Not sectioned	
21·0	♀	N24	4·0	1·4 × T4	Cords degenerating Medulla composed mainly of connective tissue stroma	Separated by stroma. Primary follicles at inner ends of cords; more numerous than in 17 cm. ♀ N21	Much interstitial material, but no interstitial cells	Primary oögonia in growth stages up to stage i	Present	Primary albuginea. Suggestion of definitive albuginea	Smaller than in males. Lumina large. Connected with gonad and with W. b	Mostly degenerated. Tubules of epoöphoron and paroöphoron remain	Present, but very small in diameter	Large. Ostium large	Present	Thick walls, muscular
26·0	♀	N25	4·5	1·4 ♀, T4 ♂, N24	Few medullary cords present Figure 11	As in 20 cm. ♀	As in 20 cm. ♀	As in 20 cm. ♀	Present	As in 20 cm. ♀	Extends only a short distance into the ovary	As in 20 cm. ♀	Atrophied	Complete. Ostium large. End of tube curves around end of ovary	Present	Thick walls, muscular
20·0	1-m	T14	2·175	1·6 ♂, T4	Some distinct cords. Some seem to be connected with rete. Medulla [illegible] cross section rete	Absent. Figure 13	Interstitial cells absent. Connective tissue stroma present		Absent	Wide, compact	Lumina distinct, separated from medulla by wide layer of connective tissue	Much degeneration. No glomeruli. Three W. tubules connecting W. duct and rete represent epididymis	Rudimentary. Ends in enlargement anterior to gonad. Present in region of uterus; discontinuous	Suggestion of hydatid. Figure 13 complete with 12.5 cm. ♀ m. T26	Reduced	No union of horns
21·5	1-m	T2	2·85	1·6 ♂, T4	Medulla large. Many medullary cords Some which resemble in inferior tubules of ♂	Absent. Figure 14	As above	A few present	Absent	Wide, compact	Large. Many cords. Lumina distinct. Not separated from rete, as in T19	Epididymis represented by a few tubules (more than in T19). Rest of Wolffian body degenerated	Complete. Position of W. duct in normal ♂. Figure 14	Absent	Reduced. See figure 20A (Lillie) Cf. figures 7 and 8 (Lillie) for normals	No union of horns
22·5	1-m	T4	2·265	1·4 × T4	Medulla relatively small (5·0 to 7·0 mm. groups resembling degenerating medullary cords	Absent. Figure 15	Connective tissue stroma large. No interstitial cells	None present	Absent	Wide	Connected with medulla cords, in a few cases. Large. Many cords. Lumina distinct	Epididymis represented by few tubules than in T13. Rest of W. body degenerated	Complete in region of gonad and in region of uterus. Discontinuous between	Absent	Represented by one large duct, closed at both ends Lillie, figure 22A, 22B	Contorted ducts, = prostatic vesicles (uteri masculini)
31·0	♂	N31	5·25	1·6 × T4	Much contorted. No lumen. Nuclei of cell, near periphery		Interstitial cells numerous	Many in growth stages up to	Absent	Wide, compact	Many tubules lined with tall columnar epithelium	Degenerated, except for large epididymis	Complete	Absent	Not sectioned	
29·5	♀	N29	7·0	2·5 × 1·6 m	Cords mostly degenerated Follicles degenerating	Many follicles growing	Stroma. A few cells may be interstitial cells	In growth stages. Those in follicles most advanced, in those in cords, d and e	Thick	Primary present. Definitive growing	Extends less than ¼ way through gland. Smaller in diameter than that of 31 cm. ♂	Degenerated, except for tubules of epoöphoron and paroöphoron	Atrophied	Large	Present	Present
28·0	6-m	T12	5·9	1·1 ♂, 2·5 ♀	Medulla not differentiated into cords	Absent	Not distinguishable	A few in stage of growth stage a?	Absent	Wide, compact	Prominent. Longer than medullary region. In position of rete in ♀	Represented by a few tubules	Atrophied, Lillie, figures 27A, 27B	Atrophied	Present	No union of horns
Killed 21 days after birth	1-m	T42	11·5	Average diameter cross section 4 mm.	Medulla relatively large and well differentiated into cords, some of which resemble secondary tubules. Degenerating follicles	Absent	No interstitial cells	None	Absent	Like ♂	¼ cross section of gonad. Resembles ♂, in structure; ♀ in position	Rete extends into region of epididymis of ♂. Rudimentary vasa efferentia—3	(.....Not examined.....)			

SUMMARY AND CONCLUSIONS

The conditions found in foetal free-martins described in the preceding pages, show the *effect of the introduction of interstitial secretion from the male embryo into the circulation of a female embryo.* The possibility of such an introduction of interstitial secretion and its effect upon the secondary sex characters has been fully discussed by Professor Lillie in his paper which precedes this. There follows a summary of the conditions resulting from the introduction of the interstitial secretion of the ♂ embryo into the ♀ embryo.

The sex cords present in the free-martin are homologous with the medullary cords of the normal female and with the seminiferous tubules of the male. In some specimens the sex cord region is simply an unorganized, homogeneous mass of cells. In the majority of embryos in which the cords are differentiated from each other, they resemble the medullary cords of the female, but in a few cases some of the cords exhibit an arrangement of cells like that of the seminiferous tubules. This latter condition is found only in free-martins in which the sex cord region is large, compared with that of other free-martins.

In no free-martin are sex cords ever formed which correspond to the second set of sex cords of the female, the cords of Pflüger. The latter are characteristic of the female and have no homologue in the male. The proliferation of the cords of Pflüger from the germinal epithelium marks the beginning of differentiation from the indifferent stage to the female condition. In the male, apparently all the primitive germ cells are carried down into the gland with the first proliferation of the germinal epithelium, leaving as the surface covering of the testis, only the thin mesothelial peritoneum from which the cords are soon separated by a compact layer of connective tissue, the albuginea. In the female, relatively few of the primitive germ cells enter the medullary cords. The primary albuginea, an irregular layer of loose connective tissue cuts these cords off from the germinal epithelium which is still a thick layer composed largely of cuboidal epithelial cells, and containing also many primitive germ cells.

The cords of Pflüger are formed by a second proliferation of the germinal epithelium, from which they become separated by the development of the definitive albuginae, just making its appearance in the older normal female embryos studied, 23 cm. and 29.5 cm. In the free-martin, but one set of sex cords is formed. The primary albuginea becomes a compact structure like the tunica albuginea of the male. Instead of the germinal epithelium of the normal female, the gonad of the free-martin is enclosed by a thin layer of flattened epithelial cells like the superficial peritoneum of the male.

The rete is present in the indifferent stage and in the early stages of both sexes, after differentiation. In the male it continues to grow larger with the testis. In the female, it gradually diminishes until in a 29.5 cm. ♀, it is of comparatively small extent. In the free-martin the rete continues to grow large, as it does in the male, sometimes to the point of becoming even larger in a certain free-martin than in a male of corresponding size.

In the youngest embryos studied, 7 and 8 cm., the Wolffian bodies differ in the two sexes chiefly in the larger size and number of Wolffian tubules of the male. The condition in the free-martin is like that in the male. In larger free-martins the Wolffian body is variable. In some, vasa efferentia connect the rete with the Wolffian duct as in the ♂, in others, there is no such connections, the Wolffian body having almost entirely degenerated as in the female.

In some free-martins the Wolffian duct undergoes complete atrophy, or at least atrophy of the entire anterior part. (Gonad to region of the body of the uterus.) In others it is present anterior to the gonad and is connected with the rete by vasa efferentia, as in the male (no such connection is ever established in the female). In some of these free-martins the Wolffian duct ends blindly, anterior to the gonad. In others it ends posterior to the gonad and in a few, is complete. In the latter two conditions it lies in a fold of the peritoneum overhanging the gonad just as it does in the male.

The Müllerian duct becomes irregular in young free-martin embryos as in young males. In older free-martins the tube has atrophied. The posterior enlargement may persist. This is homologous with the uterus in the normal female and the uterus masculinus or prostatic vesicle in the male. The two Müllerian ducts unite in the female to form the body of the uterus. In the male they do not join. In only one free-martin were they found to unite—T6—and in that case the union was for a distance of less than 2 mm. The failure of these ducts to unite to form the body of the uterus is in accord with Paton's[6] finding of non-development of the uterus in castrated female guinea pigs. In two free-martins, 20 cm. T13 and 12.5 cm. T26, a rudiment of the Müllerian duct is found near the anterior end of the gonad. This is probably the homologue of the hydatid of Morgagni, a vestige of the Müllerian duct which persists in that region in the male.

From the foregoing facts it is evident that, *1. As a result of the introduction of the interstitial secretion of the male, those organs in the free-martin which are present in the indifferent stage, develop toward the male condition* (rete, first set sex cords, primary albuginea), *and those which develop in the normal female at sex differentiation or later are inhibited from developing* (cords of Pflüger, definitive albuginea, union of Müllerian ducts to form uterus).

2. The high degree of variation found in the organs of the reproductive system in free-martins is indicative of the variability of the time at which the interstitial secretion of the male embryo may first be introduced into the circulation of the female embryo, and the amount which may be introduced; in other words, the variability of the time and degree of anastomosis of the extra embryonal blood vessels of the two embryos.

a. The fact that in some free-martins the Wolffian body and Wolffian duct have degenerated more than in a male of corresponding size, though not more than in the female, suggests a later introduction of the secretion of the male, or the introduc-

[6] D. N. Paton, Regulators of Metabolism.

tion of a smaller amount, allowing some development toward the female condition. This is also suggested by the partial union of the horns of the uterus in the 16.3 cm. free-martin T6.

b. The arrangement of cells in the sex cords of the free-martin as they are arranged in the seminiferous tubules of the male and the relatively large size of the sex cord region suggest an early introduction of the male influence, made before the beginning or early in the process of degeneration of medullary cords which normally take place in the female, or the introduction of a sufficiently large amount of male secretion to inhibit female development completely and to cause development toward the male condition. The development of the Müllerian ducts of the 22.5 cm. free-martin, T4, into contorted seminal vesicles of small diameter suggests that the male hormone was introduced into the female earlier or in larger amount in this case, than in the 28 cm. free-martin, T12, in which the Müllerian ducts are still straight ducts of large diameter.

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MICRODISSECTION STUDIES

II. THE CELL ASTER: A REVERSIBLE GELATION PHENOMENON[1]

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ONE PLATE

I. INTRODUCTION

1. Historical

The periodic appearance and disappearance of the aster in the cell, the very definite structure which it offers to the eye, and its very evident relationship to cell division make it one of the most striking phenomena in cell protoplasm.

Any idea which one may advance regarding its structure is necessarily based on a conception of the structure of protoplasm. There can be, therefore, as many interpretations of the astral structure as there are theories of the physical make-up of protoplasm. Taking for a basis the reticular theory of protoplasm, the astral rays have been considered fibrous strands whose radiate arrangement is produced by being drawn together at a point in the protoplasmic fibrous network. In accordance with Bütschli's foam theory of protoplasm the aster has been explained as an arrangement of the protoplasmic alveoli into more or less definite rows radiating from a common center.

The experimental work of Morgan ('08, '10), Lillie ('09), Conklin ('12), and others on the centrifuged eggs has shown conclusively that whatever be the structure of protoplasm the mitotic spindle at least is of such a consistency that it preserves its structure on being driven through the cell substance. The

[1] This paper was read before the American Society of Zoologists, New York City, December 27, 1916. It is based on the work done during the summer of 1916. The writer wishes to express his indebtedness to Prof. H. V. Neal, for accommodation and facilities accorded him at the South Harpswell Laboratory.

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astral radiations often persist about the poles of the spindle being apparently dragged along with them (Spooner '11). F. R. Lillie ('09), suggests that the aster about the poles of a dislocated spindle is a new configuration, and due to forces (see Hartog '05), which are focussed in the centrosomes and influence the cytoplasmic granules in situ. A significant phenomenon is the occasional occurrence of distortion of the astral rays not only in centrifuged but also in normal eggs (Mark '81, Coe '99, Conklin '02, and Painter '16). Investigators generally agree in considering the distortion to be a proof for the existence of more or less extensive movement in cell protoplasm and some conclude that the rays may be firmer in consistency than the surrounding cytoplasm.

In a recent paper Heilbrunn ('15) describes in the cytoplasm of sea urchin eggs a gelatinization produced by chemical agents which cause the eggs to undergo parthenogenetic development.

While investigating the structures of various marine ova by micro-dissection, the author found it possible to detect changes in the consistency of the protoplasm during aster formation and to demonstrate that during cell division we meet with definite reversible gelation phenomena.

2. Material and method

The microdissection method, first introduced for the study of protoplasmic structures by Kite (Kite and Chambers, '12), affords means of collecting direct evidence of the physical consistency of many constituents in the living cell. The apparatus used for holding the dissection needle is the mechanical pipette-holder of Barber. A detailed description of the instrument and of ways for making the micro pipettes and needles is given by Barber in his paper ('14). An elaboration of the method for microdissection purposes is being published (Chambers, '17). A preliminary description has been given (Chambers, '15).

The needle is drawn out of hard glass tubing. It is manipulated with Barber's instrument and projects into a moist chamber on the stage of the microscope. The tip of the

needle is bent at right angles so that it can be brought into a drop hanging from the roof of the chamber. In the drop are the cells to be dissected.

Marine eggs were selected whose development could be easily followed so as to determine their post operative viability. The eggs of Echinarachnius and of Cerebratulus lend themselves well for operative work, that of Echinarachnius because of its beautiful transparency and that of Cerebratulus because of its comparatively high resistance to injury. In Echinarachnius the presence of red chromatophores, sparsely scattered throughout the jelly surrounding the egg, is not enough to disturb one's view and the jelly and fertilization membrane are too soft and extensile, during very early segmentation periods, to afford any hindrance to the microdissection needle. The Cerebratulus egg is rather opaque and the egg membrane which rises off the surface of the egg when placed in sea water is comparatively tough. The membrane, however, can be easily removed by passing the eggs, which have stood in water for twenty minutes to one-half an hour, through a fine cambric cloth. It was also found that the eggs could be considerably flattened during cell division, so that the various cell structures are spread out in a broad, comparatively shallow area and the asters form with rays very long in the plane of flattening and very short in other directions. This offers a distinct advantage for dissection purposes.

3. Hyaloplasm and hyaloplasm-sphere

The word hyaloplasm is used in this paper to designate the continuous hyaline ground substance of protoplasm in which granules (the microsomes and macrosomes or alveolar spheres of Wilson) are embedded. This use of the word hyaloplasm, as applied to the interalveolar material of Bütschli's protoplasmic foam, is urged by Wilson ('01 a, b.) because of its priority and also because it is purely descriptive and introduces no theoretic implications.

The word hyaloplasm-sphere (Wilson, '01) is given to the central transparent area of the aster from which the astral rays radiate. In fixed material Wilson describes this area as includ-

ing the central clear zone (heller Hof of Boveri) surrounding the centrosome and the adjacent zone which contains no alveolar spheres but in which radii are closely crowded together. In the living object no trace of radiation appears in any zone of the hyaloplasm-sphere and Wilson himself raised the question whether the rays seen in its outer zone in sections may not be coagulation products. Neither is any structure resembling a central body or centrosome visible.

Eggs which have been kept flattened for some time in a shallow hanging drop tend to undergo pathological changes. The hyaloplasm-sphere is quickly affected by the abnormal conditions so that frequently cytoplasmic granules (usually the microsomes only) enter the sphere and arrange themselves in more or less regular and concentric rings. Sharply defined radiating lines appear only in coagulated eggs. In some the lines start from the very center of the sphere, in others they not reach the center leaving a central clear area which do develops a finely granular or aveolar structure. Thus several of the types of centrosomal structures figured by Wilson in his book on the cell ('00, p. 310) can be simulated in the Echinarachnius eggs by subjecting them to abnormal conditions.

In the living egg the microdissection method discloses a decided difference in consistency between the substance of the hyaloplasm-sphere and the hyaloplasm surrounding the sphere. I shall substitute in this paper the term 'sphere' for the hyaloplasm-sphere and refer to the contents of the sphere as the sphere substance or sphere liquid.

4. Protoplasm

When protoplasm is examined with transmitted light it appears as a hyaline perfectly homogeneous substance in which granules may or may not be embedded. In ova the granules are fairly uniform in size and usually so crowded together as to give to the cytoplasm of the egg the appearance of an alveolar structure. That the visible granules are not a constant feature of protoplasm may be seen in early germ cells (Chambers, '15) where the protoplasm is optically homogeneous. Very young ova also

are free of visible granules which appear only as the eggs grow when they accumulate until the egg substance is crowded with them (Wilson, '99). That the hyaloplasm is really heterogeneous in structure, although under ordinary microscopic examination it appears homogeneous, can be demonstrated if, instead of using transmitted light, the hyaloplasm be illuminated by a beam of light striking it from the side only. The presence of minute particles large enough to intercept and scatter the light rays are then revealed through the production of a cone-shaped beam of light known as the Faraday or Tyndall phenomenon.[2] If the particles are not resolvable (in which case they are known as amicrons) a hazy light is all that can be seen. If, however, the particles are large enough (when they are known as submicrons) they appear to the observer as shining spots dispersed throughout a transparent medium. In the liquid hyaloplasm, these particles exhibit active Brownian movement (Gaidukov, '10, Marinesco, '12, Price, '14). In coagulated hyaloplasm they are motionless.

Because of its ultra-microscopic heterogeneity in structure, we speak of protoplasm or hyaloplasm as existing in the colloid state to distinguish it from 'crystalloid' or true solutions, whose ingredients are too small to disperse light rays of visible wave lengths, and from true solids in which no Brownian movement is discernible. In a colloid solution the dispersed particles constitute the internal or dispersed phase and the liquid in which they are suspended is the external phase or dispersion-medium.

Of the two great classes of colloidal solutions, the class consisting of emulsion colloids[3] is of chief interest in biological phenomena. To this class belong all the colloids obtainable from organic matter, such as gelatin, glue, albumen and starch. They are colloids in which both phases may be liquid, the internal phase

[2] The Faraday phenomenon is the principle upon which the ultramicroscope is based.

[3] The other class is that of the suspension colloids in which the dispersed phase consists of solid particles suspended in a liquid dispersion-medium. Suspension colloids have no viscosity and do not, as a rule, undergo gelation. Their two phases are comparatively easy to separate and they are readily coagulated with salts. Examples of this class are the metallic suspension-colloids or suspensoids.

being probably more viscous than the external. They are not readily coagulated with salts, their two phases can be separated only with considerable difficulty and they possess the peculiar property of existing in liquid or in solid or pectinized states, either one of which is capable of being transformed into the other. The liquid state is called a sol and the solid state a gel. The ability of passing one into the other is spoken of as a reversible action. In the sol the internal phase is in a highly dispersed condition. In the gel the degree of dispersity of the internal phase is decreased to a minimum until in all probability the dispersed phase passes into a continuous phase (Hatschek, '16, p. 63), the two phases of the colloid becoming, so to speak, locked one in the meshes of the other.[4]

In protoplasm we meet with properties which are peculiar to solutions of colloids of the emulsoid class,[5] having water as the basis of its dispersion-medium. It is more viscous than a true solution, water diffuses through it with readiness (Chambers, '17), and it is capable of forming reversible sols and gels (see this paper, experimental parts).

Bütschli based his theory of the alveolar structure of protoplasm on a comparison of protoplasm with experimentally produced foam structures. He observed the striking similarity between the microscopic picture of a finely divided emulsion of xylol in a soap solution and a protoplasmic network. He also pointed out that variations in the viscosity of protoplasm could be explained by such an emulsion structure.[6] Optically homogeneous protoplasm he interpreted as possessing alveoli the walls

[4] This process may continue until a complete reversal of the phases takes place, the originally internal phase becoming the external phase and vice versa. The internal phase of an emulsion-colloid or emulsoid being more viscous than the external phase (which is aqueous), a reversal of the phases produces a solidifying of the diphasic system.

[5] It is significant that the vast majority of organic emulsion-colloids are hydrophilic.

[6] An emulsion of oil droplets in a soap solution is liquid when the oil exists in a comparatively low degree of dispersity. On sufficient shaking the dispersity of the oil is raised and the surface of contact between the emulsified oil and the emulsion's medium is so enormously increased that the resulting surface tension solidifies the mass.

of which are either so thin as to be invisible or possess a refractive index similar to that of the surrounding substance ('02, p. 217).

Regarding the gross structure of protoplasm, Bütschli's interpretation of an alveolar structure is true only for special cases where the microscopic appearance of a meshwork is due to the inclusion in the hyaloplasm of droplets or granules which are not fundamental to protoplasm (Mathews, '07, Kite, '13, Chambers, '17). In killed protoplasm the visible meshwork is distinctly a coagulation phenomenon and has nothing to do with the structure of living protoplasm (Chambers, '17). Bütschli's interpretation applies rather to protoplasm in connection with its ultra-microscopic, colloidal structure and even then probably only to its gelled or coagulated condition.

The protoplasm of certain marine ova (Asterias, Arbacia, Echinarachnius, Cerebratulus) has been described in a recent paper (Chambers, '17 a). It consists of a hyaloplasm in which clear more or less spherical bodies of 2 to 4 micra in diameter, the macrosomes, and very small granules, the microsomes, are crowded together. The hyaloplasm of the resting egg is in the sol state and is of such a slight viscosity that the nucleus and cell granules can be readily rolled and pushed about in it by the needle. At the egg surface the hyaloplasm is in the gel state and there seems to be no doubt that protoplasm owes its high viscosity, extensibility and contractility to the gelatinized condition of its surface film.

In experiments which involve tearing of protoplasm one must recognize a limit to the amount and character of the mechanical disturbance which the general protoplasm can bear without disorganization. Considerable displacement without injury to the protoplasm can be produced by a slow even movement of the needle. If, however, the movement be quick and rapidly repeated, disorganization with a change to a decidedly acid reaction occurs accompanied either by a liquefaction or an irreversible coagulation of the disorganized protoplasm.

II. EXPERIMENTAL

1. The Aster

The fully formed aster (cf. one of the asters in the illustration of the amphiaster in figure 10) in the Echinarachnius egg[7] consists of a central, hyaline, more or less spherical area, the sphere, from which radiate an enormous number of narrow rays consisting apparently of the same hyaline substance as that of the sphere. The rays are broadest at their base and gradually taper along their course to lose their identity before quite reaching the margin of the cell. The cell nucleus lies in the sphere somewhat to one side of the center (fig. 7). The granular cytoplasm surrounds the hyaline rays and projects between them into the sphere in the form of shorter or longer conical processes (fig. 1). The sphere consists of a clear non-viscous liquid. The needle may be moved about in it without a disturbance of the surroundings. Before the nucleus has increased much in size, it may be pushed about with ease in this liquid area. On the other hand, the cone-like projections of cytoplasm are very solid in comparison. They may be bent (fig. 2) and pulled about and act as comparatively rigid gelatinous structures with the cytoplasmic granules immovably embedded in them. The rays consist of a liquid similar in appearance and consistency to that of the sphere. The cytoplasm through which the rays extend is solid when contrasted with the liquid cytoplasm of resting eggs. In short, dissection of a cell containing a fully developed aster gives one the impression that the cytoplasm is in the gel state, while the sphere and its rays are liquid.

The gel state is most pronounced in the cytoplasm bordering the sphere, its rigidity diminishing as one approaches the periphery of the cell. The comparative stability of this gel state allows of considerable distortion without a destruction of the aster. At the height of the astral stage the aster may be twisted into a spiral or other distorted shapes. On removing the needle

[7] The aster in Cerebratulus, Arbacia and Asterias agrees essentially with the above description.

it may or may not resume its original shape. Figure 3 is a diagrammatic representation of a distortion produced by inserting the needle into the aster of a flattened egg and moving the needle for a short distance in a straight line. The pectinized strand-like masses of cytoplasm lying around the liquid rays are pulled at an angle by the needle, producing a like distortion in the channels which are occupied by the rays. Figure 4 shows how the gelatinized strands drag after the needle when the needle is inserted to one side of the nucleus and the nucleus is pushed along for some distance. A local destruction of a few rays may be produced by rapid thrusts of the needle, a reversal of the gel into the sol state taking place. On removal of the needle the injured area appears in sharp contrast to the surrounding area. Gradually, however, it gels and acquires its original radiate appearance.

More extensive tearing of the aster causes a complete disappearance of its ray-like structure the entire cell reversing from the gel to the sol state. If this has not injured the cell protoplasm the sphere will retain its identity as a clear area in the granular cytoplasm. This can be made to disappear by churning the cytoplasm with the needle. If the needle be then removed, or if it be held stationary, the fluid collects into a sphere, hyaline rays again appear with their bases merging into the sphere, the gel state of the surrounding cytoplasm reappears and the fully reformed aster occasionally continues its normal course of development. Usually, however, destruction of the aster is followed by an abnormal reformation, the rays being very irregular or absent in some regions. Cell division is then much impaired or completely inhibited.

Attempts were made to break up the sphere into several parts with a view of producing several asters. The centrosphere may be broken up into several areas but when left undisturbed and if the cytoplasm remains normal these areas generally fuse together to form a single aster. However, eggs which had remained in a very compressed condition in a slowly evaporating hanging drop often spontaneously produce asters. Abnormal conditions, under which class may be included the treatment of

eggs for the production of artificial 'astrospheres' (Morgan, '96, '99) and cytasters (Wilson, '01 a and others including Yatsu, '05) possibly cause a localization of the sphere liquid in several centers instead of allowing a flow into a single sphere.

2. The formation of the aster

The development of the aster was studied from the moment of its appearance on the entrance of the sperm into the egg, until the egg had segmented into eight cells and numerous dissections were made in the various stages. Because of the beautiful transparency of the Echinarachnius egg the different stages of the aster were best followed in this form and the following remarks, unless otherwise stated, apply to it.

Within two or three minutes after the entrance of the sperm and in the vicinity where it entered, a tiny aster appears which rapidly grows in size and extent. From the beginning it contains a liquid center of appreciable size. That the cytoplasm about this center has been rendered fairly solid is shown by the fact that the aster at this stage may be pushed and rolled in the surrounding liquid cytoplasm (fig. 5). The sperm nucleus is held in the gel around the sphere (fig. 6), and is therefore dragged about with the aster. The aster grows while at the same time the sphere increases in size evidently by a centripetal flow of liquid in the rays. The cytoplasm lying between the rays increases in rigidity as the gel state gradually extends in area.

During this time the eccentrically placed aster moves to the egg center. The shifting in position is possibly due to unequal pressure exerted at its periphery as the aster extends in area. An equilibrium obtains when the aster finally comes to rest at the center of the egg. As the sphere increases in size the sperm nucleus comes to lie in it. The nucleus may be dragged out of the sphere but as long as it is within the confines of the aster, it will, on being released, move slowly back into the sphere. As the aster slowly moves towards the center of the egg, its growth extension is heralded by a streaked appearance of the surrounding cytoplasm. The streaks run in the direction of the converging rays and are due to a gradual gelation of the granular

cytoplasm between the paths of an exuded liquid flowing into the sphere. The coming together of the egg- and sperm-nuclei was observed to occur in the following manner: As long as the egg-nucleus is beyond the confines of the aster, that is, while it still lies in the liquid cytoplasm, it is stationary. When the extending rays of the aster reach the egg-nucleus streaks of gelatinizing cytoplasm appear all about it. The nucleus then moves toward the sphere with increasing rate until, at the border of the sphere, it works into the liquid area where it comes to lie closely pressed against the sperm-nucleus. The movement of the egg-nucleus may be explained by assuming that there exists a centripetal current of the fluid in the rays. That such a current exists may be inferred also from the following experiment: In an egg one may occasionally see one or more small oil-like droplets 2 to 4 micra in diameter. They are possibly due to degenerative processes in the protoplasm. If one of these oil-like droplets be pushed by the needle from the liquid cytoplasm into the periphery of the aster it will move along the rays toward the center.

3. The amphiaster

The large fusion nucleus occupies an excentric position in the hyaline central area of the aster (fig. 7). The appearance of being partially enveloped by the sphere liquid as by a cap is enhanced by the gradual increase in size of the nucleus. Very soon the ray-like structure of the cytoplasm begins to fade from view (fig. 8). The disappearance of the rays commences in what will be the equator of the dividing egg and gradually extends to the two poles. That the disappearance of the rays is due to a reversal of the gel to the sol state of the cytoplasm is shown by the fact that the needle on being dragged through the cytoplasm, now carries no distorted strands in its wake but pushes the granules about as if they were embedded in a liquid which was only slightly viscous. In the cases observed a few of the rays extending from the tips of the two poles in the plane of the future longitudinal axis of the cell never faded entirely from view. During this stage, which lasts a few seconds, the nucleus

acquires a squatty bipyramidal shape with its broad equator spreading quite across the sphere. The sphere then takes on the shape of two semilunar areas lying over the two poles of the nucleus (fig. 9). The rays soon blaze out again but this time around two centers, the two polar areas, producing a brilliant amphiaster (fig. 10). The rays extend almost to the periphery of the egg and with the needle it is possible to show that the cytoplasm is again a comparatively rigid gel.

As the astral formation extends into the equator of the amphiaster the gelled strands of the two asters become irregular and distorted in shape where strands from opposite directions meet (fig. 10). The gelation figure here is a meshwork of strands rather than linear strands such as form between the rays elsewhere. In the immediate vicinity of the nucleus, however, the strands form continuous lines and since many lie directly on the nucleus, give it a fibrous appearance. No fibers of any kind could be distinguished within the substance of the nuclear spindle.

Soon after the amphiaster has fully formed the bipyramidal nucleus lengthens into a long oval body (fig. 10). Its bulging equator gradually retracts until its sides are fairly parallel. At this stage in Toxopneustes (Wilson and Mathews, '95) Wilson describes a pause of some duration during which the astral radiations become very much reduced in extent. In Echinarachnius the asters and the cytoplasm remain in the gel state although the rays appear to be less brilliant than hitherto. After a space of time (about ten minutes) a change takes place in the cytoplasm about the equator of the nuclear body which appears to be a reversal to the sol state. I was not able to convince myself on this point by the use of the needle. The cytoplasmic granules, however, which were hitherto held in a gel, become visibly mobile in the equatorial region where the rays of the two asters meet. As a constriction in the equator of the nuclear body deepens and widens a distinct flow of the granules medianward can be observed, that is, into the region of the constriction (fig. 11). Following this a constriction in the equator of the cell takes place and one gains the impression that this is

consequent to a liquefaction of the cytoplasm in the equatorial plane while elsewhere it remains in the gel state. The liquefaction is accompanied by a loss in the ray-like structure in this region. During the remainder of cell division, a one-sided appearance obtains for each daughter aster, the rays being most prominent at the polar end and absent in the region corresponding to the equator of the mother cell (fig. 13). By the time cell-division is completed all of the cytoplasm has returned to the gel state, each cell containing a single aster, the nucleus lying to one side within the sphere. This brings us back to the same figure as in the undivided egg with a single aster (fig. 7). In the segmentation of the blastomeres the process as described is repeated.

The amphiaster may be twisted in much the same way as the single aster. Figure 12 represents a distortion of the amphiaster produced by dragging the needle across the equator of the cell and pulling the highly viscous nuclear 'spindle' along with it. On removing the needle, the asters may assume their original appearance. Where a distortion persists abnormalities in cell division occur.

4. The cell nucleus

Various suggestions have appeared in print regarding the relation between the formation of the fibers of the nuclear spindle and of the aster. Mention may therefore be made here of some of the results obtained in the microdissection of the nucleus during cell-division. A paper dealing with nuclear structure will be published shortly.

The spherical nucleus, which lies between the poles of the amphiaster, possesses a plainly visible nuclear membrane. This has already been described by Wilson ('95) in Arbacia. As the nuclear body becomes bipyramidal (in the metaphase) and elongates (in the ana- and telophases) the boundary between it and the surrounding cytoplasm fades from view. It preserves its identity, however, as a structure which is optically homogeneous except for the chromosomes which lie in its substance. Manipulation with the needle gives one the impression that it is

a comparatively rigid body. It is gelatinous and somewhat extensible. Figure 12 shows how it may be pulled about on the cytoplasm dragging the poles of the amphiaster with it. The extensibility of its substance is shown in figure 16. Figure 15 represents a maturation figure in the Cerebratulus egg. The astral figure at the end of the 'spindle' directed toward the surface of the egg adheres to the cell periphery. The other end lies free in the liquid cytoplasm. In figure 16, the spindle is shown pulled out and bent by a needle which has been inserted into one end. On being released the spindle may slowly return to its original shape.

Spindle fibers have not been detected as morphological structure until death coagulation sets in. May they not be interpreted as strands produced by the coagulation of a hyaline substance in which, previous to coagulation, there existed lines of stress or diffusion?

In the telophase the nuclear mass becomes dumb-bell shaped owing to a constriction around its middle. The constriction deepens and broadens as the two halves of the nuclear body draw away from one another and a strand connecting them does not always break through until the cell itself has completed its division.

5. The aster in maturation figures

This was studied in the Cerebratulus egg. The germinal vesicle breaks down twenty minutes to half an hour after the egg has been placed in sea water. Its place is taken by a clear area in the granular cytoplasm which flows gradually in one direction to the periphery of the egg spreading somewhat as it goes. In this columnar or rather funnel shaped area appears the first maturation spindle (fig. 14). As the spindle elongates hyaline plasma collects on its two poles around which the astral rays become more and more distinct while the cytoplasm between the rays passes into the gel state (fig. 15). The whole structure, viz., asters and spindle, can be pulled and pushed about causing the rays to form curves and spirals. If the distortion be not too great, the spindle will continue its normal development irrespective of spirally curved rays which may or

may not straighten out. The ray-like structure of the aster is difficult to distinguish in the hyaline cytoplasm at the peripheral pole as the refractive index of the hyaline protoplasm even in its gel state is close to that of the rays. The rays of the peripheral aster, however, very soon reach the periphery of the egg. As the cell periphery is probably a protoplasmic gel it and the peripheral aster form a continuous gel, firmly anchoring the spindle. With the needle one may stretch the spindle (fig. 16) but it cannot be dislodged without causing disorganization.

Division of the nuclear spindle is followed by a reversal of the astral structure to the sol state. The sol state of the cytoplasm lying immediately under the surface where the polar bodies are to form is so liquid that granules pushed into it exhibit active Brownian movement. On the surface at the middle of this liquid region the egg bulges forming a nipple-like protuberance. This occurs in such a way as to give one the impression that the protuberance is due to a local weakening in the consistency of the surface where an internal pressure causes cytoplasm to flow out. As one of the daughter nuclei of the first maturation figure lies directly under this spot, it is carried into the protuberance which pinches off to form the first polar body. By pricking with the needle one can so affect this surface as to cause it to produce in succession five and six or even ten protuberances, each being pinched off in its turn and very closely simulating polar bodies. The other daughter nucleus of the first maturation figure now lies free in the protoplasm (fig. 17) and can be pushed about anywhere in the egg. If it be moved out of the hyaline area into the granular cytoplasm and left there (fig. 18), the granules immediately surrounding it gradually move away until the nucleus comes to lie again in a hyaline area (fig. 19). Granules, in the meantime, invade the original hyaline area until it is indistinguishable from the rest of the granular cytoplasm. The nucleus now elongates and migrates again towards the periphery of the egg, astral rays reappear about its poles and, when the second maturation spindle is fully formed, one finds it again firmly attached to the surface of the egg. The second polar body is thus produced some distance away from the first one (fig. 20).

III. DISCUSSION

The pioneer observers of mitotic division, for example, Auerbach, O. Hertwig, Bütschli and Fol, described the accumulation of hyaline plasma at the astral centers and suggested that the astral radiations are a result of protoplasmic currents. Later investigators, such as, Rhumbler ('96, '99), Morgan ('00), Wilson ('01) and Conklin ('02), were convinced that centripetal currents do occur in the formation of the aster. Experimental evidence described in this paper confirms these views. It is necessary, however, to make a distinction between the general hyaloplasm and the substance constituting the astral rays and sphere. The formation of the aster consists in the gelation of the hyaloplasm which comes under the influence of the astral center. A hyaline liquid separates out during the gelation and flows in innumerable centripetal paths toward the center where it accumulates to form the sphere. This centripetal flow brings about an arrangement of the gelled hyaloplasm containing the cell-granules into radial strands separated by the hyaline liquid paths. This produces the astral figure. The strands of gelatinized cytoplasm merge peripherally into the surrounding liquid cytoplasm or reach and anchor themselves in the substance of the gelled egg surface when the aster is fully formed. The liquid rays merge centrally into the substance of the sphere, the liquid of the rays and of the sphere being thus identical.

The term ray could be applied to the gelled strands as well as to the liquid paths. However, the liquid paths are tributaries of the central sphere of the aster and it appears very likely that they condition the radiate appearance of the gelled cytoplasm. It seems appropriate, therefore, to limit the use of the specific term ray to the centripetal paths as I have done in this paper.

At a meeting of the American Society of Zoologists in December, 1916, before which this paper was read, Heilbrunn ('17)[8] presented results of investigations on cell division which make

[8] Heilbrunn's paper giving his results in detail will appear with this paper, or shortly after.

his conclusions and mine confirm one another rather strikingly. By the centrifuge method Heilbrunn found that after fertilization the cytoplasmic viscosity rises gradually until it reaches a maximum. It is at this time that the mitotic spindle first makes its appearance. The appearance of the spindle is followed by a gradual decrease in viscosity and the egg cytoplasm returns to its original liquid state. With the microdissection method I was able to locate the gelatinized region in the forming aster. It is significant that Heilbrunn's conclusion, arrived at by using the centrifuge and by noting the effects of reagents which increase or inhibit cell gelatinization, and mine, by using the microdissection needle, are identical in that one of the factors concerned in cell division is a cytoplasmic gelatinization.

The interior cytoplasm of a marine egg is a viscous fluid. The viscosity is high enough to prevent any Brownian movement of the enclosed granules but low enough to allow of their free movement when pushed about with the needle. The transition from the sol to a gel state is gradual. In the formation of the asters the gel state is rigid enough to hold the granules in a comparatively constant position since strands of the gel are dragged about with the needle. It is, however, much less rigid than solid gelatin which can be cut into discrete non-glutinous masses. It is also very easily reversible, for, in the early aster stage, a slight puncture and tear with the needle will cause it almost immediately to pass back into the sol state. The gel state here is never even visibly an inert solid for one is always conscious of a very gradual almost imperceptible movement and change. The picture of a gelled strand changes from minute to minute the difference being appreciable as time passes. Living protoplasm even in the gel state, is a dynamic structure never a static one.

IV. SUMMARY

1. The sphere is a liquid region free of granules, occupying the center of the aster and increasing steadily in size until the aster reaches full development.

2. The increase in size of the sphere is apparently due to the accumulation of liquid flowing into the sphere from all parts of

the cytoplasm. The aster rays appear to be the channels in which the centripetal flow occurs.

3. The cytoplasm between the rays is in the gel state to which the rigidity of the aster is due. The gel state is most pronounced near the sphere and peripherally passes gradually into the sol state of the cytoplasm lying beyond the confines of the aster. When the aster reaches the periphery of the cell the entire cell is rendered comparatively rigid.

4. In the maturation figures of the egg nucleus the peripheral aster forms a continuous gel with the surface layer of the egg to which the figure is thus firmly attached. The confines of the central aster pass insensibly into the surrounding liquid cytoplasm.

5. A periodic reversal of the sol to the gel state and vice versâ has been demonstrated in the cell protoplasm during division. The steps taken may be divided into the following series: (a) When the aster is fully formed the greater part of the cell is a gel. (b) The cytoplasm reverses to a sol state and the astral radiations fade out while the sphere liquid collects at the two poles of the nucleus. (c) The formation of radiations about the spheres at each pole of the nucleus producing the amphiaster is accompanied by a return to the gel state. (d) A return to the sol state takes place in the equator of the cell. The nuclear spindle now divides, a constriction around the middle of the cell then follows and continues until the cell is cut in two.

6. As a general rule, one may say that the reversal of the gel to the sol state starts in the equator of the cell and spreads to the poles. On the other hand, the reversal of the sol to the gel state commences about the sphere and spreads peripherad.

7. The gel state in living protoplasm is not inert. Even to the eye, there is always a constant but very gradual change among the granules embedded in the cytoplasmic gel.

8. There are appreciable differences in the liquid state of the cytoplasm in certain regions and at various times, e.g., the interior cytoplasm of the unfertilized and fertilized egg before the aster is formed is slightly viscous, whereas, the contents of the

sphere and of the rays, also the hyaline area in the vicinity of the forming polar body, are very fluid.

9. The aster in its early stage can be made to disappear by churning the cytoplasm with a needle. This causes a reversal of the gel to the sol state.

10. From a study of cell division in the eggs of Echinarachnius, Cerebratulus, Arbacia and Asterias, it may be concluded that one of the factors concerned lies in a peculiar colloidal property of protoplasm, viz., a periodic reversibility in its sol and gel states.

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PLATE I

EXPLANATION OF FIGURES

The figures are all sketches from living eggs. Figures 1 to 13 are of the Echinarachnius egg, figures 14 to 20 of Cerebratulus. Figures 1, 2, 10, 14, 15 and 17 to 20 purport to be more or less faithful representations. The remainder are diagrams in which the astral radiations are merely indicated by lines.

The tip of the needle when shown is represented by a small circle with a central spot.

1 Enlarged drawing of the tapering tips of the gelled cytoplasm (with embedded micro- and macrosomes) projecting between bases of the hyaline liquid rays into the periphery of the sphere.

2 The same as figure 1 but with the tips bent to one side by the microdissection needle.

3 Diagram to show the distortion of astral radiations by inserting the needle into the aster and moving the needle in a straight line.

4 Nucleus in a fully formed aster pushed to one side with a needle thus causing distortion and stretching of the astral radiations.

5 Early sperm aster spirally twisted in shape by moving it through the liquid cytoplasm with a needle.

6 Inserting the needle into the sperm nucleus and pulling draws the nucleus out into a ribbon-shaped mass showing that the nucleus is held in a gelatinous mass. On being released the nucleus reverts to its original spherical shape.

7 Fusion nucleus lying to one side in the sphere so that the sphere-liquid extends like a cap around one side of the nucleus.

8 Nuclear 'spindle' becoming bipramidal in shape as the sphere-liquid begins to collect at its two poles. Rays beginning to disappear around the equator.

9 Hyaline plasma collected at the nuclear poles to form two spheres. Beginning of the amphiaster.

10 Amphiaster in living egg of the Echinarachnius. The drawing shows only the central portion of the amphiaster. The clear area extending between the two asters represents the hyaline nuclear 'spindle.' The chromosomes have been omitted in the drawing. Scattered microsomes mark off the ends of the 'spindle' from the sphere.

11 Nuclear 'spindle' beginning to take on the form of a dumb-bell. The cytoplasmic granules in the equatorial region about the nuclear constriction become visibly mobile at this stage indicating an increase in the fluidity of that region.

12 The needle inserted into the nuclear 'spindle' has pulled it to one side in the cell dragging the astral radiations along with it.

13 Egg cleavage nearly completed. The nuclear body has separated into the two daughter nuclei. Rays are absent in the equatorial region which is probably in the sol state. The sphere-liquid extends over part of each daughter nucleus like a cap.

14 Hyaline area in Cerebratulus egg with the egg nucleus preparatory to the formation of the first maturation figure.

15 First maturation spindle with amphiaster.

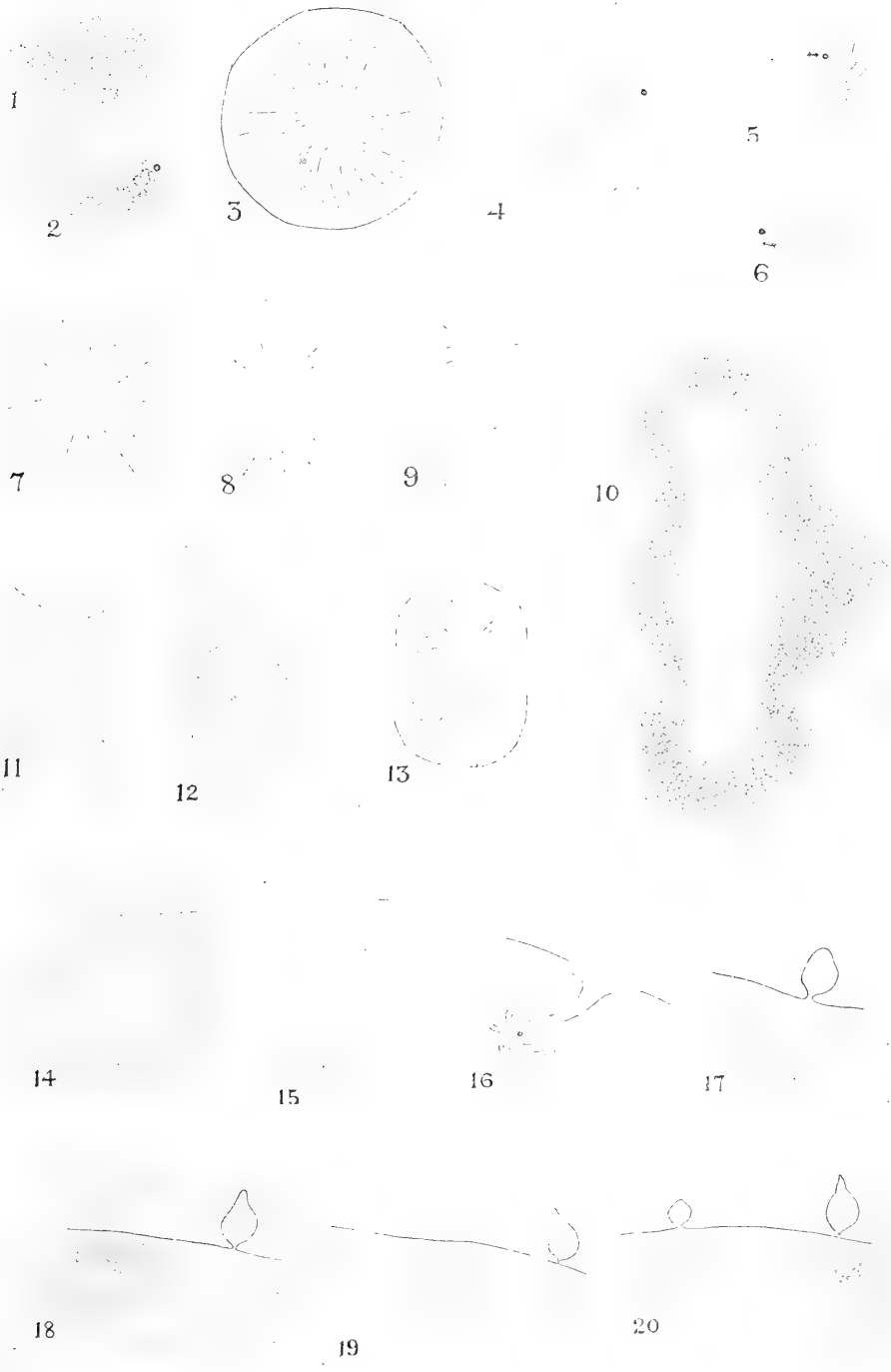
16 First maturation anaphase 'spindle' stretched by the needle in an attempt to pull it away from the egg periphery. The peripheral aster and the egg surface form a continuous gel thus anchoring the 'spindle.'

17 First polar body almost completely extruded. The egg nucleus lies in a clear area below the polar body.

18 Interkinetic egg nucleus pushed out of its normal position into the granular cytoplasm.

19 Granules in the cytoplasm receding from the dislocated egg nucleus so that it is soon surrounded by a clear area again. The area originally occupied by the nucleus is being invaded by granules so that in time it cannot be distinguished from the general cytoplasm.

20 Second polar body formed by the dislocated egg nucleus on the egg periphery some distance from the first.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THE RATE OF LOCOMOTION IN VANESSA ANTIOPA IN INTERMITTENT LIGHT AND IN CONTINUOUS LIGHT OF DIFFERENT ILLUMINATIONS, AND ITS BEARING ON THE "CONTINUOUS ACTION THEORY" OF ORIENTATION

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INTRODUCTION

Loeb in his interesting "continuous action theory" of orientation maintains that the rate of movement of the locomotor appendages on either side of a bilaterally symmetrical organism which orients depends directly upon the intensity of the stimulating agent as it reaches the receptors connected with the appendages. He maintains that if such an organism is not oriented the receptors on one side receive more energy from the source of stimulation than those on the opposite side, and that consequently the locomotor appendages connected with the receptors on this side move faster than those connected with the receptors on the opposite side. If this is true, the butterfly, Vanessa antiopa, which orients and is positive, should move faster in strong than it does in weak light.

To test this the rate of movement of 19 specimens of Vanessa antiopa was ascertained in different illuminations. Fourteen were tested in each of two lights, one nearly 500,000 times stronger than the other, and 5 were tested in each of two lights, one nearly 1000 times stronger than the other. They did not walk faster in the strong light than in the weak, as the "continuous action theory" demands. On the contrary they tended to walk faster under certain conditions in the weak than they did in the strong light.

METHODS

The experiments in which continuous illuminations differing in strength were used may be designated as experiments 1 and 2.

All the experiments were performed in a dark room. In experiment 1 the insects were tested alternately in light from a 36 c.p. automobile lamp and in direct sunlight reflected into the room by mirrors. In experiment 2 they were tested in light from the 36 c.p. auto lamp at different distances. A table 300 cm. long, covered with rough black cardboard, was divided into 20 equal spaces, each 15 cm. wide, by means of white threads stretched across the table and lying directly upon the cardboard. At one end of the table was placed a mirror upon which was thrown the desired beam of light, and from which emanated a horizontal beam, about 20 cm. wide, which extended the length of the table. Between the edge of the table and the mirror was placed a glass vessel with parallel sides, 3 cm. apart, filled with distilled water to eliminate heat effects as far as possible.

The wings of all of the butterflies used were clipped. The insects to be tested were placed in the beam at the far end of the table and facing the mirror. They soon started to proceed toward the light. As they reached each white thread the observer gave a signal which was noted by an assistant in another room. By the aid of a stop watch this helper kept an accurate record of the time taken by each insect to traverse the spaces between the threads. The animals rarely walked the whole 300 cm. without stopping or moving out of the beam. Whenever this occurred they were gently picked up by the stumps of the wings, returned to the beam, and placed behind the thread which they had last passed.

BEHAVIOR IN CONTINUOUS LIGHT OF DIFFERENT ILLUMINATIONS

In experiment 1, 14 butterflies were tested. Each was allowed to walk the whole distance of 300 cm. four times in succession, twice successively in direct sunlight and twice in suc-

cession in artificial light. In the case of 7 of the animals the tests in sunlight preceded those in weak light, while in the case of the remaining 7 animals the reverse was true. The beam of sunlight throughout the whole length of the table was approximately 5000 m.c. The beam of artificial light was 10.07 m.c. at one end of the table and 0.01 m.c. at the other. In table 1 are presented the results obtained. These results show that 10, or over 71 per cent of the insects moved faster in the weaker light, and that only 4, or nearly 29 per cent, moved faster in the stronger light. It shows also that the average rate of movement per second of all 14 butterflies in direct sunlight was only 2.831 cm., while in weak light it was 3.334 cm. This indicates that Vanessa antiopa usually moves faster in weak light than in strong light, if the difference in illumination is great.

TABLE 1

Rate of locomotion of Vanessa antiopa in two luminous intensities, one from nearly 500,000 to nearly 500 times the other at the two ends of a 300 cm. course

DESIGNATION OF BUTTERFLIES	TIME IN SECONDS TAKEN TO TRAVERSE A 300 CM. COURSE. ILLUMINATION, 5000 M.C. (APPROXIMATELY)		TIME IN SECONDS TAKEN TO TRAVERSE A 300 CM. COURSE. ILLUMINATION, RANGING FROM 10.07 M.C. TO 0.010 M.C. AT THE TWO ENDS OF THIS COURSE		AVERAGE RATE OF MOVEMENT IN CENTIMETERS PER SECOND IN ILLUMINATION, 5000 M.C.	AVERAGE RATE OF MOVEMENT IN CENTIMETERS PER SECOND IN ILLUMINATION, 10.07 TO 0.84 M.C.
	First trial	Second trial	Third trial	Fourth trial		
18-D	89	104	103	122	3.108	2.666
18-B	96	129	52	56	2.666	5.555
19-C	77	76	105	73	3.921	3.370
19-A	77	102	99	97	3.351	3.062
17-F	98	147	208	202	2.448	1.463
17-D	92	75	63	89	3.592	3.947
18-F	151	196	143	149	1.729	2.054
	Third trial	Fourth trial	First trial	Second trial		
18-G	110	129	154	104	2.325	2.510
18-C	80	94	115	144	2.316	3.448
18-A	58	65	53	97	4.000	4.878
17-E	113	139	190	166	1.685	2.380
18-E	69	74	120	126	2.439	4.195
17-G	52	60	69	65	4.477	5.357
19-B	164	171	146	233	1.583	1.791
Average rate of all fourteen butterflies.....					2.831	3.334

In experiment 2 five insects were tested in two illuminations. In one illumination the intensity in the beam varied from 0.4 m.c. at one end of the table to 1.56 m.c. at the other. In the other it varied from 1.56 m.c. to 1500 m.c. The results obtained are presented in table 2. These results show that the average rate of movement of the 5 butterflies in each of these two lights was the same, being 2.16 cm. per second. They therefore moved at about the same rate in both illuminations, one nearly 1000 times stronger than the other. This indicates that the rate of locomotion in Vanessa is within rather wide limits independent of the luminous intensity.

TABLE 2

Rate of locomotion of Vanessa antiopa in two illuminations, one from nearly 1000 to nearly 4 times the other at the two ends of a 300 cm. course

DESIGNATION OF BUTTERFLIES	AVERAGE RATE OF MOVEMENT IN CENTIMETERS PER SECOND IN TRAVERSING A 300 CM. COURSE		NUMBER OF TRIALS FROM WHICH THE AVERAGE WAS COMPUTED
	Illumination at the two ends of the course, 1.56 m.c. and 0.40 m.c.	Illumination at the two ends of the course, 1500 m.c. and 1.56 m.c.	
7-H	3.74	3.55	4
7-C	2.05	2.32	2
7-E	1.38	1.17	2
7-D	2.06	2.04	1
7-I	1.60	1.72	2
Average rate of all 5 insects.....	2.16	2.16	

The conclusion stated previously, that Vanessa usually moves faster in weak light than in strong light if the illuminations differ by a certain amount, is supported by the results obtained in the individual trials. The butterflies, as they moved toward the source of light, were being exposed to a gradually increasing luminous intensity. They should therefore, according to the "continuous action theory," gradually increase their rate of locomotion as they approached the source. That this was not the case is shown in table 3 in which is given the average rate of movement of each insect in each of the illuminations used in experiments 1 and 2 in the first 75 cm. (nearest the source), the middle 75 cm., and the last 75 cm., of the 300 cm. course.

TABLE 3

Rate of locomotion of Vanessa antiopa in three different portions of a 300 cm. course leading to a source of illumination

DESIGNATION OF BUTTERFLIES	AVERAGE RATE OF MOVEMENT PER SECOND IN CENTIMETERS						NUMBER OF TRIALS FROM WHICH THE AVERAGE WAS COMPUTED
	In first 75 cm. illumination, about 5000 m.c.	In middle 75 cm. illumination, about 5000 m.c.	In last 75 cm. illumination, about 5000 m.c.	In first 75 cm. illumination, 10.07 m.c. to 0.13 m.c.	In middle 75 cm. illumination, 0.076 m.c. to 0.027 m.c.	In last 75 cm. illumination, 0.019 m.c. to 0.010 m.c.	
18-D	3.84	3.06	2.58	2.30	3.12	2.58	2
18-B	3.00	2.50	2.20	5.76	5.76	5.55	2
19-C	3.48	4.05	4.05	2.72	3.94	4.16	2
19-A	3.12	3.41	3.75	3.00	3.12	2.94	2
17-F	2.58	2.42	2.38	1.32	1.38	1.63	2
17-D	3.48	3.48	4.28	4.05	4.41	3.48	2
18-F	2.17	2.08	1.48	1.92	2.02	2.45	2
18-G	2.17	2.23	2.11	2.02	3.06	2.77	2
18-C	3.12	2.30	1.70	3.40	3.57	3.33	2
18-A	5.17	4.68	3.19	5.17	5.35	4.68	2
17-E	1.72	1.68	1.64	2.54	2.60	2.17	2
18-E	2.60	3.00	2.20	5.35	4.28	4.16	2
17-G	4.05	4.16	5.55	5.17	5.55	5.35	2
19-B	1.41	1.56	1.48	1.74	2.05	1.61	2
Average rate of above 14 insects...	2.993	2.900	2.756	3.318	3.586	3.347	
Illumination	1.56 m.c. / 0.11 m.c.	0.87 m.c. / 0.624 m.c.	0.52 m.c. / 0.40 m.c.	1500 m.c. / 20.76 m.c.	11.34 m.c. / 4.15 m.c.	2.96 m.c. / 1.56 m.c.	
7-H	3.919	3.544	3.347	4.225	3.75	2.857	4
7-C	2.272	1.666	2.272	2.380	2.727	1.724	2
7-E	2.586	3.125	2.727	2.238	2.142	1.363	2
7-D	1.785	2.083	2.272	2.777	1.923	1.744	1
7-I	3.333	3.000	2.631	4.245	3.488	2.830	2
Average rate of above 5 insects...	2.779	2.683	2.649	3.173	2.806	2.103	

This table shows that, of the 14 insects tested in the light ranging from 10.07 m.c. at one end of the table to 0.010 m.c. at the other, 6 moved less rapidly in the first 75 cm. than in the

last 75 cm. and 12 also moved slower in the first part of the course than in the middle of it. Moreover, the average rates in the three portions of the course of the five animals tested in the light ranging from 1.56 m.c. to 0.40 m.c. were, respectively, 2.779, 2.683, and 2.649 cm. per second, while the average rates in the stronger light were, respectively, 3.173, 2.806, and 2.103 cm. per second. These results show that the butterflies tend to move slightly slower as they come nearer the light, i.e., as the illumination increases.

Mast ('11, p. 186) obtained similar results in experiments on the relation between the rate of movement and intensity of light. He showed that fly larvae which crawled at the rate of 0.321 cm. per second in a luminous intensity of 7 m.c. crawled at a rate of 0.345 cm. per second in an illumination of 3888 m.c. Thus, they moved only 0.024 cm. per second faster in the very strong light than they did in the weak light. It is evident that all of these results militate strongly against the "continuous action theory" of orientation.

Table 2 also shows another interesting fact, namely, that after the butterflies have traversed a few centimeters they tend to move at a somewhat faster rate. Out of the 14 insects tested in both sunlight and in weak light, 10 moved faster in sunlight in the middle 75 cm. than in the last 75 cm., while in weak light 11 butterflies moved faster in the middle of the course than in the last part of it. This phenomenon is very probably analogous to the phenomenon of 'treppe' seen in stimulated isolated muscles and in increased efficiency in athletes due to 'warming up.'

BEHAVIOR IN INTERMITTENT LIGHT

The results obtained in the experiments now to be described present some evidence in favor of the theory that orientation depends upon the time rate of change of intensity. This evidence consists in the fact that Vanessa seems to move faster when exposed to intermittent light of a certain frequency of interruption than when exposed to continuous light.

The method used in ascertaining this was as follows. A circular disc of black cardboard fastened to a wheel and turned

by a small motor was placed vertically at one end of the table described above. The motor rested upon a thick pad of woolen cloth to prevent vibration as far as possible. Sectors of various sizes were cut out of the disc. A 36 c.p. automobile lamp was used as a source of light. It was placed in a black box in one end of which was an adjustable slit. This source of light was placed behind the disc. From the lamp emanated a horizontal beam which extended the length of the table. By means of a rheostat inserted in the circuit the rapidity of the rotation of the disc could be regulated, and so intermittent light of the frequency of interruption desired could be secured. By moving the disc to one side a beam of continuous light was obtained. The table was marked off into sections as in the previous experiment. The rate of movement of the insects was measured as before. Three different experiments were performed which may be designated as experiments 3, 4, and 5.

TABLE 4

Rate of locomotion of Vanessa antiopa in intermittent light of a frequency of interruption of 10 per second, the periods of illumination and non-illumination being equal

DESIGNATION OF BUTTERFLIES	AVERAGE RATE OF MOVEMENT IN CENTIMETERS PER SECOND IN TRAVERSING A 300 CM. COURSE			NUMBER OF TRIALS FROM WHICH THE AVERAGE WAS COMPUTED
	In continuous light, the illumination at the two ends of the course being 1500 m.c. and 1.56 m.c.	In intermittent light, the frequency of interruption being 10 per second. The illuminations at the two ends of the course were 750 m.c. and 0.7 m.c.	In continuous light, the motor being allowed to run (see text)	
7-A	2.531	2.898	2.448	2
7-B	3.10	2.87	2.66	2
7-C	1.948	2.479	2.439	2
8-H	2.479	2.702	2.4	2
8-G	2.214	1.739	2.489	2
8-D	3.26	3.26	3.33	2
8-C	3.658	3.896	3.592	2
8-F	3.076	2.870	2.620	2
8-A	2.702	3.37	2.510	2
8-E	1.744	1.764	1.666	2
Average rate of all 10 insects	2.6712	2.7848	2.6154	

In experiment 3 the rate of locomotion of Vanessa in continuous light and in intermittent light of a flash frequency of 10 per second was ascertained. One-half of the disc previously described was removed. An insect was first given one trial in continuous light, that is, it was allowed to walk over the 300 cm. course toward the source of light and its rate of movement was ascertained. It was then given a trial in intermittent light. A third trial followed in continuous light. During this trial the motor was allowed to run. This third test was given to determine the effects, if any, of the 'hum' of motor. This series of three trials was then repeated a second time, thus giving two trials for each insect under each of the three different conditions. The illumination in the trials in continuous light ranged from 1500 m.c. at one end of the table to 1.56 m.c. at

TABLE 5

Rate of locomotion of Vanessa antiopa in intermittent light of a frequency of interruption of 16 per second. The period of illumination is 3 times as long as the period of non-illumination

DESIGNATION OF BUTTERFLIES	AVERAGE RATE OF MOVEMENT IN CENTIMETERS PER SECOND IN TRAVERSING A 300 CM. COURSE			NUMBER OF TRIALS FROM WHICH THE AVERAGE WAS COMPUTED
	In continuous light, the illumination ranging from 1500 to 1.56 m.c. at the two ends of the course	In intermittent light the frequency of interruption of which was 16 per second. The illumination at the two ends of the course, 500 m.c. and 0.52 m.c.	In continuous light, the motor being allowed to run (see text)	
6-E	1.61	1.78	1.59	2
6-D	3.84	3.72	4.13	2
6-C	3.37	3.29	3.04	2
6-B	2.67	4.61	4.08	2
6-A	1.88	3.94	2.58	2
5-A	1.85	2.52	2.07	2
5-B	3.75	4.28	3.80	2
5-C	1.84	2.13	1.64	2
5-D	3.17	3.06	2.87	2
5-E	1.74	1.73	1.50	2
Average rate of all 10 butterflies.....	2.572	3.106	2.73	

the other end, and the illumination in the trials in intermittent light was one-half of this.

In experiment 4 the rate of locomotion in continuous light and in intermittent light of a flash frequency of 16 per second was ascertained. The mode of procedure was exactly that in experiment 3 except that one-fourth of the disc was removed during the trials in intermittent light. The illumination in the trials in continuous light ranged from 1500 m.c. to 1.56 m.c. at the two ends of the table. The illumination in the trials in intermittent light was one-third of this.

The object of experiment 5 was to ascertain the rate of movement of Vanessa in intermittent light in which the frequency of interruption was very high and very low. One-half of the disc was removed. An insect was first given one trial in continuous light, then one in intermittent light with a flash frequency of 22 per second, then one in intermittent light with a flash frequency of 1 per second, and finally one in continuous light with the motor running. This series of four trials was repeated a second time, giving two trials for each insect under each of the four different conditions. The illuminations used were the same as those used in experiment 3.

The results obtained in these three experiments are presented in tables 4, 5, and 6.

As is shown in table 4, intermittent light of a flash frequency of 10 per second seems to stimulate Vanessa, since 6 of the 10 insects tested moved faster in intermittent light of this frequency than they did in continuous light. Moreover, the average rates of movement of all 10 insects in continuous light and in intermittent light were 2.67 cm. and 2.78 cm. per second, respectively. That vibration from the motor did not produce this result is proved by the fact that 83 per cent of the insects which walked faster in intermittent light moved slower in continuous light when the motor was allowed to run than they did in continuous light when the motor was not running. The running of the motor therefore seems to retard movement.

That Vanessa also tends to move faster in intermittent light of a flash frequency of 16 per second than in continuous light is

TABLE 6

Rate of locomotion of Vanessa antiopa in intermittent lights, the frequencies of interruption of which were 22 and 1 per second. The periods of illumination and non-illumination were equal

DESIGNATION OF BUTTERFLIES	AVERAGE RATE OF MOVEMENT IN CENTIMETERS PER SECOND IN TRAVERSING A 300 CM. COURSE				NUMBER OF TRIALS FROM WHICH THE AVERAGE WAS COMPUTED
	In continuous light, the illuminations at the two ends of the course being 1500 m.c. and 1.56 m.c.	In intermittent light, the frequency of interruption of which was 22 per second. The illuminations at the two ends of the course, 750 m.c. and 0.78 m.c.	In intermittent light. Frequency of interruption, 1 per second. Illuminations at the two ends of the course, 750 m.c. and 0.78 m.c.	In continuous light, the motor being allowed to run (see text)	
12-A	4	3.75	3.84	4.05	2
12-B	5.30	4.19	5.17	4.37	2
16-A	4.65	4.02	4.19	4.31	2
16-B	3.14	2.72	2.31	2.69	2
9-C	1.75	2.11	1.96	2.07	2
11-A	3.77	3.79	3.01	3.55	2
14-B	4.47	3.48	3.31	3.77	2
14-A	3.61	3.82	2.66	3.06	2
Average rate of all 8 insects...	3.83	3.48	3.30	3.48	

shown by the results of experiment 4 which are given in table 5. From this table it is evident that 60 per cent of the 10 insects tested moved faster in intermittent light of this frequency than they did in continuous light. Moreover, the average rate of movement of all 10 insects in intermittent light was greater than the average rate in continuous light, being 3.106 cm. per second in the former and only 2.572 cm. per second in the latter. That vibration from the motor did not produce this result is shown by the facts, that the average rate of movement of all 10 insects was 0.376 cm. per second faster in intermittent light than it was in continuous light when the motor was allowed to run, that one half of the insects moved faster and one half moved slower in the continuous light while the motor was running than they did in continuous light when the motor was not running, and that only one insect moved as fast in the continuous light while the motor was running as it did in the intermittent light.

When however the flash frequency of intermittent light is very rapid the effects of continuous light and intermittent light upon the movement of Vanessa seem to be the same, while if the frequency of interruption is very slow the butterflies seem to move at a slower rate than they do in continuous light. This conclusion is drawn from the data presented in table 6. As is shown in this table, the average rate of all of the 8 insects tested was exactly the same in intermittent light the frequency of interruption of which was 22 per second as it was in continuous light when the motor was running, thus showing that the interruption of the light had no effect on the rate of movement. This table shows also that 7 out of the 8 butterflies moved more slowly in intermittent light of a flash frequency of 1 per second than they did in continuous light. That vibration from the motor did not produce these results is indicated by the fact that while 6 out of the 8 insects moved more slowly in continuous light while the motor ran than they did in continuous light while the motor was not running, 7 moved slower in intermittent light of low flash frequency than they did in continuous light while the motor was running.

That this increased rate of movement in intermittent light of a certain frequency of interruption is not due to the fact that the illumination was greater in the tests made in continuous light than it was in those made in intermittent light is shown by the fact that Vanessa tends to move at about the same rate in continuous lights of different illuminations unless the difference is much greater than was that between the continuous illumination and the intermittent illumination. Even in illuminations differing by nearly 100,000 per cent this butterfly tends to move at about the same rate, as is shown in table 2. Yet in none of the tests in intermittent light did the illuminations differ by more than 300 per cent.

Consequently, the three experiments described in the preceding paragraphs seem to show that Vanessa tends to move at a faster rate in intermittent light of a frequency of interruption of 10 and 16 per second than it does in continuous light, while it tends to move at a slower rate in intermittent light of a flash frequency

of 1 per second than it does in continuous light. That intermittent light of a certain frequency of interruption acts as a stimulus upon Vanessa indicates that orientation in light in this butterfly may be due to the time rate of change of intensity.

I wish to express my very sincere appreciation of the kind interest, numerous suggestions, and unfailing aid of Prof. S. O. Mast in this work. It is a pleasure also to acknowledge my indebtedness to Dr. H. E. Howe for the loan of apparatus and to my brother, S. B. Dolley, and my student, W. A. Wightman, for valuable assistance in conducting the experiments.

SUMMARY

Vanessa antiopa does not move faster in strong light than in weak but on the contrary tends to move faster in weak light than in strong, if the difference between these illuminations is sufficiently great. This behavior is not in accord with that which is demanded by Loeb's "continuous action theory" of orientation. The results in general support those described in an earlier paper which indicated that orientation of Vanessa in light can not be accounted for on the basis of Loeb's theory.

Vanessa moves faster in intermittent light of a frequency of interruption of 10 and 16 per second than it does in continuous light. This supports the contention that orientation in light in the mourning-cloak butterfly is due to the time rate of change of intensity.

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EFFECTS OF LIGHT AND DARKNESS ON THE EYE OF PRORHYNCHUS APPLANATUS KENNEL

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THREE TEXT FIGURES AND ONE PLATE

The eye of Prorhynchus applanatus Kennel consists of but two cells. One of these cells is the pigment cell and the other is the visual cell or retinula (figs. 1 and 2). The earliest investigators in this field recognized that a pigment-cup was associated with the visual elements of turbellarian eyes. In 1864 Leydig described in part the contents of this cup; but it was not until Hesse's ('97) work that we get a satisfactory account of the structure of the visual cells and pigment-cup and the relation between them. In one respect the work of Kepner and Taliaferro ('16) has carried the knowledge of the cytological details of the eye of this rhabdocoele beyond Hesse's observations, as will be later indicated.

The material and methods of fixation of the specimens for this work were on the whole similar to those used by Kepner and Taliaferro. The specimens have been collected by bringing into the laboratory pads of Vaucheria and other plant masses from the face of a dam over which water continually flows. These masses of algae and higher plants were placed in large glass vessels in tap water. The animals within forty-eight hours made their way to the surface in such aquaria where they were readily collected and transferred to embryological watch glasses that contained spring water. One part of each collection was placed during the day (from 9.00 a.m. until 5.00 p.m.) in the northern light of the laboratory from time to time during the months of April and May. At 5.00 p.m. each day a 40 Watt, 110 volt, Fostoria Mazda light lamp was turned on as it hung with its glass wall 21 cm. above the surface of the water

of the watch glass. No effort was made to eliminate the heat factor. In our preliminary experiments it was found necessary to isolate the ones that were to be subjected to light; for they showed a great tendency to hide beneath one another, if they were not separated. It was also found that they were killed by exposure to the open northern sky. The specimens were, therefore, placed ten feet away from a window in the laboratory. The second lot of animals of each collection was placed in a vessel of spring water and carried into a dark room. After forty-eight hours each series was fixed for two to five minutes in chrome-aceto-formalin. A given lot of fixing fluid was divided and one-half of it was used on the animals of the light series and the other portion of the fixing fluid was used for fixing the animals that had been kept in the dark. Of course the fixing of each series was done in the light or the dark as determined by the condition under which the members of the series had been kept. After the fixing all other steps were carried on in the light. The specimens from the fixing fluid were washed quickly in four changes of tap water and quickly carried through the alcohols, xylol and paraffin; so that the animals were in a paraffin block within one half hour after they had been fixed. The sections were stained in iron hematoxylin in a few cases, but Mallory's connective tissue stain was for the most part employed.

The relative position of the visual element and accessory cell in material so handled can readily be made out when immersion lenses are employed. It can thus be seen that, as Hesse has observed, the rhabdomes of the visual cells were not directed towards the light as it entered the eye, but away from the light and towards the cells of the pigment cup, the light passing through a great part of the visual cell before encountering the visual rod or rhabdome. It is interesting to note that this inverted position of the retinal elements is encountered in vertebrates, some molluscs and turbellaria. In these three groups we must, therefore, have eyes that represent either (a), a suppression of this type of eye through many intervening phyla to appear atavistically in these three groups; or (b), a retention of this type in certain mollusca and all vertebrates

from their direct ancestral stock, thus making the inference that these forms arose from a much lower level on the phylogentic tree than is usually suggested—lower even than Hubrecht's nemertean ancestry for the vertebrates; or (c), we must have in these three groups three cases of parallel evolution. We have suggested these alternatives by way of indicating the fields of general interest into which this study has led us, without committing ourselves to any of them. The analogy however, between these eyes and those of vertebrates is so striking that we have become interested in trying to find an analogy of function between the visual elements of the vertebrate and Prorhynchus.

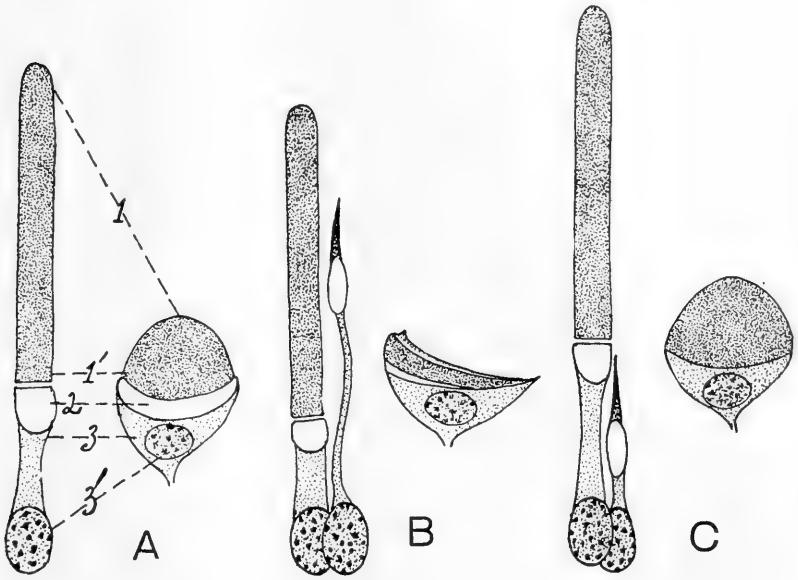
The pigment cells of vertebrates are influenced by light and its absence. "In general it may be said that light induces an expansion and the absence of light a contraction, of pigment cells;" Arey ('16 b, p. 360).

The accessory cell or pigment cell associated with the visual cell of Prorhynchus is closely applied to the rhabdome or receptor of the retinula (fig. 1). In eyes that have been subjected to alternation of day and night conditions, when the specimens are free to seek and find optimum conditions of illumination, this cell in each eye is found to have a uniformly stratified cytoplasm. Little change is to be observed in these cytoplasmic strata when the eyes have been illuminated continuously for forty-eight hours or longer. Figures 10, 11, 12 show the closely crowded strata of cytoplasm in the pigment cells that have been continuously illuminated for two nights and two days or longer. It is to be seen here that, if anything, these eyes show a contraction of the pigment cells. We have met with one apparent exception to this type of reaction on the part of an illuminated pigment cell. This exception is shown in figure 9. Here the cytoplasmic strata are less crowded and lie less regularly about the contour of the rhabdome. Examination shows that this is the right eye of the animal and that the left eye has a typical light adapted pigment cell. It is suggested by this that the right eye might have been shaded in some manner. When we first undertook to expose animals to the continued illumination, it was found that the specimens would frequently disgorge some

of the contents of the enteron and then bring its anterior end to lie beneath this ejected food mass. Care was taken, then, to remove such material whenever it was present, but it is possible that a small piece of shading material might have escaped our attention in this particular specimen. Unless in this or some other manner this exceptional eye had been shaded we have no way to explain its departure from the type of a light adapted eye. This one exceptional case, however, constitutes a low coefficient of variation when we consider that twenty animals or forty eyes of this series of light adapted eyes were studied.

On the other hand, all dark exposed pigment cells have shown that the lamellae of their cytoplasm move apart with the result that the cell itself becomes greatly expanded (figs. 2 to 8). The section adjacent to and belonging to the section shown in fig. 1 also shows a greatly expanded pigment cell. The reaction of the pigment cell of the eye of Prorhynchus, therefore, stands in sharp contrast to that of the eye of a vertebrate, in that light causes a contraction and dark an expansion of it, while the reverse is the reaction of the pigment cell of a vertebrate eye. The response of the pigment cell of Prorhyncus's eye to light is similar to that of the photoreceptor's pigment cell of Amphioxus, as Crozier ('17) describes.

In the retinula or visual cells of some vertebrates the following cytoplasmic details have been observed: (1), an inner segment, the myoid, with which the nucleus is directly associated; (2), a middle region, designated the ellipsoid, which is to be found in the eye of fish, amphibia, reptiles and birds; and (3), the outer segment, the visual rod or rhabdome (text-fig 1). Kepner and Taliaferro ('16) found three cytoplasmic regions in the retinula of Prorhynchus; (1) the inner one most closely associated with the nucleus; (2) the middle segment, a lens-shaped region, that in a freshly dissected eye was highly refractive, thus strongly resembling the ellipsoid of a vertebrate visual element; and (3) an outer segment, the rhabdome. These authors recognized, at the time their paper had appeared, that they had carried the knowledge of the histological details of a turbellarian eye a step beyond where Hesse ('97) had taken it. However, it was not



Text-fig. 1 A. Shows a retinula of the frog (left) and the retinula of Prorhynchus (right). Three cytoplasmic regions are to be seen. Lines *1* and *1'* indicate the receptors or rhabdomes; line *2* connects the ellipsoid of a frog with its analogue in Prorhynchus; lines *3* and *3'* connect the myoid of a frog's retinula with its analogue in the retinula of Prorhynchus.

B. Two dark adapted retinulæ of frog (left). Observe elongated myoid of cone and contracted myoid of rod and no change in other cytoplasmic segments. One dark adapted retinula of Prorhynchus (right). Anterior end to right. Note the relative small size of the middle cytoplasmic segment and the low, trough-like rhabdome.

C. Two light adapted retinulæ of frog (left). Observe contracted myoid of cone and elongated myoid of rod; no change in other segments of the cytoplasm of these cells. One light adapted eye of Prorhynchus (right). Observe absence of middle segment of cytoplasm and the high, rounded rhabdome. The drawings of rods and cones of frog taken from Arey ('16b).

until the appearance of Arey's ('16) work that the authors of this paper realized that the analogy, if not the homology, between a visual element of a vertebrate and that of Prorhynchus was so evident. Text figure 1 was made at the suggestion of Mr. W. H. Taliaferro.

This structural analogy suggested the problem of learning whether or not there was to be found a functional analogy.

The functional changes of visual cells in certain vertebrates have recently been well described and the literature of the subject reviewed by Detwiler ('16) and Arey ('16).

Englemann (85, p. 500) found that in the eye of the snake Tropidonotus matrix, which contains no rods in the retina, the cones contracted but little; also that in Testudo graeca it is doubtful whether any contraction takes place. Angelucci ('94) however, claims "that in Testudo marina contraction of the cones does take place, though less in extent than in the frog. . . ." Chiarni ('06) also reports that in the eye of L. agilis the cones shorten when the eye is brightly illuminated, but only slightly, for the cones measure in dark eyes 25 to 35μ, in light eyes 23 to 30μ. Finally Garten ('07) found also a very slight contraction (not more than 1.2μ) in the eye of Chamelion. (Detwiler (16) p. 166–7.)

In speaking of his own work, Detwiler says:

Light causes a migration of the pigment and a contraction of the cones in both the tortoise and lizard retina, the extent of migration in the tortoise averaging 3.6μ, and in the lizard 3.1μ. The extent of the contraction of the cones in the tortoise averages 2.3μ (p. 186). "To the contractile portion of the cone's inner member Englemann applied the significant term 'myoid'. . . . The contractility of the myoid is extraordinary, since in some fishes light produces a shortening of this part to 10% of the length which it assumes in darkness. If effective at all, light always causes a shortening and darkness an elongation of the cone cell" while the "myoid of the rod-visual cell elongates in light and shortens in darkness" in all investigated vertebrates.

Arey ('16-b), p. 441. The latest observations, therefore, indicate that a shortening or an elongation of the retinulae takes place by means of a modification of the myoid. The change in the size and shape of the rhabdome has nothing to do with the total length of the visual cell. No change in the ellipsoid of the visual cell of the vertebrate has been recorded.

The functional changes to light as judged from our study of the fixed condition in the eye of Prorhynchus, stand in sharp contrast to the changes in the retinulae of vertebrates. The reaction of the visual cells to light and darkness is more pronounced than that of the pigment cells of the eyes.

No nuclear change has been observed in the retinula of Prorhynchus by us. The change in the cytoplasmic segment, that is

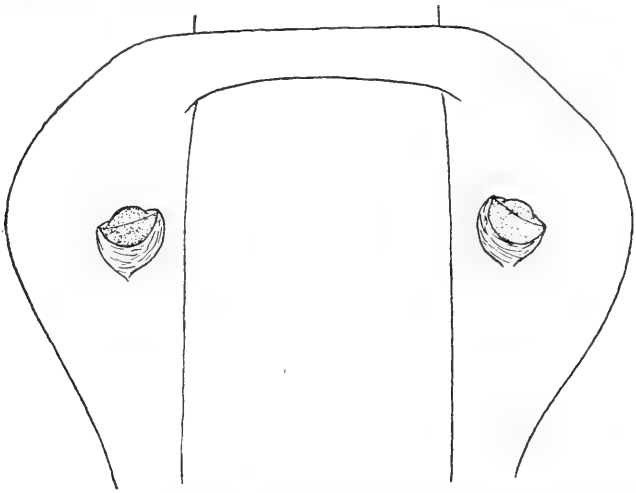
associated with the nucleus and hence the analogue of the vertebrate visual cell, seems to be passive and conforms to that of the other segments of the cytoplasm. Continuous illumination causes the middle refractive segment, which is analogous to the ellipsoid of the vertebrate eye, to disappear in the fixed material. No trace of it is to be seen in the eyes shown in figures 11 and 12. The striation to be seen at the base of the rhabdome in figure 10 may represent the vestiges of it.

Figure 8 shows the most conspicuously dark adapted eye that we have found. Here there is a trace of the middle, cytoplasmic segment to be seen. This middle region of cytoplasm is depicted as the lightest region of the rhabdome in figure 8. It is to be observed in this frontal section that the middle region is thickest at its posterior end; the relative thickness of the middle segment is also shown in the figures 6 and 7, which too are frontal sections. Figures 2, 3, and 4 show transverse sections taken at the level of the posterior end of this middle segment of the retinula or visual cell. In figure 1 we have a section shown that is exceptional. This section was only somewhat transverse. The adjacent section of this eye does not show the middle segment so conspicuously. This particular eye is exceptional as a dark adapted one in that it has shown no departure in its middle segment from that of an eye that has been subjected tonight and day alternation of light and dark. The rhabdome in this section also departs little from that of an illuminated eye; the adjacent section, however, gives us a typically dark adapted rhabdome.

The reactions of the rhabdome to light on the one hand and darkness on the other, as seen in the fixed condition, are the most conspicuous changes of all those observed in this study. In eyes that have been subjected to day and night alternation in laboratory light the rhabdome has a low cone-shaped contour both in transverse and longitudinal sections (Kepner and Taliaferro, '16). The rhabdomes of eyes that have been illuminated continuously for forty-eight hours have relatively high axes and relatively small diameters. The free margin of such rhabdome is uniformly curved and directed laterally (figs. 10, 11, 12). The

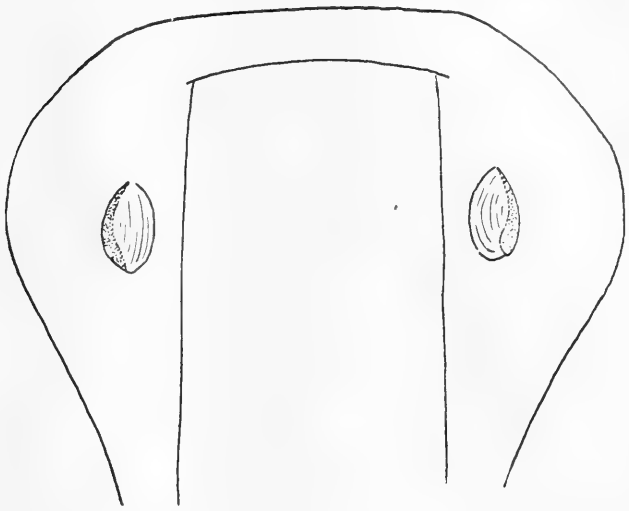
frontal section of a dark adapted eye shows a rhabdome that is relatively low and greatly elongated;—the posterior end being the thickest (figs. 5, 6, 7, 8 and 9). Transverse sections show that these rhabdomes are concave (figs. 2, 3, and 4). Thus we have the visual rod of this eye becoming low and elongating in the dark and becoming high and rounding up in the light.

Pigment cell and visual cell, therefore, of Prorhynchus in the dark seem to make an effort to locate light by increasing their receptive surfaces.



Text-fig. 2 Outline drawing of dorsal ganglia and eyes of light adapted eyes. Note the rounded contour of eyes and their forwardly directed axes. Taken from living specimen.

In addition to this reaction of the part of the elements of the eyes, the eyes themselves change their axes with reference to that of the body as a response to light and darkness. A living specimen under strong illumination has the axis of its eye anteriorily directed at an angle of about 45 degrees from the axis of the body; while the eye of a dark adapted animal has its axis laterally directed at right angles to the axis of the body of the animal (text-figs. 2 and 3). The dark adapted eye shifts its position as if in this way also light were being sought.



Text-fig. 3 Reconstruction of dorsal ganglia and eyes of dark adapted eyes. Note the elongation of eyes and their laterally directed axes.

CONCLUSIONS

1. Stimulation by light results in a contraction of the accessory cell or pigment cell.

In sustained darkness the cytoplasmic lamellae of the pigment cell open up or move apart, resulting in the expansion of the cell.

2. The three cytoplasmic regions of the retinula or visual cell show more or less marked changes in response to light and darkness. The nucleus-bearing part of the visual cells somewhat widened in the dark. The refractive, middle segment (analogous to an ellipsoid of a vertebrate retinula) disappears in continuous illumination and is most conspicuous in eyes that have been subjected to optimum illumination. The rhabdome in light adapted eyes is a rounded cone-shaped body, while in dark adapted material it is an elongated trough-shaped structure with its long dimension directed parallel to the axis of the body of the animal.

3. Despite the analogy that is apparent between the structure of the retinula of a vertebrate and that of a Prorhynchus, there is no analogy in functional changes shown. In the former it is

the myoid that most markedly changes form, in the latter it is the rhabdome that is most conspicuously modified in response to light and darkness.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

1 Approximately transverse section of right eye. Dorsal side up. Dark adapted (?). × 1500

2 Nearly transverse section of left eye. Dorsal side up. The mate of eye shown in figure one. Dark adapted. × 1500

3 Transverse section of left eye. Dorsal side up. Dark adapted. × 1500

4 Transverse section of right eye. Dorsal side up. Dark adapted. × 1500

5 Frontal section of left eye. Anterior end up. Dark adapted. × 1500

6 Nearly frontal section of right eye. Anterior end up. Dark adapted. × 1500

7 Nearly frontal section of left eye. Anterior end up. Dark adapted eye. × 1500

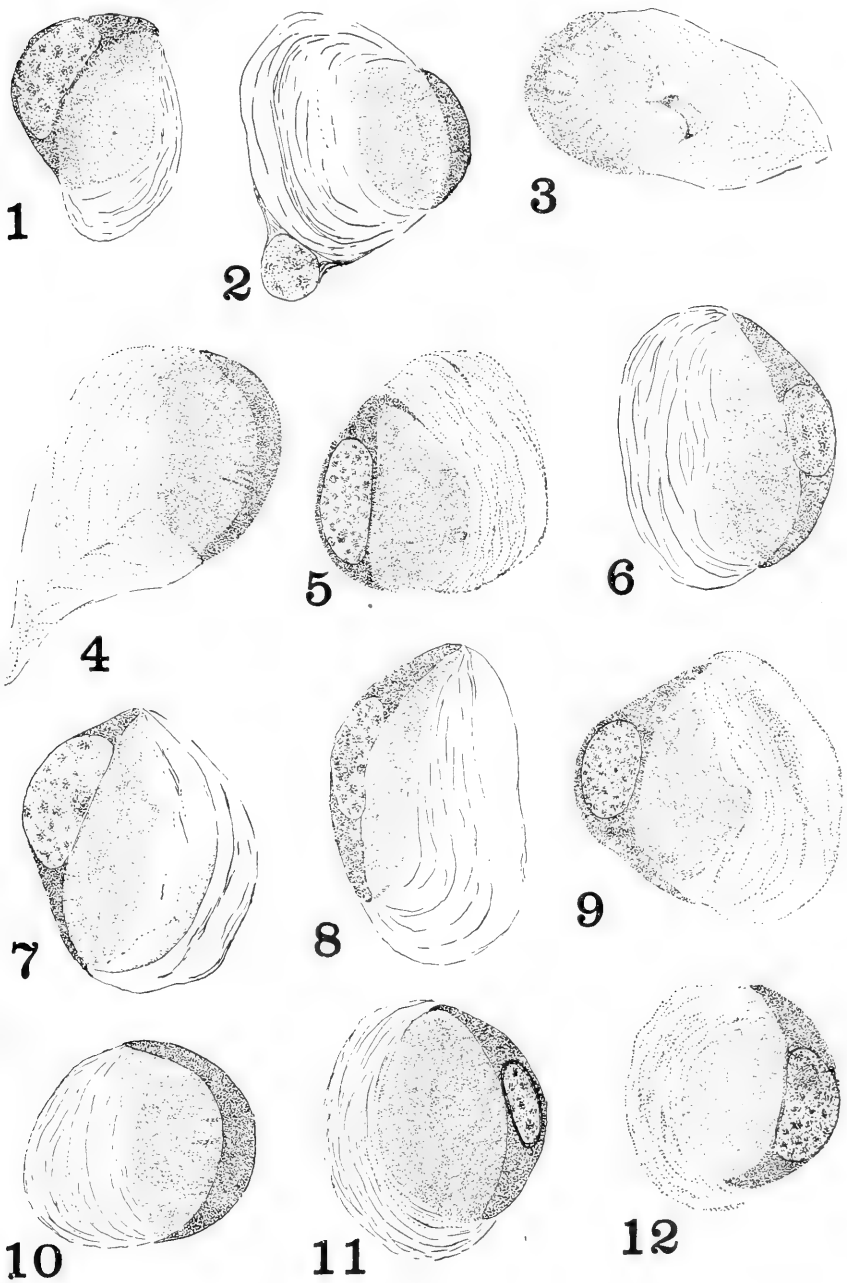
8 Nearly frontal section of left eye. Anterior end up. Dark adapted eye. × 1500

9 Nearly frontal section of right eye. Anterior end up. Light adapted eye. × 1500

10 Approximately frontal section of right eye. Anterior end up. Light adapted eye. × 1500

11 Nearly frontal section of left eye. Anterior end up. Light adapted eye. × 1500

12 Nearly frontal section of right eye. Anterior end up. Light adapted eye. × 1500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STUDIES UPON THE BIOLOGICAL SIGNIFICANCE OF ANIMAL COLORATION

I. THE COLORS AND COLOR CHANGES OF WEST INDIAN REEF-FISHES

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EIGHT FIGURES (ONE PLATE)

CONTENTS

INTRODUCTION

Zoologists have long been uncertain how to interpret the vivid coloration of some animals. Many consider the advantage it confers measured by its conspicuousness. Others deny its utility; ascribe its appearance to the vagaries of metabolism; regard it as an expression of tendencies determined by racial constitution, or refer it largely to the action of external factors. To only a few the conspicuousness of animals of high color seems too lightly assumed, and they maintain that belief in its existence rests chiefly upon failure to appreciate the obliterative effect of bright or strongly contrasted hues when they are displayed under natural conditions. Thus it is apparent that confusion prevails, and that new methods are required to rehabilitate a subject of investigation that has fallen somewhat into disrepute.

A study of the interrelations of the gulfweed fauna was in progress, when the prevalence of Sargassum colors upon animals living in it focussed attention almost at once upon issues raised by this agreement. It appears that no less than 12 species (4 fishes, 2 crabs, 2 shrimps, 3 gastropods, and a planarian) are marked with the brown and yellow of the algae, with or without the white of their adherent bryozoan skeletons and worm tubes. No animals normally restricted to the floating weed distinctly display other colors, and the convergent evolution of the group is indicated in addition by the fact that several have organs so shapen that their resemblance to parts of the plants can scarcely be ignored.

These observations suggest many problems whose direct presentation reinvests them with the freshness and interest they possessed when pioneers of Darwinism first attempted their solution. Among them few seem more fundamental, than why in some habitats all the animals should wear dull colors, while in others conspicuousness should appear at a premium, and no hues be too gaudy to serve in attaining it.

It is noticeable that in the former case the animals' colors tend to repeat those of their surroundings. In the present instance one is impressed besides by the fact that agreement in color is correlated with coincidence in range, and exaggerated resemblance to surrounding objects with intimate association with them; for the gulfweed animals are essentially inhabitants of small oceanic islands, and are cut off from racial points of origin by open water. But, if a tendency toward uniformity in coloration characterizes groups of sluggish animals living in surroundings whose color-characters are relatively invariable, it is possible that the colors of more active ones living under less uniform conditions may obey the same laws of distribution without the fact being obvious. For in one case all the colors which the animals wear may surround them at all times, while in the other this may not be so, and the fact of repetition may thereby escape detection. Hence, it was accepted as a working hypothesis that, in general, the colors which may appear upon animals are rather strictly conditioned by the environment in which they live.

The diversity of coloration occurring among reef fishes led to the testing of this assumption by its application to them, but in such an undertaking no advantage is to be won through multiplying sporadic instances of 'protective' or 'warning' coloration which may attract one's attention upon the reefs. It is necessary rather, if any should appear to exist, to determine the system or law of distribution of color among the fishes of a given region, in the hope of discovering relations of general validity, facts which, regardless of preconceived opinion, shall make the same appeal to all observers to whose attention they may come. It is not clear that there is more than one way to approach such a problem: the foundation of all later researches, pursued by whatever method, must be laid by long continued study of living specimens under absolutely normal conditions.

It may be an incorrect conclusion based upon ignorance of other animals, but tropical reef-fishes seem superior material upon which to carry out such a preliminary study as the present. In the richness of their coloring they vie with birds and butterflies. Many species may be observed in one circumscribed region. Of these a fair proportion are common. The great majority of them are not very shy and are little disturbed by observation from a boat, and perhaps as little, or even less, by one wading among them waist-deep in water or going down among them at greater depths with diving equipment. They possess, as a class, large power of color-change, which, correctly interpreted, is of the highest significance, for it provides an unmistakable clue to much that has remained obscure. They may be studied in a perfectly natural environment, unaffected by human activities as most terrestrial habitats are. Finally, their food is such that its nature may usually be determined without difficulty from analyses of stomach contents, and conclusions drawn from observation of the creatures' movements be checked readily against information of this sort and validated or disproven.

Regarding methods of observation little need be noted in detail; such information as is necessary or of interest will appear in its appropriate place with the statement of results obtained. The fishes have been viewed from every angle above and below, by

day and night, free and confined, wholly beyond the control of the observer, or deliberately led from place to place upon the reefs. So many of the Teleosts that are common at the Tortugas during the summer have been studied, that it is believed that false conclusions flowing from the study of unrepresentative material have been avoided. The minimum amount of apparatus has been used with full appreciation of the fact, that under such circumstances charges of manipulation may not be urged with any such show of reason, as that with which they have been directed against Thayer's clever devices.

The research has been in progress for about five years, yet no one realizes more clearly than the writer that the intensive study of one class of animals in a single region, though the species studied be considerably more than a hundred, and the stations at which observations were made, more than a thousand miles apart, is little more than a reconnoissance in the field of so large a problem. Still it seems that two conclusions may be safely accepted: There is method in Nature's madness, and that method is perfectly demonstrable by approved and conservative modes of analysis.

The results achieved speak for themselves. They might have been obtained with great difficulty, if at all, with other support than that of The Carnegie Institution of Washington, yet owe such merit as they possess, far less to material assistance generously placed at my disposal, than to the unfailing interest and support of Dr. Alfred G. Mayer, Director of the Department of Marine Biology, to whom I remain permanently indebted.

DETAILED STATEMENT OF RESULTS

Countershading

Countershading is the definite gradation of pigment from darkest on the mid-dorsal, or upper, to lightest on the mid-ventral, or lower line. Its effect in destroying the solid appearance of an object when it is exposed to uniform lighting from the direction of its darkest surface is clearly demonstrated by Thayer's countershaded models. Nevertheless, F. C. Selous

('08), the well-known English hunter of big game in Africa, states that he is quite sure that to a South African Bushman there is in nature no such thing as protective coloration.

In so far as this remark has reference to the effect of countershading (and it must have such reference, since the part is included in the whole), Mr. Selous is affirming an apparent impossibility. The native tracker may be vastly superior to his white master in his ability to interpret certain sense-impressions, but whatever reduces their distinctness militates against his success, and this is exactly the effect of countershading. For of much light falling upon the darker surface of a strongly countershaded object little is reflected, while of the little reaching its shaded underparts a mere fraction is absorbed. But, if much subtracted from much and little from little leave the same net result, no eye, savage or civilized, can gather from the appearance of the countershaded object that evidence of its solidity upon which every observer is forced largely to rely.

Thayer's theory of obliterative coloration was begotten through understanding the necessary effect of countershading. Even if it should appear that errors have been made in its application it remains an eminent contribution to the literature of its subject; yet Dewar and Finn ('09) fail to review it upon its merits, and justify their reference to it by the fact that it was enunciated in reputable journals, and has been noted with approval by a leading student of the color problem. They intimate that this is an hypothesis based upon the assumption that animals see with an artist's eye. Their view is apparently in part a direct result of Mr. Thayer's over-insistence upon artistic sensibility as a prerequisite to appreciation of the laws of animal coloration. It involves confusion of ideas, however, and failure to distinguish between the necessary effect of countershading upon image-forming eyes, and comprehension of the principle of its operation.

Countershading appears almost universally upon reef fishes, and its absence, or relative deficiency, seems to be definitely correlated with some unusual habit or peculiar form. The remora (Echeneis naucrates) may have any side uppermost, as

it happens to attach itself to one surface or another of large fishes to which it is commonly found adhering. Its essential lack of countershading is therefore correlated with the fact that it maintains no constant position with reference to the source of light. Fierasfer lives within the cloaca of large holothurians upon the reef flats, and not only has no countershading, but has even lost its external pigment. Fishes which habitually live in dim light, e.g., the glass-eyed snapper (Priacanthus cruentatus) are only slightly countershaded, and the same is true of such fishes as the Chaetodontidae, whose thickness is little in comparison with their depth, and whose sides as a consequence are nearly vertical.

Obviously all these facts and many more, such as the reversed countershading of Agalena naevia, a common spider which hangs in its web ventral side uppermost, that of the caterpillar of Automeris io which feeds in an inverted position, and the absence of external pigment in the crab (Pinnotheres) found in the mantle cavity of the oyster, may be referred to one system, which involves the production of dark pigment in any region in direct proportion to the average intensity of the illumination of that part. They may be equally well explained by supposing that the pigmentation observed is the effect of insolation, or the result of selection directed toward the development of inconspicuousness.

The second hypothesis may unquestionably be attacked from the vantage ground of Cunningham's ('91, '97) observations and experiments, which show that, if flounders be illuminated from beneath, pigment will develop upon their lower sides. To argue that this is scarcely a fair test, since the lower side of a flounder is not a true ventral side really lacking dark pigment through unknown generations, and that these experiments show the power of light to recall, but not to induce the creation of pigment anew in tissues in which it had not appeared during the racial history, would be of little avail. But the hypothesis that countershading is the direct effect of illumination is after all more vulnerable than the other. We may admit that we do not understand the change in the remora's pigmentation; we do not know that it has been effected through natural selection, but there is an inter-

esting difference, between the sexes in some crabs, which shows that the explanation which refers the origin of countershading to the direct action of sunlight is incapable of general application.

Examination of a number of Tortugan Brachyura makes it clear that their sexually dimorphic coloration is definitely correlated with other characters. It is absent in the ghost crab (Ocypoda arenaria), the orchid crab (Gecarcinus lateralis), in Grapsus grapsus,[1] Chorinus heros, Mithrax hispidus, etc., but in none of these species is the abdomen of the ovigerous female exposed in dorsal view. In the Oxyrhynca mentioned the sternum is deeply excavated and the eggs are virtually borne in a brood chamber. In Ocypoda and Gecarcinus the abdomen of the female is hinged to the thorax in such fashion that it is impossible for its exposure to occur under normal conditions—an observation confirmed by examination of ovigerous specimens of the former species. On the other hand, in all the Portunidae which have been noticed, Portunus sayi, ventralis, depressifrons, and sebae, Callinectes ornatus and marginatus, and Charybdella ruber, the abdomen has greater freedom of movement, and is exposed in large part by the female when carrying her eggs. In all these the pigmentation is as indicated in the following paragraph, though the distinctness of the difference between the sexes varies as the species is dark or light, being more clearly defined in the case of the former.

The variable ground color of the gulfweed crab (Portunus sayi) is always some shade of brown. This is marked with lighter brown which may verge toward white. The ventral surface of the thorax and the exposed face of the abdomen lying beneath it are in the male dull, waxy white. The same is true of the female with the exception noted below. In the male three abdominal segments visible in dorsal view are each crossed transversely by a band of brown, with which similar bands correspond in the other sex. But upon the following three segments, invisible from above and uncolored in the male, the female has three additional bands. These appear before sexual maturity, and

[1] The two sexes of this species are not of the same color throughout, but their differences are of no importance in the present connection.

consequently before the female abdomen has been exposed in any attitude differing from that assumed by the male. With the onset of reproductive activity the pleon is elevated and covers the egg mass which the female bears. It then continues the dark color of the thorax posteriorly down over it into the shadow with delicate countershading.

The point to be established is of such importance, that the reason for believing that color differentiation precedes sexual maturity, i.e., that difference in coloration antedates difference in exposure, must be stated in full. The facts are as follows:

When a large collection of the crabs is made the two sexes are taken in approximately equal numbers. The males are all of one sort differing only in size, but the females are of two types. The larger ones, some of which may be bearing eggs, fall into one group. The others ranging from medium to smallest sizes, are intermediate between males and adult females in several respects. The abdominal margins of such specimens are straight instead of convex, and the contour of the abdomen is therefore triangular rather than broadly rounded. The groove in the sternum into which the pleon fits is relatively deeper and retains it more firmly than in females of the other type. Finally, the openings of the oviducts are so small as to be practically obsolete, though they are conspicuous in mature specimens of the same size. That these are not abnormal individuals appears from the fact that every female apparently passes through this stage, as all the smaller ones show these characters.

If then there are animals, such as these, whose countershading is a congenital character, and others on which the same gradation of pigment appears, with possibility of the same explanation, one risks much who asserts with assurance, that the condition observed in the second group is due to causes entirely different from those operative within the first. That it is not clear in what way the countershading originated and whether it is, or has been preserved by natural selection, will not warrant such action. The observations upon crabs, therefore, justify the rejection of insolation as a universal immediate cause of development of dark pigment upon animals' surfaces that are habitually turned

toward the light. They also justify the appeal to ignorance in the case of the remora's unusual coloration, which may not be inconsistent with the development of countershading through natural selection.

That illumination alone cannot account for the distribution of their dark pigment, is shown with additional interesting implications by many countershaded animals, perhaps by all that wear distinct patterns. There are fishes, for example, with alternating light and dark horizontal or vertical stripes. In the former case the dark elements may be so graded that the countershading is largely applied in a series of discrete patches, rather than in a mass appearing unbroken to the naked eye.

The bass (Roccus lineatus) shows the pattern in question. It is an interesting detail that some of its dark stripes decrease in intensity posteriorly, as they pass more and more into the shadow of the caudal peduncle. The fish might be described with some truth, as dark in color, perfectly countershaded, and marked with a series of horizontal light stripes. This pattern is constant within the species, and hence inherited; or it might be urged that what is inherited is a difference in material in the regions covered by the two sets of stripes, so that one is darkened by sunlight while the other is not. In any case even an indirect effect of light in controlling countershading through the inheritance of acquired characters is excluded; for there is obviously a fundamental internal condition upon which the development of the whole system of pigmentation depends, and it cannot be so simply explained.

But if countershading is no necessary effect of the mere presence of pigment (Echeneis), and is neither directly nor indirectly induced incidentally by external factors (Brachyura and Roccus), and if, in addition, this system of pigmentation is positively known to contribute most decidedly to the inconspicuousness of its possessors under certain normal conditions, it would seem that if any characters be maintained by natural selection this should be one. Therefore, from a consideration of all the facts, unless indeed one assumes that the countershading is vestigial and antedates the appearance of bright colors, to the conspicu-

ousness of which it remains antagonistic, it is difficult to avoid the conclusion, that, no matter how glaring the colors with which it is associated, its presence is constructive evidence of the concealing function of the coloration observed.

Color change

Color change in fishes has been studied extensively by physiologists and histologists.[2] Much has been learned regarding the mechanism which produces and controls it. It has also been shown, in a few cases, that its general effect is to assimilate the organism with its environment. Yet the observed facts are not adequate tests of the hypotheses which postulate conspicuousness; for the changes that have been most fully investigated occur rather slowly in fishes whose hues are not particularly gaudy, and with no known exception have been induced in artificial environments. But, granted that an animal's ability to change its color under laboratory conditions is demonstrated, in every case it remains to be seen whether when they occur in nature its color changes conform to the same or other laws. It is then for the first time possible to estimate their probable effect in diminishing visibility, warning off enemies, or otherwise advancing the interest of the individual that displays them.

Concerning the color changes of fishes in their natural habitat few facts are available. It is known that the trout, for example, may exhibit a dark or light phase according to the character of its haunts, but no statement of the rate at which one may succeed the other has been encountered. Knowledge upon this point seems to differ only in degree from that we have regarding Bodianus fulvus and punctatus, which have been considered by systematists different subspecies or species. The two forms described have been observed, however, by C. Tate Regan ('09) in the New York aquarium, and by the writer, upon the reefs in Porto Rico, to be merely alternative color phases of one creature, which may replace one another instantaneously.

Other cases comparable with these may be gleaned from the literature. Gourret ('93) describes the color varieties of the

[2] For a review of the literature of the subject see van Rynberk ('06).

Labridae of Marseilles and assigns them to different stations. Noé and Dissard ('94) find that Labrids are brown among rocks and green in algae. Jordan and Evermann ('98) note that Iridio bivittatus is variable in color, and Reighard ('08) records the same of this species and of Thalassoma bifasciatus; but in every instance one would infer from the context that the observers were impressed by what they believed comparatively fixed color types, rather than by any marked power of color change in individuals.

That notable color changes do occur rapidly in many species of tropical fishes, may fairly be described as a matter of common knowledge among zoologists. Those acquainted with the fact, however, place very different interpretations upon it. Some idea of the diversity of opinion which prevails will appear from the following considerations.

Dr. Charles H. Townsend, Director of the New York Aquarium, has studied fishes confined there in exhibition tanks, and has published detailed descriptions of a large number of them. While he finds that coloration is determined to some extent by the construction of the tank and by the position of the fishes in it, Dr. Townsend states ('09, '10) that frequent changes are dependent upon activity, rest, play, fright, temperature, food and so forth, and thus suggests the association of the various color phases with specific psychic states. Dr. F. B. Sumner ('11) thinks that these changes are probably of no more utility than blushing and various other evidences of emotional disturbance in ourselves. Prof. S. J. Hickson ('10) believes, upon the contrary, that there can be little doubt that some are phases of protective coloration, of which the excitement phase is not one, and that much work must yet be done before it can be determined whether any phase has definitely a warning significance. Mr. Thayer ('09), convinced of the truth of his theory of animal coloration, but, as he informs me, without other ground for his opinion, has hazarded the suggestion that the chief use of such changeableness is adaptability to various backgrounds. Finally, Professor Poulton ('08) states categorically that rapid adjustable protective resemblance is suited to wandering forms which must

in the course of their lives continually pass and repass over environments of different colors, and adds that it is widely found in fishes.

For at least thirteen West Indian species it appears that Thayer's and Poulton's hypothesis is fully confirmed. This implies only that in nature the color changes of the animals vary with their environment, with which they tend to assimilate them. It has not been demonstrated that the power of adaptive color change actually confers any advantage upon its possessor.

The reason the truth above has remained obscure is not far to seek. One studying fishes goes naturally, especially if time be limited, first to those places where, upon the whole, species are most abundant. This will be almost invariably some mixed bottom covered in part by algae, gorgonians and living coral, in part covered by bare sand, coral skeletons and stone. But over such a substratum confusion prevails, for the color of a given fish at a given station is not always the same, since it may be modified by a number of factors. The color of the underlying bottom, the size of the patch over which the fish is at the moment and the rate at which it is moving across it, as well as the level at which it is swimming, are all effective in determining its appearance. Above all the fish's recent experience confounds the observer, for two individuals that have just come from environments of different character may temporarily show the color phases induced there over a third type of bottom to which neither is adjusted.

Months of study of the problem under the conditions described may leave one with the feeling that there is, upon the whole, some evidence of adaptive color change manifested vaguely in a few species. When this appeared to be the case with Iridio bivittatus, a specimen was placed in each of two 5-litre aquaria, which had been wrapped in black paper after the bottom of one had been covered with white sand and that of the other with a mixture of brown and green algae. A little manipulation then showed, when the jars were set out in the sunlight, that either fish might be made the darker or lighter within a few seconds by making proper disposition of the pair, the difference in color

between the two phases experimentally induced being probably greater than any consciously observed upon the reef up to that time.

Following the clue afforded by the experiment it gradually appeared that where the character of the bottom is uniform over large areas, there is close agreement in color between individuals of certain changeable species, or, in other words, that definite color phases are correlated with simple environments of different kinds. With species that will gather about food provided for them it soon became clear that particular phases may be demonstrated at will by leading the fishes from one place to another appropriately chosen. The color phases of others may eventually be seen succeeding one another in the same way, if one undertakes the patient observation of uncontrolled specimens passing from one type of bottom to another.

The first method has been applied to five species, and it appears that their color changes are definite, and that they involve the repetition upon the fish of the dominant color of the environment, either uniformly, except for countershading, or in distinct spots, stripes or bands. The second method has been followed with four species whose color changes may be predicted with accuracy, and are marked by similar convergence toward the color of the background that induces them.

Finally, with fishes of small or medium size it is possible to test for and to demonstrate adaptive color changes under laboratory conditions by using combinations of colors occurring normally in the environment of the animals experimented upon. For example, the bottom of a tank 28 inches long and 13 inches wide and deep, inside measurement, was covered to a depth of several inches with coarse sand and fragments of corals and shells from the beach. In the sand at one end over half the bottom turtle grass (Thalassia testudinum) was planted until a dense green covering had been secured. The other end was left bare. Fishes that had been seined upon reef flats covered more or less thickly with the same kind of plants, with bare spaces appearing at intervals among them, were then placed in the tank, and it was discovered that a number changed regularly

TABLE 1

Adaptive color changes in reef fishes

A. Phases appearing when fishes are led into selected natural test environments

SPECIES	CHARACTER OF BOTTOM			REMARKS
	Gray sand	Green turtle-grass	Brown algae, gorgonians, etc.	
Epinephelus striatus	Cream color with dark bands that may nearly disappear	[3]	Light olive with very dark brown bands	Color adjustment commonly very striking; requires only three or four seconds
Iridio bivittatus	Very pale gray	Distinctly green	Decidedly brown	Color changes readily controlled in confinement
Iridio maculipinna	Grayer than when over brown bottom	[3]	Dorsal side wholly olive	
Lachnolaimus maximus	Chiefly pale gray	Not often seen; then merely darker than over gray	Solid red-brown or mottled brown and cream color	
Thalassoma bifasciatus	Dorsal surface gray	[3]	Dorsal surface yellow	The record applies only to the juvenile form, which is described as T. nitidus

B. Phases appearing when uncontrolled fishes pass freely into selected natural test environments

Neomaenis griseus	Gray	[3]	Dark brown	As observed in fish about the laboratory dock the change from gray to brown or *vice versa* are most marked in the early morning and evening. It requires about three seconds

Sparisoma abildgaardi	Gray	[3]	Dark brown; top of head sometimes washed with yellow	
Sparisoma flavescens	Gray, uniform or mottled	[3]	Dark, uniform or with a broad brown stripe on the side	This fish is uniform or mottled green among turtle-grass in the tank
Sphyraena barracuda	Gray	A single young specimen seen among green algae near shore was distinctly green	Body crossed by about seven dark bands covering about half its surface	This species is most commonly seen near the surface, hence these notes refer to individuals in rather unusual positions
C. Phases appearing in reconstructed natural test environments				
Monacanthus hispidus	Gray	Green	Not tested; see next column.	A good many of the specimens seined on the grass flats are taken in a brown phase
Scorpaena plumieri	Very light	Not tested	Very dark	This species is variegated in color. Has many dermal outgrowths simulating the appearance of algae and hydroids. Its color changes, at least in confinement, are slow, but the fish is sluggish, and such perfect adjustment is attained that specimens may be in the centre of one's field of vision, yet be overlooked
Siphostoma mackayi	One small specimen almost white; response commonly less definite	Same specimen green among turtle grass. Response unusually clear	Not tested	Seined among turtle grass on sandy flats this species is usually green, but may be brownish olive, or green with a gray dorsal surface
Sparisoma hoplomystax	Gray	Green	Not tested	Color adjustments very striking and rapid, almost instantaneous

[3] No record; species seldom or never seen in the environment indicated.

from gray to green, or vice versa, as they swam slowly from one end to the other.

It is not necessary to appeal to such methods as the last in support of the thesis that the colors of tropical fishes are of an adaptive character, but this was useful in determining what species might be studied most profitably upon the reef, and the occurrence of what types of change might be anticipated with the greatest confidence. Some results of these experiments, not verified in the field, are incorporated with other observations in the following table, which summarizes incompletely the information gained regarding color change. Failure to follow up suggestions springing from these preliminary tests was due in part to lack of time, and in part to the relative scarcity of the animals, or difficulty in observing their habits.

The facts embodied in table 1 should be considered merely representative. No other species, to be sure, has been studied even as closely as those listed, but half as many more have been seen making adjustments as accurately as any mentioned. Reference to these forms is omitted from the table, because no serious attempt has yet been made to follow the gamut of changes through which they run. The reserve subject to draft, whenever it seems expedient to extend the list, includes the red grouper (Epinephelus morio), rock fish (Mycteroperca venenosa), yellow grunt (Haemulon sciurus), the tangs (Teuthis caeruleus and hepatus), red goatfish (Upeneus maculatus), sea robin (Prionotus sp.[4]), and the razor fish (Xyrichthys, sp.[5]).

All the changes noted in the first two sections of the table may be observed from a boat. Some of them, especially those of I. bivittatus and Thalassoma, may be followed in specimens that will gather about one in 3 or 4 feet of water, but the diving-hood is almost invaluable on account of the ease and accuracy with which it permits the investigation of the fishes' color changes to be conducted.

This piece of apparatus is essentially an inverted, weighted, metal cylinder with a plate glass window in front. It rests upon

[4] One specimen only; passed from my possession before being identified.

[5] One specimen only; accidentally lost before being identified.

one's shoulders, and air pumped into it through a hose reaching a boat above continually replaces that which is exhausted and escapes in turn below its rim. A total weight of about 80 pounds is necessary to steady the diver in 15 to 20 feet of water, where excursions may be made up to at least half an hour, and probably much longer, without discomfort. For the prosecution of a research such as the present the hood has all the advantages and none of the objectionable features of a regulation diving suit. With a wax-coated slate and sharp-pointed nail ample records may be kept and transcribed at leisure.

Some of the fishes one encounters when diving are so little afraid that they will almost feed from one's hand. Hence, even minor changes in their coloration may be observed to advantage. This encouraged an attempt at submarine photography which was at least as successful as might reasonably be anticipated when working under novel conditions with untested apparatus.

A No. O Graphic camera, taking a picture $2\frac{3}{8}$ by $1\frac{1}{2}$ inches, was enclosed in a brass box, windows in which permitted the use of the finder, while the necessary manipulation of shutter and film was accomplished by means of a screw and plunger protected by water-tight packing. Since the lens was of universal focus adjustment for distance was unnecessary. Some of the pictures secured are reproduced herewith, and serve to illustrate the striking changes in pattern which the fishes undergo, and suggest also how slight ground there is for considering the animals conspicuous. Much remains to be done, but in the light of present experience it is apparent that adequate exposition of the facts should be possible by the photographic method.

Regarding color change it seems perfectly safe to go much farther than Townsend ('10), who notes that his fishes showed more color phases as their surroundings were more varied, i.e., that their environment played at least a subordinate part in determining their aspect; farther even than Mast ('14), who states his conviction that adaptive color changes in fishes occur not only in marked degree, but that they are rather widespread. There is, indeed, within the limits of my own experience almost

no evidence that in nature any other factor than the color and shade of the surroundings exercises notable control over those changes in coloration which occur by day, modifications in pattern excepted.

Fishes in tanks, it is true, often show color phases which baffle interpretation, but when they are unconfined this is not commonly so. This suggests that in the former case the behavior is not entirely normal, and two sets of observations lend some support to this conclusion. In the first place, the whole behavior of some species seems to be modified by restraint, to which they do not become accustomed in weeks of confinement. It is also known that in some animals processes underlying growth, secretion, etc., are directly affected by comparatively slight disturbances in their environment. The rate of growth of rats, for example, is distinctly modified, if unfamiliar operations are carried on by strangers in or about the creatures' quarters. That is to say, modified color reactions may be anticipated with reason in fishes in confinement, since they sometimes show its effects plainly in other ways, and since, in addition, in other animals processes apparently no more complex than those concerned in adaptive color changes are inhibited by no more evident stimuli, and without more marked departure from normal behavior in other respects.

But wholly apart from complications which might originate in disturbance of organic function through confinement, there is a possible source of error in experimentation upon fishes under such conditions. This lies in the fact that the color-complex in which most captive fishes find themselves, only remotely resembles that in which they normally occur, and to which they are able to adjust themselves accurately. But when to match a given background color is wholly impossible, and there is bound to be maladjustment in any case, much greater variation is to be expected in one fish at different times, and in different ones at the same time, than if a stereotyped response well within the creatures' capacity were demanded. Finally, exhibition tanks such as those in the New York aquarium unquestionably fall under the head of mixed environments, in

which, even under the most favorable conditions, the least intelligible results are obtained. Therefore, when all is considered, the surprising thing is not that the significance of color change should have been imperfectly understood, but that its meaning should have been grasped at all.

Except as banded patterns sometimes appear upon fishes as they come to rest, unconfined specimens give one little reason to believe that psychic states, special activities, or uncontrolled internal stimuli determine their color reactions to any appreciable degree. The following records are rather exceptional. A hogfish (L. maximus) feeding in the gray phase over light colored bottom is noted as flushing momentarily and then returning to and remaining in its previous condition. Similarly a Nassau grouper (E. striatus) lying in a dark phase in the shadow of a large coral head came into the open over clear sand (fig. 3), turned pale as it crossed it (fig. 4), and darkened somewhat (fig. 5) as it commenced to feed upon the half of a crawfish (Panulirus argus) which had been provided for it. Such irregularities in behavior seem inconsistent with the impression conveyed in the preceding pages, but they are apparently mere swirls upon the great current of evidence, and move on with the tide they seem to oppose.

Flounders adapted to black or white bottom will reverse their coloration completely, while the whole body rests upon the color to which it conforms, if the head is upon the other (Mast, '14). Sparisoma flavescens reacted for me in the same way in a tank with a slate bottom, half of which was covered with white sand. It was observed repeatedly swimming slowly toward the white, where for a moment its passage was impeded at the border. Then, with only a part of its head across the line, it assumed in full the color and pattern it regularly showed over the light material before it. The whole system of chromatophores in the most changeable species seems to be in a state of highly unstable equilibrium. Hence, dominance for even an instant by a dark object in which interest might be centered for the moment might very well induce the exact aberrations which have been noted in the preceding paragraph.

How largely independent of activity, 'states of mind,' and internal stimuli the color phases are, may best be illustrated by reference to specific instances.

On July 28, 1915, I broke open a large, long-spined sea urchin (Centrechinus) over clear sand in a small bight surrounded by large coral heads, which were at no place within 8 or 10 feet of the bait. A small hogfish about 10 inches long came and circled about many times, for the broken test was lying spines uppermost, so that it could get nothing, although smaller fishes could go under and feed from the inside. Finally it found a detached spine which it picked up and masticated base foremost. It remained consistently in the gray phase throughout the whole period during which it was under observation (fig. 6).

I next moved to a dense gorgonian patch 3 or 4 feet in diameter, and placed food beneath the brown branches of the clustered colonies. Within a few minutes a larger hogfish, 18 or 20 inches in length, went in under them and displayed an almost uniform brown color, or, as it moved about, replaced this by a mottled pattern of rich reddish brown and gray (fig. 7). When it was driven out over the open bottom, it turned gray, and swam off in that phase with its mouth full of food. It went under gorgonians nearby and turned brown for the second time and finally swam away low down over mixed bottom in its mottled livery. Coming back after a little, it approached the original station, ate and swam about in brown phases. Then a broken Centrechinus was placed about 10 feet away on bare sandy bottom; the fish was driven out to it with a long-handled dipnet, and assumed its gray phase in the open. The food was moved back and forth a number of times, and color and pattern were changed regularly with each significant change of position, but neither variation in activity, nor any incipient alarm engendered by the repeated seizure and removal of its food seemed to have any effect in modifying the fish's appearance. The facts regarding the color changes of the other species mentioned in table 1 are perfectly comparable with these and seem to point to only one rational conclusion.

Whether or not a familiar object minus its color might by association induce a color change appropriate to its normal but

unsuited to its modified condition seemed worth investigating. The turtle grass (Thalassia) provided suitable material for making the test. The rootstock of the plant is usually deeply imbedded in the sand. The leaves spring from short offshoots, and for half their length, on the average, are not exposed to the light. Their buried portions are white, therefore by cutting off the green parts and making shallow plantings of the basal segments it was possible to manipulate the materials in a small tank, so that the fishes were exposed to two sets of surroundings, whose essential difference was in color.

In the white grass Iridio bivittatus, Monacanthus hispidus, Siphostoma mackayi and Sparisoma hoplomystax all reacted as they did over bare sand, except that one young Siphostoma was even lighter in color than in that case. To the green they responded as indicated in table 1. Hence color changes in fishes appear to be induced directly by the color of surrounding objects,[6] and not indirectly through suggestion derived from familiar forms. There is, therefore, nothing here to lend collateral support to such hypotheses as that of Steinach ('01), rejected indeed by Cowdry ('11), that the chromatophores in the octopus are controlled by reflexes from the suckers, and that, as a consequence, the texture of the bottom, rather than its color, determines the color changes that occur.

At this point one should refer for a moment to an idea one frequently encounters, and which seems in fair way to become an article of faith in the matter of animal coloration. The reasoning upon which it rests is wholly illogical, as the reader will observe. Sometimes it is simply affirmd *ex cathedra:* "Absence of movement is absolutely essential to protectively colored animals." (Beddard, '92, p. 90.) . Sometimes it is stated with some attempt at justification: "No color whatever could make a flying butterfly invisible to its enemies, because the background against which its body shows is continually changing during its

[6] In view of what is known of the path of nerve-impulses, the fact that the fishes are able to match different colors proves, of course, that they possess color vision. The point has already been made by Mast ('14), but the demonstration of adaptive color changes in additional unrelated species emphasizes a conclusion that has been commonly mistrusted.

flight, and, moreover, the movement alone is enough to betray it, even if it is of a dull color." (Weismann, '04, p. 74.) "No observer of Nature can have failed to remark how the least movement on the part of an animal will betray its whereabouts, even though in color it assimilates very closely to its environment. . . . Thus in order that protective coloration may be of use to its possessor the latter must remain perfectly motionless." (Dewar and Finn, '09, p. 200.) The same sentiment is expressed by Werner ('07), Selous ('08), Palmer ('09), and is quoted from Beddard with approval by Roosevelt ('10, p. 493). It reappears in Allen's ('11) review of Roosevelt's Revealing and Concealing Coloration in Birds and Mammals, yet is diametrically opposed to the just inference from the facts noted in the present section of this paper. It is one of the 'obvious' things, the number of which used in constructing theories of coloration is so great, that if all were eliminated, the skeleton remaining would be scarcely recognizable. It is so inconsistent with the fact that an unusually active fish, such as Iridio bivittatus, which seems never to rest by day, possesses three color phases, which it changes appropriately as it passes from one environment to another, that farther comment is unnecessary.

Some of the species whose color changes have been discussed in the preceding pages wear bright colors and bold patterns commonly considered conspicuous. Five are included in and constitute 25 per cent of a list of fishes for which, among other animals, Reighard has felt it desirable, if not imperative, to restate and extend Wallace's ('67) hypothesis of immunity coloration. There is some reason for believing that adaptive color changes may yet be demonstrated in other listed species. Therefore one feels safe in saying that the advocates of the color hypotheses which postulate conspicuousness must face the fact, that in an important group of animals, whose colors and patterns rival those of any other group in brilliancy and contrast, many which to the casual observer seem among the most conspicuous possess, in addition to countershading, an elaborate mechanism which enables them to reproduce upon their own bodies the dominant hues of the different environments in which they normally find themselves.

Correlation of color with habit

When it stands alone, the inconspicuousness implied by the occurrence of countershading is insufficient to force the abandonment of current explanations of the significance of bright colors; for once grant that there are 'conspicuous' animals, and it is incontestable that in a species springing from an inconspicuous line countershading, having outlived its usefulness to the ancestral type, may persist as a vestigial character. But when countershading and adaptive color change are coupled with one another, as they are in many highly colored species, the pressure brought to bear upon the hypotheses which assume that certain types of coloration must be conspicuous is greatly intensified, for the interpretation naturally placed upon the two phenomena is the same, and is opposed to this conception. The second, moreover, can scarcely be explained as a survival from another age, for its manifestation depends upon the efficient coöperation of several of the most highly differentiated organs or organ systems in the body. Yet the facts of adaptive color change are not of general application to bright colored animals. Hence it is difficult to exaggerate the importance of a successful attempt to discover a rational system of distribution of the colors themselves, which the different species wear, and with which countershading and adaptive color change are associated.

If the attempt to define such a system should fail, it is still possible that the creatures are as inconspicuous as may be under the conditions in which they live. If, upon the contrary, the effort should be successful, and a consistent relation between color and habit be demonstrated, such that conspicuousness might thereby be supposed to be reduced to a minimum, the uniform suggestion flowing from this fact and from countershading and adaptive color change will constitute as strict proof of the concealing function of color as may be had, short of unimpeachable feeding experiments.

The first attempt to determine whether the reef fishes repeat the colors of their environment, as the Sargassum fauna does, was made by analyzing their patterns and listing the few shades that stand out at a distance of feet, rather than the great variety

appearing in flecks and vermiculations upon closer scrutiny. The method is crude; allowance for the personal equation of the observer must be large, and the number of species examined was neither great nor thoroughly representative. Moreover, as will appear later, the implicit assumption that all hues that accord with those of the environment must repeat those of the bottom is without foundation. The results obtained are not, however, without interest, and may be stated as follows:

Upon 30 species considered, yellow occurred nineteen times; brown and gray, sixteen times each; blue, eight times; red and green, five times each; and black, three times.

These facts might seem to warrant the conclusion that, roughly speaking, colors occur upon the fishes in the same proportions as those in which they appear upon the reefs; for the gray of bare sand and dead corals, and the brown of large algae, or the microscopic ones living symbiotically in some corals, and the brown gorgonians, are the commonest colors from the shore line to depths where the bottom becomes invisible. Turtle grass is abundant over some parts of the reef flats, and at places upon the reefs its color is supplemented by the vivid green of Zooanthus or Halimeda. Yellow heads of Porites astraeoides and yellow gorgonians are common upon the rougher bottoms where fishes most abound, and red and blue are not wholly absent, though forming only an infinitesimal part of the color mass as a whole. But the conservative inference that, at least, no positive evidence appears that different laws prevail in the distribution of color upon animals in the Sargassum and on the reef, is all that is permissible; for it may be shown that some greens, most blues and apparently all true reds are not related at all to the colors upon the bottom, but have another significance.

It was noted next that the remarkably large eyes of the squirrel fish, Holocentrus ascensionis, and of Priacanthus cruentatus are correlated in each case with red body color. This observation, and the knowledge that the upper limit of the range of certain red, deep-water animals is also the limit within which most of the sun's rays are absorbed (Murray and Hjort, '12, p.

664), led to careful study of the habits of the five species of red fishes which occur in the shallow waters of the inner reefs at Tortugas.

The conclusion that these animals belong to a well defined ecological group was soon reached, and is supported by the following facts.

The fishes are very rarely seen in the open by day. Priacanthus cruentatus has not been seen fully exposed, of its own initiative, except at or after twilight. One specimen of Amia sellicauda was observed a few inches from cover, and the squirrel fishes, Holocentrus ascensionis, siccifer and tortugae, are little less retiring in habit, for weeks may pass without one being noted outside the shelter of the coral stacks,[7] although the observer may be on the alert to catch them.

The five species may be secured in the daytime by blasting with dynamite at almost any station among the stacks in whose crevices they lie hidden. As many as six individuals, representing four of them, have been taken at once, and complete failure rarely followed when using dynamite among the heads. In attempting to visualize what this means regarding the comparative abundance of hidden and exposed specimens one should bear in mind that the explosive, in the quantity used, is effective up to a distance of only 4 or 5 feet from the point of discharge. But the fishes taken are not uniformly distributed throughout its 'sphere of influence,' which lies largely outside the heads among which they lurk. This is apparent from the fact that where the work was done the water averaged little more than 8 feet in depth. In addition, it was always necessary to place the shot within a yard of the outer face of the stack, which in water of the depth mentioned, commonly rises nearly vertically to a height of 5 or 6 feet. If this precaution were not taken, the fragments of coral, failing to be thrown out into the open, would fall in a heap from which the specimens could not be re-

[7] The coral stacks are masses of heads rising nearly vertically from the bottom in water from eight to fifteen feet deep. They are, in respect to the shelter they afford to fishes of small and medium size, upon a level with a pile of boulders loosely cemented together.

trieved; but, obviously, each condition limits the space in which the fish secured must have lain.

Still more vivid impressions of the actual abundance of the red fishes, in spite of their infrequent appearance by day, may be obtained by watching them come out of hiding at dusk. If one goes at that time to a suitable place, and flashes a strong light down into the water, it is possible to demonstrate that they are more in evidence than all other species combined. One may easily have several in sight at once through a glass-bottomed pail. Just before dawn the fishes may be seen again with the same frequency in the same places, as they are about to retire into seclusion. In neither case is there any possibility of undue concentration of individuals being induced by the light, for the first flash may reveal the situation described.

Farther and decisive evidence to the same effect may be obtained by studying their feeding habits, which have not yet received the attention they merit. As matters stand, an examination of the stomachs of nine specimens gives no reason for supposing that they feed at all by day. The facts are stated in detail in table 2. It is worthy of note that the nearest relatives of these red fishes, also red, are large eyed and live at considerable depths. Red and yellow appearing during the breeding season of fishes which spawn at depths which the sun's rays of those colors do not attain (Hess, 1913) may have the same significance as the other reds under discussion.

These discoveries, if it is proper to use such a term in connection with facts which may be common knowledge among fishermen, but do not seem to have been used in any attempt to arrive at an understanding of the function of animal coloration, lead one to oppose the application to the red fishes of hypotheses of warning or immunity coloration, or signal or recognition marks. But it is not really probable that any one will maintain that, natural selection having failed to curb the tendency of color to run riot in this group of animals of the same habit, the same color has been produced independently and accidentally in each case; and this is the essence of the immunity hypothesis. Nor is it more likely that any one can be found who will seriously assert

that through the operation of natural selection, in view of their undemonstrated possession of disagreeable characters, there has been developed in five species of one habit, and without any direct relation to it, a single type of warning coloration which has not reappeared once among many warningly colored species of other habits. That this hypothetically conspicuous color should be that one of all possible shades which first loses its distinctive quality in dim light, in which the animals that bear it habitually live, is an additional difficulty.

The five species under discussion represent three families of two widely separated groups, and even the two families of the same group are not closely related (Jordan and Evermann, '96, p. 1237). Their common color does not, therefore, seem to be due to common descent, unless it is held that they resemble a very ancient ancestral type, from which all related families have diverged. Even so the question remains, why types of one habit should have been stable, or persistent, while others underwent modification. There are apparently only two possible solutions to the problem: either the environment through some direct action controls the pigmentation of these creatures, or red, under the conditions in which they live, possesses selective value hitherto unrecognized. In any case the correlation of red color with a definite habit renders it highly desirable to have detailed information regarding the habits of fishes in general in order to determine whether this is simply a sporadic instance, or a striking example of conformity to a general law.

The knowledge desired is the answer to the question, where and how the species investigated normally spend their time. One must narrow the inquiry, however, so effort has been concentrated upon the determination of three sets of facts. These seem to be of fundamental importance and may be ascertained with comparatively slight difficulty. When the investigation is completed, one should be able to say when the different species normally feed, where they are usually found by day, and at what level they habitually swim.

Nocturnal feeders are little given to diurnal wandering, differing decidedly in this respect from those that feed during daylight.

Hence, if large numbers of a species of such fishes can be definitely located by day, one may have almost complete confidence that an inconsiderable proportion of the whole is in other surroundings, searching for food and subject to exposure which might result in adaptation to an environment differing from that in which the great majority lies.

The importance of knowing the exact range of the animals studied should be self-evident, for if one undertakes to ascertain whether their colors repeat those of their normal habitat, it would seem to be axiomatic, that the nature of the places over or through which they wander should be accurately determined. But some writers take it for granted that the observation of individuals of any number of species in what we may loosely describe as the same place at the same time, proves that they have the same habit. This uncritical attitude is manifested in extreme form by Dewar and Finn ('09, p. 88–89), who argue that because the moose, Greenland whale, and a farther miscellaneous assortment of mammals, including seals, narwhals and musk-oxen, are colored, the prevailing whiteness of the arctic fauna has been greatly exaggerated, and that the common impression that adaptive coloration is dominant in that region is misleading. This means that to these authors the difference in habit and habitat between terrestrial animals ranging south to the latitude of northern Spain, aquatic animals and polar bears, is so inconsiderable, that their difference in color demonstrates its essential lack of correlation with habit and its slight biological significance.

It is of importance to know at what level fishes commonly swim, for, as they rise or fall, the proportion of the finny population in whose sight they appear against a background of blank water, or against the variegated bottom or its vertical excrescences, is continually changing.

As a first step toward the separation of the fish fauna of the Tortugas into ecological groups, the time of feeding has been conclusively determined for a number of species by examination of their stomach contents at different times during the day. The record appears in the following table. A few observations are

TABLE 2

Data upon fishes' time of feeding

SPECIES	TOTAL TAKEN 5 A.M.	CONDITION OF ALIMENTARY CANAL		TOTAL TAKEN 5 P.M.	CONDITION OF ALIMENTARY CANAL		REMARKS
		Full[3]	Empty		Full	Empty	
Anisotremus virginicus (Porkfish)	17	17		2		2	Not common, seems to school about fixed stations by day. One taken at 8 a.m. was full
Haemulon macrostomum	1	1		6		6	Schools in and about coral stacks by day
H. parra. (Sailor's choice)	2	2		7		7	Schools by day
H. plumieri (Common grunt)	16	13	3	36		36	The great majority of individuals of this species school about coral heads and among gorgonians during the day
H. sciurus (Yellow grunt)	45	45		38		38	This species gathers about coral heads or in gorgonian patches by day
Neomaenis analis (Mutton fish)	19	16	3				Large specimens are seen singly on the open reef during the day, but those of medium size (7 to 14 inches in length) school about the heads with other species of the genus. Seven additional specimens, of which three were empty, were taken at 8 a.m.
N. apodus (School master)	28	27	1	2		2	Schools closely about heads, or rocky places along shore by day. Twenty-four additional specimens, of which ten were empty, were taken at 10 a.m.
N. griseus (Gray snapper)	114	95	19	45	4	41	Gathers about definite foci by day. These are more varied than in the preceding species. There are also more stragglers here than there. Twenty additional specimens, of which seven were empty, were taken at 8 a.m.

TABLE 2—*Continued*

SPECIES	TOTAL TAKEN 5 A.M.	CONDITION OF ALIMENTARY CANAL		TOTAL TAKEN 5 P.M.	CONDITION OF ALIMENTARY CANAL		REMARKS
		Full[3]	Empty		Full	Empty	
Ocyurus chrysurus (Yellow-tail)	57	33	25	8	3	5	Very common over reefs by day, singly or in schools. May be seen and taken about sunken bodies, but is decidedly a frequenter of open spaces
Upeneus martinicus (Yellow goat-fish)	4	4		11		11	Given to schooling about heads by day.
Holocentrus tortugae (Squirrel fish)	2	2					Striped and washed with red; nocturnal, lies closely hidden by day. One taken at 10 a.m. were full.
Priacanthus cruentatus (Glass-eyed snapper)	2	1	1	1		1	Red, changeable; sometimes shows pale bands. May turn to almost pure silver, when stimulated by injury. Three taken at 10 a.m. full.
Epinephelus morio (Red grouper)							This fish is solitary and common on rough bottoms. It seems to lie concealed for a large part of the time, but appears rather regularly, when opportunity to feed presents itself. Full specimens were taken at 12.20 a.m., 1.45, about 8.00 (2), between 2.00 and 3.00 (2), 3.00 and 4.00, 5.00 (2), 8.30 and 8.45. Empty ones were secured at 5.00 a.m., about 8.00 between 2.00 and 3.00 (2), 3.00 and .00, and at 9.35 and 9.45.
Mycteroperca venenosa (Rockfish or yellow-finned grouper)	1	1					Comments upon *E. morio* apply here, except that this species is less common. One taken at 9.00 a.m. was empty.

Kyphosus sectatrix (Chub)	3	3		3	3		Not very common; school by day about a few isolated points at Tortugas.
Teuthis caeruleus (Blue tang)	1		1				Common; one taken at 5.00 a.m. fairly well filled with algae—no microscopic examination. One each taken at 9.30 and 10.00 a.m. full of readily identifiable algae.
Pomacanthus arcuatus (Black angel fish)							One taken 5.00 a.m. had large amount of food in advanced stage of decomposition in lower intestine. Another taken at 9.00 a.m. gorged with freshly eaten food, including three species of algae and two sponges. The lower part of intestine full of disorganised, slimy refuse, as in the case above.
Chaetodon Ocellatus	1		1				This and the two species above are diurnal reef-rangers.
Microspathodon chrysurus	1		1				Very active by day; seldom goes more than a few feet from the shelter of the coral stacks.
Caranx ruber (Runner)							Very common in schools. Active all day long. Twenty specimens were taken from one school at 9.00 a.m. Five contained minnows. None had other food.

[8] As used in this table the term full really means containing identifiable food, even in comparatively small quantities.

included which are insignificant in themselves, but gain interest through their relation to others

Despite its brevity a number of trustworthy inferences may be drawn from the table.

The five Haemulidae, which group includes Anisotremus, are nocturnal, and given to schooling about coral heads or among gorgonians during the day. Brachygenys chrysargyreus belongs to the same family, schools regularly with its relatives at some of their rendezvous, and scatters with them at night throughout the open spaces. It may be taken after dark with a gill net as it swims along shore, where it never appears in daylight. It belongs to the bionomic group of which the grunts are notable members.

The snappers (Neomaenis spp.) studied select food which differs markedly from that of grunts, but secure it at the same time. The stomachs of 87.1 per cent of the 208 specimens examined were full in the morning or empty late in the afternoon; yet it is clearly an understatement to say that that proportion of their feeding is done at night, for identifiable food was found in 85.7 per cent of those taken in the morning and in only 8.7 per cent of those captured twelve hours later. This is equivalent to saying that, if equal numbers were examined in the two cases, 90.8 per cent of the full specimens would occur among the former.

Even this higher percentage fails to express the truth fully, for both the amount of food and the number of organisms contained in the fishes' stomachs are greater in the morning than in the evening. The possibility that some whose stomachs contain food in the late afternoon have eaten nothing since morning must also be taken into consideration, but at present too little is known of the rate of digestion in the snappers to permit one to speak with assurance upon this point. The only observations which bear upon the matter are these, that in the three hours between 5.00 and 8.00 a.m. the proportion of empty stomachs in the total catch increased from 14.3 per cent to approximately 32.2 per cent although in the interest of accuracy a slight correction is necessary, since the three species do not bear exactly the same ratio to one another in the two cases.

The feeding time of the yellow goatfish (Upeneus martinicus) seems to coincide with that of the grunts and snappers, though during the day individuals are sometimes seen stirring the sand with their barbels, as though searching for food. Holocentrus and Priacanthus may also be included in this group with slight probability of error. The number of these red fishes examined is small, but the results are consistent and a great mass of collateral evidence supports this interpretation.

What fishes feed by day is not made clear by the tabulated record; but this may be supplemented by observations which permit one to state with some confidence that most Labridae, Scaridae and Chaetodontidae, to mention no others, are diurnal.

From daylight to dark the activity of Iridio and Thalassoma is scarcely interrupted. Not one broken sea urchin is exposed upon the reefs, that they do not gather about and feed upon. The same is true of Lachnolaimus, except in so far as its comparative rarity limits its appearance. Scarus may be seen floating listlessly near the bottom at daybreak, and combining forces with Sparisoma a dozen common species grub industriously upon it all day long. At dusk the activity of all the Scarids changes; all disappear, except as an occasional one in its nocturnal color-phase lies half-revealed beneath the imperfect shelter of coral, gorgonian or projecting stone. Darkness also modifies the behavior of the Labrids which, perhaps favored by their smaller size, disappear even more completely. Species of Thalassoma, Iridio and Xyrichthys, three labrid genera, when confined in tanks, if the bottom be covered with loose sand to a sufficient depth, burrow into it and remain in hiding until the dawn.

To burrow or partially cover themselves with loose material is not uncommon procedure among fishes. Gnathypops aurifrons and maxillosa use their jaws for digging and their mouths for carrying away excavated material from narrow pits they make and occupy. Halieuticthys aculeatus, a batfish dredged in 45 fathoms, backs into the sand and uses its pediculate pectoral fins to throw an incomplete but almost impenetrable screen over its flattened body from its postero-lateral margins. Syno-

dus foetens lies flat, and may sink with one convulsive wriggle till only the dorsal surface of its head—or nothing—is visible beyond the depression beneath which it is buried. Flounders too, when resting, commonly bed themselves and diminish their visibility. In these various species each reaction, though their result be one, is performed in distinctive fashion.

The Labrids commonly use the pectorals alone in swimming, but when they are about to bury themselves, all their trunk and tail muscles are called into play, and in an instant they plunge head foremost out of sight. That the behavior of three genera should be consistent each with the other, and yet be without observed parallel beyond the family, is significant. The expedition with which the reaction is carried out, its regular recurrence at nightfall, the ease with which it may be evoked at any time of day by darkening the tank, and a tendency to perform it at a stated time, even in the absence of essential conditions, are also significant, for all alike emphasize its habitual character. Unfortunately it has not been noted in nature, but as that might occur only through some lucky accident, there can be little doubt that it is a normal response of the animals, and explains, at least in part their nightly disappearance.

The Chaetodons, Angel fish and Tangs are diurnal. The most interesting record concerning them in the table is that regarding Pomacanthus arcuatus. This shows that in the very long intestine of some fishes that ingest great quantities of food during the day much may remain many hours later. What may fix their feeding time, is not the presence or absence of material in the alimentary tract, but its apparent freshness. The grouped observations upon these fishes are consistent with the idea that they feed by day. They fit in well with what has been noted of their diurnal activities, and the fact that at night they are not on the open reef, but are more or less definitely sheltered among the heads.

It does not appear that the activity of all fishes varies so distinctly at different times. The yellow-tail and red grouper seem to feed when food is available, and to be governed by no other condition. From superficial agreement in habit with Epine-

phelus morio the same may be inferred for E. striatus and Mycteroperca venenosa. The chub (Kyphosus sectatrix) apparently schools by day in definite places, and one should expect to find it full in the morning and fasting in the late afternoon. At the moment, however, the records are too few to serve as a basis for sound judgement in the matter, and are not supplemented by decisive observations.

The nocturnal fishes fall into various subclasses according to the stations which they frequent by day. Some are rarely seen. Others congregate about the coral stacks, where all day long they lie idly at some little distance above the bottom; or swim leisurely through the openings between the heads, but in no sense seclude themselves in their recesses. Others, again, confine themselves less definitely to the region of the stacks, but, nevertheless, make such places distinct foci of their activity.

Those whose concealment is so complete that under natural conditions one would scarcely suspect their presence, and assuredly never appreciate their abundance, are the five red fishes. There are others that are rarely seen in the open. These are the green moray (Lycodontis funebris) and the ribbon-fish (Eques acuminatus). Of the former only one, and of the latter three have been encountered in the daytime; but these may not be significant observations, for not many of the morays have been taken at any time, and the other has also been obtained but rarely. Six were secured once when blasting at dusk, and one under similar conditions during the day. As collateral evidence tending to justify the exclusion of the two species from the bionomic group indicated, it may be stated, that the spotted moray (L. moringa) is not uncommonly seen with its head protruding from holes in the coral, and that the widow-fish (E. pulcher), though rarely met, seems also to have no aversion to appearing in the open when the light is strong.

The second subclass of the nocturnal fishes includes the grunts and their allies, Anisotremus and Brachygenys, the two snappers (N. analis and apodus) and the yellow goatfish (U. martinicus).

The gray snapper (N. griseus) represents the third subclass, and the chub may eventually find its place in this group. In

passing, it is worthy of note that there is a marked difference in the ratio of full to fasting specimens in N. apodus and N. griseus taken at 5.00 a.m. Empty stomachs are 3.6 per cent of the total examined in the former, while the proportion rises to 16.7 per cent in the latter. This may indicate that in nocturnal fishes irregularity in feeding is correlated with failure to conform strictly to the custom of schooling in closely restricted areas by day.

There are also obvious lines of cleavage among the diurnal fishes. Some are surface feeders from whose environment the bottom colors are so remote as to be practically non-existent. Others, such as Microspathodon chrysurus and Eupomacentrus fuscus, haunt the coral stacks, swim freely in and out of their interstices, but seldom venture more than a yard or two away. Finally, there are the wandering Labrids, Scarids, etc., which differ from one another both in the horizontal and vertical limits of their range. Knowledge of these distinctions must precede appreciation of the correlation of color with habit which prevails among reef fishes.

Two lists are displayed in table 3. The first includes species which may be observed moving freely in the mixed environment of the open reef. The second comprises others whose diurnal activity seems definitely centered in the coral stacks. Within the limit of present experience each is complete, and is approximately correct, though later modification may appear desirable. Hence it is instructive to analyze both with reference to the distribution of sandy gray among their members, since the two environments differ widely in the proportion of this color they contain.

The three blue-gray species differ sufficiently from the others to justify their exclusion from consideration in the present instance; yet to have passed them over without comment might have led to misunderstanding. Taking the remaining records as they are, we may say, then, that the color of three of 21 species in series II repeats that of sandy bottom. But in series I, which is only 50 per cent longer, there are almost five times as many which show evident adaptive color changes under the same conditions, or have permanent gray markings.

TABLE 3

Distribution of sandy gray in two groups of reef fishes of contrasted habit

SERIES I		SERIES II	
Species	Remarks	Species	Remarks
Angelicthys ciliaris		Abudefduf saxatilis	Blue gray; banded with dark brown or black. Upper third of light interspaces may be yellow
Calamus arctifrons	Gray, with yellow wash fading ventrally and posteriorly from frontal and occipital regions. Specimen with yellow suppressed came to food placed on clean white sand	Anisotremus virginicus	
		Amia sellicauda	
		Brachygenys chrysargyreus	
Caranx ruber	Largely bluish gray	Elacatinus oceanops	
Chaetodon capistratus		Eupomacentrus fuscus	
Chaetodon ocellatus	Gray; banded with brown	Haemulon flavolineatum	
Chaetodon striatus		Haemulon macrostomum	
Epinephelus morio	Has a gray color phase which is appropriately displayed, but does not appear as often as one should anticipate	Haemulon parra	A slaty or gray fish, which has, I believe, an adaptive gray color phase
		Haemulon plumieri	
Epinephelus striatus	Adaptive gray color phase	Haemulon sciurus	

TABLE 3—*Continued*

SERIES I		SERIES II	
Species	Remarks	Species	Remarks
Iridio bivittatus	Adaptive gray color phase	Holocentrus ascensionis	
Iridio maculipinna	Lightens in color over gray bottom	Holocentrus siccifer	
Iridio radiatus		Holocentrus tortugae	
Lachnolaimus maximus	Adaptive gray color phase	Lycodontis moringa	
Mycteroperca venenosa	Same as E. morio	Microspathodon chrysurus	
Ocyurus chrysurus	Blue gray; yellow lateral band expanding posteriorly to cover caudal fin.	Neomaenis analis	
		Neomaenis apodus	This species is capable of making an appropriate adjustment over gray bottom. It is commonly brownish yellow, with or without darker bands, and with stronger yellow upon its fins.
Pomacanthus arcuatus			
Pseudoscarus sp.			
Scarus (five species)			
Sparisoma (five species)	*Sparisoma abildgaardi* and *flavescens* have an adaptive gray color phase	Neomaenis griseus	Adaptive gray phase. This species is really intermediate in some respects between the average members of the two series

Sphyraena barracuda	Usually seen at intermediate or upper levels. When it sinks and rests near bottom, even the largest (four or five feet long) assume a banded pattern. In this state all sizes adjust themselves very distinctly to the shade of the underlying materials	Priacanthus cruentatus Upeneus martinicus	
Teuthis caeruleus			
Teuthis hepatus	Has a gray color phase shown over sandy bottom. Case resembles that of *E. morio*		
Thalassoma bifasciatus, *juv.* (T. nitidus and nitidissima.)	Adaptive gray color phase		
Upeneus maculatus	Gray; three dark spots on the side. Marked power of color change, but despite comparative abundance has not been observed except in its gray phase, and over bottom dominated by that color		

It may be urged that the fact that these lists are incomplete, or may reflect some unconscious prejudice of their compiler, detracts from their significance. But, whatever may be the case regarding the second suggestion, the first is invalid. Farther observation will merely emphasize the disproportionate occurrence of gray in the two series. Though this is not the dominant color among the heads and close about them, it occurs in both places, as well as upon the open reef. Hence perfect contrast between the two groups may scarcely be anticipated. Therefore, no conclusion seems more legitimate than that we have in the distribution of gray markings and color phases an additional instance of correlation of color with habit among fishes.

Brown is so common in the environment of the species of both series that it is scarcely worth while attempting to secure definite suggestion regarding its meaning by reexamining them. Other lists might be compiled that would yield results comparable with those above, but their presentation seems unnecessary.

Wholly aside from the frequent appearance of brown upon the fishes and in their environment, it is interesting to note that many species show a brown phase in its presence. To limit one's choice of examples arbitrarily, the following may be cited from the forms mentioned in table 3: E. morio and striatus, I. bivittatus and maculipinna, L. maximus, M. venenosa, Sparisoma abildgaardi and flavescens, S. barracuda, T. hepatus, N. griseus, and N. apodus. But, if brown may replace gray as an essential self-color, as it does in L. maximus and N. griseus when they change their environment, no functional conspicuousness may be ascribed to either, under the conditions in which it appears. Nor may this be imputed more rationally to any quality of contrast inherent in a combination of the two colors, such as appears in E. striatus (fig. 2); for the relative intensity of the two elements in the fish's pattern may vary continuously, as the proportion of light to dark in its environment changes with its change of position. Color photographs alone can convey the impression an observer receives in the field.

Although those of the common sort seem to show, that the phases at the ends of the graded series are obliterative, since

it appears that either would be more conspicuous than the other, if displayed in its stead. But the extreme phases of coloration are not essentially different from the intermediate ones, and may be separated in time by no more than five seconds, and, in space, by as many feet. Is the dark fish under the head less immune from attack than when it is a yard from shelter, that it should assume a more conspicuous pattern as it moves from beneath it? Or is this contrastive design in brown and gray a warning combination, whose serviceableness diminishes as the fish moves still farther into the open? It seems impossible to answer such questions in the affirmative; yet, if all are aspects of obliterative coloration, no combination of brown and gray that may appear upon the reef fishes in an unchangeable pattern may be assigned to another category, until it is proven that its possessor's normal range lies beyond the bounds within which the changeable forms show comparable phases.

Yellow is not of common occurrence in large patches upon the reefs, so it is virtually impossible to make extended observations upon its effect in evoking yellow phases, which, by the way, are not frequently encountered among the fishes, in spite of the fact that yellow marks or washes appear in abundance.

The red parrot fish (S. abildgaardi), with its notable power of color change and capacity of adjustment to light and dark backgrounds, in its darkest phase is distinctly washed with yellow over the whole top of its head. The immature blue-head (T. bifasciatus) has a gray phase with no yellow, assumed over sand, and a yellow phase which it resumes when it returns to more variegated surroundings. There are also indications that the porgy (Calamus arctifrons) dons and doffs its yellow with reference to the character of the underlying bottom. Thus yellow may occur in combination with brown and gray, colors to which no conspicuousness attaches in themselves. In some species it is suppressed, when its bearer passes into an environment to which the color is foreign, and reappears only upon its departure from that place Hence in its bionomic relations it seems to differ in no wise from the colors already considered.

The study of blue has yielded results that are unsurpassed in suggestiveness, but it is much more difficult in some respects than other equally important undertakings, and has been neglected in proportion.

This color seems to occur in nature in prominent markings, or as a self-color, upon no fishes which lie close upon the bottom, although Mast's ('14) flounders distinctly developed it under experimental conditions. Similarly, it is wanting upon those living just beneath the surface film, except as there are bluish reflections from the silvery lateral stripe of some of he Esocidae, for example. It appears in a great variety of tints and shades, ranging from the blue-gray of Abudefduf saxatilis and Ocyurus chrysurus through the pale blue of Gnathypops aurifrons and Scarus caeruleus to the richer blue of Thalassoma bifasciatus. It even attains the blue-black sometimes worn by Teuthis caeruleus and constituting the permanent ground color of a large Pseudoscarus that seems undescribed by systematists. Kyphosus sectatrix, Caranx ruber and Elacatinus oceanops complete the list.

It is noteworthy that a number of these fishes, and particularly the blue-gray types, have one habit in common that is as distinctive as their color. Abudefduf is equally at home breaking the water's surface above or near the coral stacks on quiet days, or deeper down among the heads themselves. Caranx and Ocyurus pursue schools of minnows and make the water fairly boil about them in calm weather. But the former may enter one's traps upon the bottom on occasion, and the latter feeds commonly upon Crustacea that never leave it. Kyphosus, which is distinctly darker and less blue than the others, has the same vertical range, but is neither seen at the surface as frequently, nor appears to come at all commonly into as shallow water. At Tortugas full-grown specimens appear by day, typically at mid-level or slightly lower, in water approximately 20 feet deep, over one small patch of broken bottom, or about a mass of sunken wreckage.

It seems more than accident that four out of ten species grouped by color should react habitually in a manner uncommon among

fishes as a whole. There are 52 species, for example, listed in table 3. Of these eight are included in the group under consideration. Among the forty-four remaining there is only one whose vertical range is coextensive with those above. It is questionable, indeed, whether this much should be conceded, for the gray snapper has been observed only twice actually breaking the surface as the others do. Each occasion was in the very early morning when the water was teeming with Annelids—the palolo worms—in their annual swarm. But even if the record be accepted without qualification, the proportion in the two cases stands as 40 to 2.27 per cent, or, in other words, the reaction in point occurs more than seventeen times as frequently among fishes of the blue series as it does among others selected at random.

There are additional observations which tend to support the hypothesis that a correlation exists between the habit of swimming well above the bottom and the development of blue pigment.

Gnathypops aurifrons (Jordan and Thompson, '04), a species known from only one specimen, taken at the Tortugas, is not very common, but one sometimes chances upon a colony of these interesting creatures in water 8 to 10 feet deep, or even shallower. They seem to spend a large part of their day floating inactively well up over their burrows. When undisturbed, they maintain themselves at an angle of 70 to 80 degrees with the horizontal for indefinite periods. Upon being alarmed they retreat into their shelters tail foremost, and remain in these tiny vertical pits until the disturbance has subsided. Even if unmolested they seem to withdraw toward dusk, and probably remain under cover all night. Hence, in spite of their fossorial and tubicolous instincts, it would appear that they are preeminently fishes of the middle depths, living in moderately shallow, open water, above the bare bottom in which all but one of some dozens of burrows observed were located.

Thalassoma bifasciatus and Iridio bivittatus occur so commonly together, that of them, if ever, one might say, "Here are two animals living in the same environment, but differing in color and pattern, therefore, it is perfectly apparent, that if one type

of coloration is protective, the other is not." Yet it appears that the ranges of the two species differ both in horizontal and vertical extent.

The Iridio occurs on the turtle-grass flats, to whose color it is able to adjust itself, but the Thalassoma does not range over the larger areas of this character. Not one has been taken among scores of Iridioes caught with the seine on grassy bottoms, nor do they come about baits set out in such places. In other respects the horizontal ranges of the two specise seem to coincide.

It was a long time before difference in their vertical distribution was noticed which was sufficient to suggest an appeal to experiment, but when this was undertaken it was demonstrated with ease that the Thalassoma rises readily to higher levels than the Iridio. If food be placed on coral heads of different height, Thalassoma will arrive first at the higher stations and may be the only visitor, although Iridioes swim about at lower levels. Again, if in a tank filled with water to a depth of 15 inches a vertical partition a foot high be erected, and specimens of the two species be placed on one side, a Thalassoma will swim over the obstruction first, and may recross twenty times, before the first Iridio finds its way over. Each experiment, therefore, clearly confirms the suggestion that led to their performance.

But though there is correlation between blue color and a fish's habit of swimming at intermediate levels in water of moderate depth, its meaning may not be apparent, for Wallace ('77) writes, and the explanation is approved by Poulton ('90), that "looking down on the dark back of a fish it is almost invisible, while, to an enemy looking up from below, the light under surface would be equally invisible against the light of the clouds and sky." However, at a depth of 15 to 20 feet in clearest water, under a tropical sky, one does not see the fishes above against a mere light or whitish background. The sky seems to have fallen, for one stands beneath a dome of blue, paling toward the zenith and deepening toward the horizon. Little is visible at 20 feet. The 'atmosphere' of the painter is thick; it is apparent almost at arm's length, and softens the outlines of all but the nearest objects.

In this Lilliputian world one has a demonstration of the effect of color and pattern which supersedes all argument. Fishes come from nowhere and vanish into nothingness, not because they have gone beyond the range of vision, but have wheeled at right angles. The secret involved in their disappearance is largely this, that, as they turn, foreshortening reduces, even to zero, tell-tale lateral markings upon which the eye had been fixed. Blue-gray bodies, or blue-gray bands upon bodies of other colors, are utterly resolved into the blue-gray haze which surrounds them, and one is wholly unable to perceive the outlines of objects easily within visible distance, as the distinctness of certain markings testifies.

Figure 8 illustrates the effect of differential visibility of two colors combined in simple fashion. It shows, in other words, the mode of operation of Thayer's 'ruptive pattern,' when it appears upon a countershaded body. It is so efficient in the case of Abudefduf, that when the fish is above the level of the observer's eye, its silhouette becomes indistinct, or is interrupted, at a distance of 4 or 5 yards, through the assimilation of some of its color elements with the watery background against which it appears. If it were wholly of the gray color, suitably countershaded, it would be almost invisible at that distance, except as its eye might reveal its position. Some simply colored species of the upper levels, when appropriately viewed, are, indeed, the veriest ghosts of fishes. Tylosurus is a notable example, and Sphyraena, though a large fish, has defied the camera times without number, largely on account of its lack of contrast with its background.

At this point it may appear to the reader, that the presence of the dark bars of Abudefduf, which prove so clearly that its partial invisibility is attained well within the limit of vision, is inconsistent with the argument that is being developed. For if natural selection guides the evolution of such characters, why, it may be asked, should the fish retain these marks, if its visibility might be farther diminished by their suppression. This fair question may be answered most readily by calling attention

to some differences in habit upon the part of the three species last mentioned.

The coloration of Tylosurus raphidoma is simple and apparently unchangeable. Its surroundings are equally simple and almost as invariable, for it has not been observed at any time more than a few inches beneath the surface, where it may lie for considerable periods, scarcely covered and almost motionless. Sphyraena has much in common with Tylosurus, but sometimes sinks to the bottom. As has been noted, under such conditions it changes in shade if necessary, and assimilates itself with the substratum. The vertical range of Abudefduf exceeds that of Sphyraena, and its relations with the bottom are more intimate, yet, in spite of the fact that its color is inconstant, definite adaptive color changes have not been observed in the species The fish bears instead a relatively stable color combination, which is compounded of characteristic tones from its environment.

There is, therefore, no inconsistency in Abudefduf's retention of its dark bands. Its coloration, like that of most other fishes as yet studied carefully in their relation to a complex environment, is a patent compromise. Its range is extreme, and as a result the vicissitudes to which it is exposed are many. But the risks it runs are well distributed; for its pigmentation is such that it is at once both more and less distinctly visible than it should be, if it were all of any one of the several colors perfectly suited to a limited portion of its range.

To conclude, there is undoubtedly correlation between at least the lighter blues and the probability of being seen from below against an aqueous background. Moreover, the interpretation which it seems necessary to place upon this fact is in no wise opposed by the appearance of others in combination with 'water colors' in one pattern.

There is little novelty in the facts concerning the distribution of green among the fishes studied. They are added in the interest of completeness and are thoroughly consistent with what has gone before. This color appears upon surface-swimmers, or upon bottom fishes in correlation with the occurrence of green

in their environment. It dominates the coloration of such types as Atherina, Stolephorus, Tylosurus and Hemirhamphus, which typically occur at high levels. Among representatives of this group at Tortugas the simple green color is distinctly interrupted only by a silvery lateral stripe, which seems to occur nowhere except upon fishes of this habit, and suggests that pattern, no less than color, may not be wholly fortuitious.

The correlation of green with its presence in the normal habitat of the animals displaying it is best shown by study of those seined upon reef flats covered by turtle grass. These are not of clear and uniform green color. The plant's flat, narrow leaves may be too short, and the vertical branches of the rootstock from which they spring too far apart for the sand to be completely concealed. In each tuft the older, outer leaves, whose death and detachment approach, are brown. While they retain their connection, or lie scattered among the others, their contribution to the color complex is important.

In this composite environment of which greenness is, nevertheless, the distinctive quality, fishes have been taken as shown in the following table.

The table includes twenty-four species. Eleven of these are wholly or largely of an unchangeable green color, or have definite green phases, which are assumed in the midst of green surroundings. That this should be true of so large a proportion of the total catch among the grass, is perhaps more than might have been anticipated. It certainly demonstrates that green, like the other colors, is distributed according to system, for with the exception of one group there is probably no other in which it occurs with such frequency. Yet the statement above is mechanical and expresses the truth imperfectly; the facts certainly merit more careful analysis.

These twenty-four species are not members of one bionomic association. Some of them may be excluded from consideration after brief review of the evidence.

Synodus and the flounder may both be seined on sandy bottoms adjacent to the grass flats. Both are accustomed to bury themselves more or less completely when resting. Both are marked

TABLE 4

Fishes seined on turtle-grass flats

SPECIES	REMARKS
Actaeis moorei	Small, brown and gray banded blenny; variable in coloration. Very common on rocky bottoms and about coral heads. Two taken
Amia sp.	Small, about one inch long, very dark brown. One taken
Calamus arctifrons, juv.	Yellowish with brown bands. Taken on a number of occasions
Corythroichthys cayorum	Brown and gray pipefish. Two specimens, female and male with eggs, taken at the same time
Flounder sp., juv.	Gray flecked with darker gray or brown. Two small specimens about one inch in length secured. Same species of the same size taken also on sandy bottom near by
Haemulon plumieri, juv.	Brown with faint blue or gray longitudinal stripes. Taken frequently; occurs elsewhere
Iridio bivittatus	Large power of color change; green, gray and brown phases. Ranges widely over almost all sorts of bottom. All sizes taken among the turtle grass
Iridio kirschii	Essentially a green fish. Seen or taken quite commonly upon the grass flats. Has not been observed elsewhere
Lactophrys bicaudalis, juv.	Dark green; no evidence of ability to change color. Taken repeatedly, but not encountered elsewhere
Lactophrys tricornis, juv.	Light green; otherwise as above
Monacanthus hispidus, juv.	Marked power of color change; appears in green, brown and gray phases. Both green and brown phases are seined in abundance among the grass. Same species of same size sometimes taken over sandy bottom
Neomaenis synagris, juv.	Yellow and silver in narrow longitudinal stripes. Taken rather commonly. Not seen elsewhere
Ocyurus chrysurus, juv.	Blue-gray and yellow, same as adult. Taken rather commonly. Young specimens of the same size are sometimes seen in other places

Scorpaena sp.	Small, marked with brown, gray and red. One specimen only
Siphostoma jonesi	Green, narrow brown stripe through the eye. Not noted elsewhere
Siphostoma mackayi	Green or brown, with dorsal surface commonly gray. Some half-grown specimens have large power of color change, and may turn almost uniform gray. Catch included mature females and males with eggs. None taken elsewhere
Sparisoma flavescens (?) juv.	Has large power of color change with green and gray phases. S. flavescens is common over the open reef; this fish is common on the flats
Sparisoma hoplomystax	Possesses very great power of color change with green, gray and other phases. Adjustment to green is made almost instantaneously. Taken frequently on the grass flats; not secured elsewhere
Spheroides spengleri	Green; dorsal surface turns gray when the fish swims over, or comes to rest on gray sand. All sizes taken, up to about 2.5 inches in length. Common on the grass flats; not taken elsewhere
Sphyraena barracuda, juv.	Several specimens taken 1.0–1.5 inches in length. Brown and gray; common elsewhere
Synodus foetens, juv.	Gray, marked with darker gray or brown. Taken also on sandy bottom.
Teuthis hepatus, juv.	One specimen; brown
Xyrichthys sp.	One specimen; green and gray phases
Xystaema cinereum, juv.	Gray, marked with darker gray or brown. Seined commonly over clear, sandy bottom near shore, as well as upon the grass flats

with colors which are repeated upon other fishes and upon some Crustacea (Portunus ventralis and Gonodactylus oerstedii) which may be taken in the same sandy region. They do not belong to the grass-flat fauna strictly defined, but are apparently swept up and gathered in from minor barren patches, which may be wholly bare. With perfect fairness both may be disregarded in the present connection. Xystaema should be rejected with them. Its colors are much the same as theirs; young specimens are common near shore, and others that are full grown may be seen frequently idling over sandy bottom along the beach during the day. Actaeis is no more justly considered a member of the group we are attempting to define, for it seems to be much more abundant in other places. Of those remaining Sphyraena at least may be seen and has been taken as commonly elsewhere, in the same early stages of development, with less efficient implements. This revision, every step in which is justifiable, leaves, then, nineteen species, of which eleven (57.9 per cent) repeat, or are capable of repeating the green color characterizing the environment in which they were secured.

There is another correction that may be applied. Fishes frequently encountered are, upon the average at least, more justly considered typical members of the local fauna than those that appear in the haul only rarely. Upon this basis, Amia, Scorpaena, Teuthis and Xyrichthys may be, or must be denied consideration until more complete records of their distribution are available. It may be that one of these or even more should be retained, but the relative frequency with which green occurs upon the fishes of the grass flats may probably be measured more accurately if all four are dropped, than if all are included in the calculation.

To prune the list no farther, although such action would not be wholly unreasonable, there remain fifteen species which may be regarded tentatively as characteristic of the locality in which they were taken. Ten (66.6 per cent) of these repeat the distinctive color of their environment. Except among surface fishes green occurs in no such ratio, and if these be excluded in

addition to the present group, it is probably impossible to name as many green fishes as are mentioned here, though the entire fish fauna of the Tortugas be drawn upon. Since this includes more than two hundred species (Jordan and Thompson, '04), the statement above is highly significant.

There is no reason to believe that all typical fishes of the grass flats are green. However, one fair inference may be drawn from the facts presented, i.e., the more nearly a species is restricted to these places at a given stage of its development, the more probable it is that its pigments include that color. It is apparent, therefore, that there is intimate correlation between the colors and habits of tropical reef-fishes. It is scarcely necessary to repeat that this fact, which has been demonstrated in the individual cases of red, gray, the lighter blues and green, is of the utmost importance in its bearing upon the problematic significance of animal coloration.

The working hypothesis enunciated in the introduction seems adequately sustained. Much more has been done, indeed, than test it by applying it to the reef fishes, but positive results in this phase of the investigation support the suggestion of the obliterative effect of color and pattern, which flows from the all but universal existence of countershading and the common occurrence of adaptive color changes within the group.

DISCUSSION OF RESULTS

Fishes which range by day amid the most varied surroundings display the greatest variety of colors. They may also possess the best developed means of defence. In this event there would be secondary correlation of vivid color combinations with armament and 'distastefulness.' Whether this is so must be determined before appraising the fact above as a confirmation and extension of the partial truth, or a demonstration of error existing in the color hypotheses that postulate conspicuousness.

In the diversity and richness of their coloration no large group of fishes surpasses the Labrids and Scarids. These are, therefore, fit subjects for the present inquiry. If their distasteful-

ness is fictitious, this should appear from the analysis of the stomach contents of potential enemies. It was with this idea in mind that the feeding habits of snappers were examined more closely than those of other species, for none seemed more likely to exact its toll from unprotected forms.

From the snappers' stomachs several perfectly recognizable specimens of Iridio bivittatus were taken. The jaws and pharyngeal teeth of fishes of this genus, and probably of this species, are found very commonly with vertebrae and other structures which are slowly digested. Four specimens of Scarus punctulatus which were only slightly macerated were secured. What appeared to be remains of the same species was obtained in a number of cases. Jaws and pharyngeal teeth, differing sufficiently among themselves to suggest that others of the genus are freely eaten, are frequently recovered, and one Scarus croicensis was doubtfully identified. Sparisoma hoplomystax was represented by one specimen, and another in an advanced stage of decomposition was assigned to this species. If this determination is incorrect another must be added to the list of edible forms. One Sparisoma flavescens was also obtained.

Five species of the Labrids and Scarids in Jordan and Evermann's Fishes of North and Middle America were described from types, or were known to the authors only from specimens found in the stomachs of snappers or groupers. When these are added to the five mentioned in the preceding paragraph it makes a total of more than 11 per cent of edible forms in the two families. Though many of the Scarids and some of the Labrids when full grown are too large to be preyed upon by the snappers the list presented is certainly capable of extension. It already includes some brilliantly colored types, hence it seems incredible that these creatures are protected by distastefulness advertised by warning coloration. It is also clear that they are not secure from attack at night, and doubtful whether they ever enjoy immunity unaccounted for by their speed, alertness and inconspicuousness, for some at least wander far from the shelter of the heads, and do not instinctively fly to cover when alarmed.

TABLE 5

An annotated list of 'Conspicuous' fishes

SPECIES	REIGHARD'S DESCRIPTION	REMARKS
Abudefduf marginatus (saxatilis)	Yellow and black banded	Photographs show, as the observer sees, that when the fish is viewed from below its blue-gray ground color dissolves and is lost in the water surrounding it. Reighard's excellent plates show that its dark bands may operate in the same way under other conditions. Three full-grown specimens were taken from snapper's stomach
Angelicthys ciliaris	Body chiefly bright blue, fins chiefly bright yellow, *preopercular spines*	
Amia sellicauda	Scarlet, two black spots	This species is very common, yet has not been seen in the open by day. One specimen noted in full view in a broad cleft among the heads. Three individuals taken from snapper's stomachs, one engaged in oral gestation
Anisotremus virginicus	Yellow, with two black stripes, black caudal spot	The adult is differently marked, but with essentially the same colors. Its pattern is changeable, but under what conditions has not been satisfactorily determined
Caranx crysos	Silvery, yellow stripe	Reighard states in another connection that this species may be protectively colored
Chaetodon capistratus	White, black bands and black spot; *large dorsal and anal spines*	
Chaetodon ocellatus	Silvery ground, two wide black bands. Fins yellow in part. *Large dorsal and anal spines*	Reighard's Plate III, figure 5, shows this fish in a typical setting. The focus is perfect, for even the smudge of dark color at the base of the dorsal fin and the spot at its posterior angle are clearly shown, yet it seems fully as inconspicuous as the gray snappers over sandy bottom near shore (Plate 4, fig. 9).

TABLE 5—*Continued*

SPECIES	REIGHARD'S DESCRIPTION	REMARKS
Chilomycterus schoepfii	Greenish, heavy black stripes. *Body armed with spines*	Have never observed this species. Reighard had only four specimens
Echeneis naucrates	Broad stripes, nearly black and white	Comparatively little difference in the shade of dorsal and ventral surfaces. Maintains no constant relation to the source of light
Elacatinus oceanops	Broad light and blue-black stripes	
Eques pulcher	Black and white conspicuous stripes	Not common at Tortugas; only two or three observed
Haemulon flavolineatum	Blue and yellow stripes	Capable of changing its color to some extent
Haemulon sciurus	Blue and yellow stripes	Nocturnal feeder; schools about heads and among gorgonians during the day. Has marked ability to change its color, as Townsend ('09) states, but has not been observed showing more than one phase upon the reefs. Another was displayed by specimens about the dock and in the moat at Ft. Jefferson. The extent to which the fish's color changes are controlled by its surroundings is not yet clear. Lycodontis funebris eats this and other species of grunts; one trapped specimen disgorged six of medium size
Teuthis (Hepatus) hepatus	Black; lancet on caudal peduncle	Color very variable; ranges from black to pale gray. Is most frequently seen in an olivaceous phase among algae of much the same color
Iridio bivittatus	Gray, dorsal line and sides with broad dark brown stripes	Has adaptive gray, green and brown phases. Is very commonly eaten by snappers. Variability noted by Reighard, who considered it less conspicuous than most of the species in this list
Neomaenis (Lutianus) griseus, juv.	Yellow and black banded	The adult gray snapper exhibits very distinct adaptive changes in shade; I am not sure, however, that the form to which reference is made is not the young of N. apodus

Eupomacentrus (Pomacentrus) leucostictus	Half orange, half blue or black	The species of this genus are not readily identified. There is a blue and orange, and a black form, whose ranges are distinct. The former occurs in the open and the latter among coral heads. None have been seen changing their color in nature, though Mr. Mowbray of the New York aquarium informs me that the brighter specimens of E. leucostictus turn dark in the exhibition tanks there
Eupomacentrus leucostictus	All black	
E. planifrons	Half orange, half blue or black	
Pomacanthus arcuatus	Black, five conspicuous yellow bands; blue spots. Preopercular spine	The adult is decidedly a dull colored fish, with some ability to change its color
Sparisoma flavescens	Mottled olive and brown (protective colors)	This should perhaps be omitted from a list of 'conspicuous' fishes. Its adaptive color changes are very definite—incidentally, it is the first in which they were observed—but it is not superior to all brighter forms in the fitness of its coloration or its ability to change it. It is eaten by the snappers
Thalassoma bifasciatus	Blue head, green body, black band between	One specimen alone of many hundreds observed conformed to the terms of Reighard's description. Commonly the blue head and yellow green body are separated by a series of three bands each approximately a centimetre in width. The first and last are blue black, and the intermediate one light blue. Only one color phase has been observed during the day, but at night the dark bands are much less distinct or may wholly disappear. This is not a conspicuous fish when viewed from a lower level
Thalassoma nitidus	Very variable, usually green with lateral purple stripe	This I believe to be the young of T. bifasciatus. It has gray and yellow phases that may be induced at will by leading it into appropriate surroundings; green ones have not been observed. Reighard considered this and the preceding species less conspicuous than the others except I. bivittatus, S. flavescens, and Caranx crysos

The reader may justly challenge the assumption that the two families cited fairly represent fishes of high color. No exception may be taken, however, to an argument based upon analysis of the facts regarding a list of species compiled by one familiar with them, convinced of their conspicuousness, and unaware of the use to which his product is to be applied. Such a list taken from Reighard's ('08) Field Study of Warning Coloration, appears in table 5. In the second column its author's comments upon the included species are given in full. The remarks in the third column require no explanation.

Warning significance has been imputed to the colors of animals for many reasons, such as assumed offensiveness in taste or odor, or possession of poisonous qualities, or unpalatability depending upon scaliness, hairiness or the presence of some form of sting. But no claim has been made that one sort of disagreeable attribute rather than another may be more fitly associated with types of warning coloration. It is apparent, therefore, that an additional method of testing whether color combinations of distinctive quality are correlated with distastefulness or immunity lies in the examination of species which possess the one sort of defensive attribute whose occurrence may be determined with approximate accuracy, i.e., organs capable of inflicting painful bodily injury.

The fishes which may be fairly included in this group are listed with their salient characteristics in the following table. All are countershaded.

Fishes of high color are freely eaten; 'conspicuous' and specially protected types reduce their visibility as commonly as others by adapting their hues to their surroundings; and, in pigmentation, the species enumerated in tables 5 and 6, do not differ from groups of the same size selected at random. Hence, in this class of animals there is not even secondary correlation of bright color with special means of defence, and, in so far as appears from the evidence, the hypothesis of obliterative coloration remains in undisputed possession of the field.

This situation should surprise no one familiar with the literature of animal coloration, since a student of the subject must be

TABLE 6

Coloration of fishes provided with special means of defense

SPECIES	APPROXIMATE AVERAGE SIZE	REMARKS
Angelicthys ciliaris and isabelita	10 inches	Scales firm; fin spines stout and sharp; long sharp, flat spine at the lower angle of the preopercle, with shorter ones above it. Olive and yellow in color with a few minor blue markings; little or no power of color change
Diodon hystrix	10 inches	Body capable of inflation and covered by strong, sharp, erectile spines. Spotted and blotched with brown on a somewhat lighter ground color
Holocentrus ascensionis	8 inches	Fin spines strong. Every free bony margin of cranial or opercular elements or scales sharply serrate. Chiefly red; common but rarely seen on account of its strict nocturnal habit
Lycodontis funebris, moringa and verrili	3 –5 feet 2.5–3 feet 2.5– feet	All have strong, sharp teeth and are pugnacious. L. funebris is almost uniform, rather dark green in color; L. moringa, mottled brown and yellow, and L. verrili dark brown, its fin margins black edged with white, and its body marked with fine brown spots darker than the ground color
Pomacanthus arcuatus	10–12 inch	Strong preopercular spine; fins and scales much as in Angelicthys. Color dark gray-brown; a patch of lemon yellow on the posterior face of each pectoral fin. The young are marked with alternate yellow and brown bands in the ancestral Chaetodontid pattern. The adult is able to change its color within rather narrow limits
Prionotus sp.		Has a well developed coat of mail and additional, stout, sharp spines about the head. Colors dull; only one specimen: seemed to have very marked power of instantaneous, adaptive color change
Scorpaena plumieri	9–10 inches	Sharp spines about the head. Color changeable and adaptive; pattern complex. Dermal outgrowths simulating algae, etc., aid in concealment
Sphyraena barracuda	3–4 feet	Very powerful sharp teeth; a veritable sea-wolf. Coloration changeable and adaptive
Teuthis caeruleus	10 inches	Has a movable lancet on either side of the caudal peduncle. Color very changeable; ranges from bluish gray almost or quite to black. Color changes not studied in relation to the fish's environment
Teuthis hepatus	8–9 inches	Armament as in case of T. caeruleus. Color ranges from gray to black; usually seems adapted to the creature's surroundings

impressed by the uniform absence of effort to demonstrate that that conspicuousness exists, whose occurrence it is undertaken to explain.

Wallace's ('91, p. 228) statement, that to color for recognition we owe most of the variety and much of the beauty of the colors of animals; that it has caused at once bilateral symmetry and general permanence of type, and that its range of action has been perhaps equally extensive with that of coloration for concealment, ultimately rests upon what "a little consideration will show." Reighard has experimental evidence of the insufficiency of the hypothesis of warning coloration as applied to certain tropical fishes, but his belief in immunity colors is based upon the alleged fact that "even casual observation shows that they are highly conspicuous." Roosevelt ('11, p. 176) also declares that "the most elementary study of prongbucks amid their natural surroundings shows that there is not even the smallest foundation for Mr. Thayer's theory."

That the conspicuousness so lightly assumed is a subjective phenomenon is capable of demonstration. Bristol ('03) has expressed his opinion of seven or more Bermudian species, all of which occur at the Tortugas, although only three are included in table 5. Chaetodon capistratus he considers protectively colored. Midway between such extremes as the squirrel fish and the hind he places the angel fish and the tangs (Teuthis spp.), yet to Reighard all three seem highly conspicuous. In addition Bristol's distribution of his material points the same moral as what has gone before. He recognizes, first, types of warning coloration with bright hues, simple patterns and little ability to change their appearance; second, protectively colored species, whose scale of coloration is not so high, whose pattern is complex, and whose power to change color is great. But to an intermediate position he assigns others whose "color is medium, pattern not complex, and range of color change less than in the second group." Again, of the seven or more species which he mentions the tangs, for example, have very great power of color change, but their pattern is as simple as that of his warningly colored forms. Neither, I think, is the blue parrot placed in the

second class more able to change its color than the green one which is assigned to the first, and the complexity of its pattern is distinctly less than that of Sparisoma viride, if the reference is intended for that species. Hence upon his own testimony it would appear that Bristol dealt with a series in which there are no natural breaks.

The ease with which observers become convinced of the conspicuousness of some animals seems to rest upon a twofold error which may be illustrated by reference to fishes, for the presence of red and blue species in surroundings where these colors do not commonly appear, and the occurrence of forms of different color side by side may be justly held responsible for much of the persistent misunderstanding that has prevailed. In the first case, seeing nothing in the background the repetition of whose tones one might suppose to minister to the inconspicuousness of the creatures upon which they appear, one is led to believe that there is nothing, and might even infer that the colors of the animals are out of keeping with their surroundings and, therefore, conspicuous. In the second instance one is tempted to conclude that color is not of great importance, since all sorts of fishes seem to mingle freely at one station.

There is a natural fish-trap, or weir, formed by a hook at the tip of a small promontory beside the eastern entrance of the channel cutting off San Juan from the mainland of Porto Rico. Atthis place, in an area measuring perhaps 50 by 75 feet in extent, there were counted in the few minutes it required to wade about and examine it thirty-six species of fishes, including all types but the red. In viewing such a motley assemblage, since many of the shades noted obviously fail to repeat local coloring, one who does not realize the unnaturalness of the situation can scarcely be blamed for concluding that many of the hues are either functionally conspicuous, at at least without adaptive significance.

The same condition prevails in less marked degree on every coral reef under circumstances concerning which no plea of abnormality may be entertained. But one gradually learns that first impressions are unreliable. The wealth of species and

diversity of coloration to be observed at particular points is due simply to the fact that most extensive overlapping or stratification of specific ranges occurs there; they are foci at which different habitats converge or interdigitate in greatest numbers. About the coral stacks, for example, the diurnal reef-rangers meet and mingle with fishes, which all day long swim in and out through the interstices between the heads. An occcasional red recluse is seen dimly or emerges momentarily from the shadow, and conveys the impression that its kind associates closely with those which lie at ease or move sluggishly about, as digestion of last night's gains goes on apace. Others rising or falling, breaking the water's surface or lost in the gaping darkness of the reefs, like animated shuttles bear the weft that binds them in an almost indissoluble whole.

When one becomes steeped in such facts as these, much of the current literature of animal coloration is hopelessly unconvincing. One reads without enthusiasm such sentiments as the following:[9]

> On every Indian lake three species of kingfisher pursue their profession cheek by jowl. . . . It is obvious that all . . . . of these diversely plumaged birds cannot be protectively coloured. It may perhaps be objected that their piscatorial methods differ in detail. We admit that this is the case, but would maintain, at the same time, that these comparatively slight differences in habit do not account for the very striking differences in plumage.

In view of established fact one feels that the significant idea expressed above is precisely that which its authors dismiss as immaterial; but, hereafter, although the correlation between color and habit is conclusively demonstrated in only one class of animals, the burden of proof of such statements as the second in the preceding paragraph lies with those who make them, whether they refer to distinct species or to the two sexes in dimorphic forms.

It may not be settled conclusively at present whether the correlation between color and habit demonstrated in this paper is due to natural selection or the action of the environment, since intensive researches upon the degree of protection afforded their

[9] Dewar and Finn ('09), p. 202.

possessor by its color and pattern under natural conditions have not been undertaken. Young's ('16) feeding experiments, which among those coming to my attention, a few imperfectly reported by Poulton and Sanders ('98) excepted, alone possess positive value, suggest, however, that the latter has directed the development in question.

It is not clear that all advocates of the hypothesis of direct action would maintain its sufficiency to account for such detailed agreement in color as appears to exist, between organism and environment in some cases. Allen ('77, '07), referring chiefly to North American birds, writes:

> The southward increase of color correlates with an increase of atmospheric humidity and temperature, and consequently with the protective influences of luxuriant arboreal vegetation and clouds; and, conversely, loss of color accompanies excessive aridity, a scanty vegetation, and an almost cloudless sky, the conditions, in short, of all others the most powerfully effective in the blanching of color; and again, the somber, dusky tints of the northwest coats accompany the most humid conditions of climate and the conditions generally most favourable for the protection or preservation of color.

This excerpt seems to imply appreciation of no such agreement in color between birds and their environment as exists in general among the reef fishes; yet it may, perhaps, be fairly inferred from Chapman's ('94) observations upon the birds of Trinidad that the truth regarding them is the same. If the inference is correct, it does not appear that Allen's hypothesis can explain existing conditions, for it involves nothing more on the one hand than reduction of color through blanching, and the preservation, upon the other, of any hues, congruous or otherwise, which may be developed in the process of metabolism. Packard's ('04) conception of the relation between organism and environment, upon the contrary, postulated more definite control of the creature's chromogenic function, since he held that alleged cases of Müllerian mimicry might be explained by convergence due to the action of similar physical or climatic causes.

All the facts which the present research has yielded conform to the terms of Thayer's hypothesis of concealing coloration. How, then, has it happened that twenty years has failed to bring

recognition of its essential truth? Biologists may have been surfeited with theories, and its first presentation ('96 a, '96 b) was certainly inadequate for a concept of such revolutionary tendencies. Still, it never lapsed into obscurity, and has even aroused more opposition in some quarters as its exposition has become more detailed.

Thayer's work must remain the inspiration—and despair—of many who will eventually accept its main contention. It is full of suggestion of things yet to be done, but the keen eye and cunning hand of a master workman have established an almost unapproachable standard of excellence, and his active mind has anticipated the results of investigation in almost every major field of inquiry within his province. But, with all its merit, it has one fundamental defect, which explains its failure to win general approbation. It is many-sided, yet is predominantly a work of exposition or deduction. As Gerald H. Thayer has it in the introduction to Concealing Coloration:

> For the most part, we do not draw hypothetical conclusions from facts; but we *reveal* certain beautiful facts hitherto unknown; we disclose and explain the remarkable power of several naturally applied laws of optical illusion—as these applications stand, by whatever causes produced, and as all may see them. That is, we show and analyse the *concealing* power of the colors of animals as they exist today.

That countershading is the prime factor in the etherealization of solid bodies, is not an abstraction based upon the study of animals of unsubstantial appearance; it is the conclusion of a purely deductive process of reasoning. By shading a plane figure we endow it with the semblance of solidity; by countershading a solid object, appropriately lighted, we invest it with unreality, and leave it lacking only fitting background in order to become wholly invisible. A system of countershading appears upon the bodies of animals; hence, and this is Thayer's chief contention, their coloration is obliterative, since it is established upon this principle. This is probably true, but sceptics maintain that the fitness of coloring necessary before the deception may be complete is not proven.

Thayer's second thesis is:

> Colors, patterns, and appendages are the most perfect imaginable effacers under the very circumstances wherein such effacement would most serve the wearer. . . . Patterns on animals' coats are the utmost that nature can do in opposition to the potent vicissitudes of silhouetting. . . . Their bold coloring minimizes, not increases their conspicuousness.

The optical principles involved in this series of propositions are explained with precision, and their necessary effects demonstrated by a multitude of devices of surpassing ingenuity. Yet, when all is done, the most friendly critic, who has not undertaken supplementary researches, hesitates to believe, for it is perfectly clear that, if Thayer's reasoning is correct, the colors of animals should repeat those of their environment, and this is not known to be so. Great numbers of positive illustrations are offered, to be sure, but there are greater hosts of animals to draw upon; it is not difficult to find among others explanations that seem fanciful, and one animated by nothing more than a spirit of scientific doubt may suspend judgment, rather than become the victim of a plausible hypothesis supported by selected and unrepresentative material.

It is at this point that Thayer's method reveals its intrinsic weakness. If he had been able to supplement his demonstration of the effect of countershading with proof rather than suggestion that color is intimately correlated with habit, his theories would probably have displaced those that preceded them, as soon as they could be disseminated among zoologists. As it is, when an explanation of sexual dimorphism among birds is required, he falls back upon difference in the nesting habits of the sexes, which is certainly permissible when these are known to be different; but Dewar and Finn ('09) assert that there is dimorphic coloration is species in which the male shares the burden of incubation. Now and hereafter the existence of other differences in habit may be inferred tentatively, but this has not been legitimate heretofore, since it would have involved the support of one hypothesis by another for which no shadow of justification appeared.

Similarly, there has been no conclusive reply to Roosevelt's ('11) objection—which is not original, but is stated recklessly by him in its most uncompromising form:

> The claim that a large number of birds each coloured entirely unlike the others both in tint and pattern and all living in substantially the same surroundings are all concealingly coloured can never be tenable. . . . . Here near my house, for example, there is a hedge or tangle running on one side of the garden. . . . In this are to be found thrashers, towhees, catbirds, chats, indigo buntings, and Maryland yellowthroats (together, of course, with other birds). The fact is, not one of these birds really has a concealing coloration.

Unprejudiced inquiry has exposed the fallacy involved in these remarks, but Thayer's hypothesis will require the labor of many professional naturalists, before it assumes the position to which it seems justly entitled. As a contribution to its sounder establishment it is a pleasure to state that the coloration of tropical reef-fishes is correlated with their habits, repeats in general the color notes of their environment, and is obliterative under natural conditions.

SUMMARY

Fishes are countershaded; color changes, which are common even among the most gaudy, tend to assimilate them with their environment; and, in general, their colors repeat those of their surroundings. Specially defended types are not unlike others in pigmentation, nor inferior to them in their ability to effect adaptive color adjustments. Finally, there is no evidence that bright colored species enjoy greater immunity from attack than their fellows, for they constitute a large proportion of the food and may be readily identified in the stomach contents of predaceous forms.

These statements which rest upon a great body of verifiable observations, are consistent with the Darwinian hypothesis, but inconsistent with the assumption that animals of high color possess more than minimal conspicuousness under natural conditions. They impel one to reject the hypotheses of warning and immunity coloration, signal and recognition marks, and

sexual selection, at least in so far as they may ever have been supposed to apply to these forms. Upon the contrary, they confirm Thayer's conclusions regarding the obliterative function of color and pattern, emphasize the common occurrence of adaptive characters among animals, and suggest that their evolution has been guided throughout by natural selection.

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PLATE 1

EXPLANATION OF FIGURES

1 Nassau grouper (Epinephelus striatus) in a dark banded phase beside gorgonians, of which the most conspicuous are the finely divided Gorgonia acerosa,[10] and the fan-shaped G. flabellum whose color varies between dark brown and blue. The bottom is gray and covered with coarse fragments of dead coral, largely Acropora cervicornis. The fish's dark markings are brown, the ground color, gray, more or less distinctly suffused with yellow.

2 The same fish, as it appears upon leaving such surroundings. A colony of Plexaura flexuosa is seen in the background, and two large sea urchins (Centrechinus setosum) in the upper left hand corner of the picture. Attention may be called to the fact that this and the following figure show that the fish above is fully exposed, in spite of all suggestion to the contrary.

3 to 5 were taken in rapid succession, when upon another occasion the same individual as above was led into the open by offering it food.

3 This shows essentially the same phase as the preceding figure, though it is perhaps a little lighter.

4 The fish is now essentially gray, and its visibility could scarcely be diminished. The creature seems less substantial than its shadow, yet is in such perfect focus that in prints made directly from the negative the dark line of the depression between its closed lips is perfectly apparent, as is also the pupil of its eye. The bottom is clean white sand. There are several specimens of Iridio bivittatus in the foreground.

5 This records a change that occurred when the fish had approached the dark bait closely. It became even more pronounced, but at present its meaning is not positively determined (p. 551).

6 Two hogfishes (Lachnolaimus maximus) over bare sandy bottom. They are uniformly gray except for a dark spot at the base of the soft dorsal fin, and a dark wash over the frontal and occipital regions. The large fish in the centre of the picture is so definitely in focus that its individual fin rays may be distinguished.

7 A specimen of the same species photographed among brown gorgonians (Plex aura homomalla). Its color is now dark reddish brown mottled irregularly with light. Its fin spines also are visible. The change from the phase here shown to that presented above, or *vice versa*, requires only an instant, and is subject to control by the experimenter. A specimen of Iridio bivittatus appears just behind the larger fish.

8 A branching cora[1] (Porites) growing upon a massive Orbicella head. The picture, in which five specmens of Abudefduf saxatilis appear, was taken from the bottom looking up on a line tangent to the head and making an angle of perhaps 30 with the horizontal. One or two fishes a little to the left of the middle show the effect of blue-gray ground color in blotting out their contour, as it appears against the watery background. Four or five dark stripes with alternating light ones alone mark their position. Another near the face of the coral head and toward the lower part of the picture faces the observer. Its stripes are little in evidence, and from it one may get some idea of the way in which individuals vanish when they wheel at right angles.

Several small specimens of Haemulon sp. are also shown more or less distinctly.

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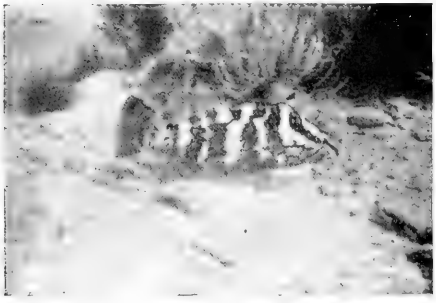
[10] The names of the gorgonians are kindly supplied by Dr. L. R. Cary of Princeton University.

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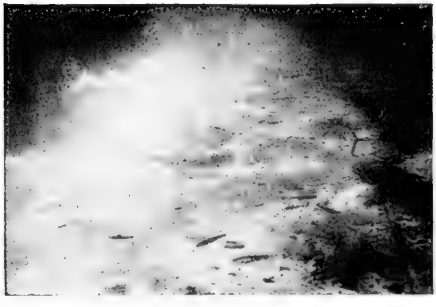

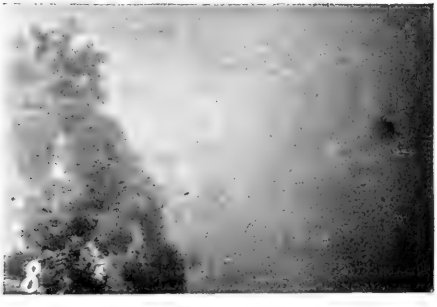
8

SUBJECT AND AUTHOR INDEX