

STUDIES IN NUCLEAR CYTOLOGY

INTRODUCTION TO WORK SUBMITTED FOR EXAMINATION FOR  
THE DOCTORATE OF SCIENCE, EDINBURGH UNIVERSITY.

by

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My work falls broadly into three groups of subjects :

1. Studies on the determination of secondary sex characters in invertebrates : papers 2, 3 and 4.

These papers describe research on one of the first problems which engaged my attention : the causal determination of the differentiation of secondary sex characters in invertebrates. While I was a student at Naples in 1938/9 I was impressed by the problem of parasitic castration and sex reversal in crustacea. The problem has great physiological and genetic interest it has also great complexity. This became apparent to me when I found that the effects of epicarid isopods on their prawn hosts is quite unlike the action of similar isopods on Upogebia, this latter animal showing all the familiar features of sex reversal when parasitised. The castration of unparasitised prawns by means of X-rays gave evidence that the cyclically developed female secondary sex characters are dependent on the presence of a maturing ovary for their differentiation : the same situation was found, though not published, for the adult female characters of the crab Pachygrapsus, though this animal undergoes sex reversal, male to female, on being parasitised. It was hoped that castration of males might be accomplished by means of X-rays since this would supply crucial evidence for the resolution of the whole problem. However, too high a level of irradiation is necessary for castration of male crabs, which die before completing a further moult. Surgical castration likewise proved impracticable and the problem was left in this state.

2. Studies in chromosome cytology : papers 5,6,7,8,9,10, 11,14,(16,17),19,20 and 21.

My interest in chromosome cytology, originally aroused by Dr J.R.Baker and Dr E.B.Ford, F.R.S., at Oxford

and further stimulated by contact with Dr C.D. Darlington, F.R.S., then of Merton, London, led me to study a number of problems in this field. It is with these problems that the main bulk of my papers are concerned.

Paper 6 demonstrated that a multiple chromosome mechanism determines sex in the earwig. The situation is complicated by the fact that males may have two alternative chromosome constitutions and that the Y chromosome in this species is dicentric; the mechanism is considered to be related to the abnormal sex ratios in earwigs which are found in the wild.

Paper 8 describes the behaviour of a supernumerary autosome at meiosis in the grasshopper Mecostethus.

Paper 9 describes how a fertile plant hybrid has arisen by the hybridisation of two autopolyploid species: this mechanism is an alternative means of arriving at an allopolyploid constitution.

Paper 10 describes the discovery of low temperature sensitive heterochromatic segments of the chromosomes of newts both at mitosis and meiosis and leads to the inference that desoxyribose nucleic acid ~~is~~ plays an important role in chromosome spiralisation.

Paper 11 describes the upsets to the spindle mechanism at mitosis which can arise as a result of exposure of newt cells to low temperature or to the action of colchicine.

Paper 14 demonstrates that the centromere is not necessarily a barrier to chiasma interference at meiosis, as was previously held to be the case. This was first shown for the mosquito Culex and has subsequently proved true for many other animals.

Paper 19 shows how chiasma interference across the centromere is suppressed when multivalent chromosome associations are formed at meiosis.

Papers 20 and 21 describe the reduction in chiasma frequency and the localisation of chiasmata in hybrids between geographical races of newts. Evidence is also given of the origin of a chromosomal translocation which distinguishes the chromosome complement of one race from the other two and which can act as a barrier to genetic leakage between these races.

Papers 16 and 17, which do not rightly fall under the heading of chromosome cytology, are nevertheless related to this theme since they are concerned with the possibility of a connection between heterochromatic chromosome segments, ribose nucleic acid synthesis and

growth rate determination.

3. Experimental work with giant nuclei : papers (12, 13), 15, 18, 22, 23.

Partly as a result of a controversy over the distribution of nucleic acid in the cell (papers 12 and 13) and more generally because, having worked for some years in the field of descriptive cytology and cytogenetics, I was impressed with the need for an experimental attack on the many outstanding and general problems concerning the cell nucleus, I turned my attention three years ago to the exploitation of giant nuclei.

A great deal of this work is not yet ready for publication : it is the general line of research in which I am most actively engaged at the present time. Since, however, a number of observations have already been made, these are summarised in a final manuscript entitled " Experimental studies on amphibian oocyte nuclei". This should be read as an introduction to my published papers in this field.

LIST OF PUBLICATIONS

H.G.Callan

1. 1938. "Cell size in millipedes". NATURE, 141, 247
2. 1939. "The absence of a sex-hormone controlling regeneration of the hectocotylus in Octopus vulgaris Lam.". Pubbl.Staz.zool.Napoli, 18, 15
3. 1940. "The effects of castration by parasites and X-rays on the secondary sex characters of prawns". J. exp. Biol., 17, 168.
4. 1940. (with F.G.W.Knowles) "A change in the chromatophore pattern of crustacea at sexual maturity". J. exp. Biol., 17, 262.
5. 1940. "The chromosomes of the cymothoid isopod Anilocra". Quart.J.micr.Sci., 82, 327.
6. 1941. "The sex-determining mechanism of the earwig, Forficula auricularia". J.Genet. 41, 349.
7. 1941. "Determination of sex in Scalpellum". NATURE, 148, 258
8. 1941. "A trisomic grasshopper". J.Hered. 32, 296.
9. 1941. "The cytology of Gaulthetia wisleyensis (Marchant) Rehder: a new mode of species formation." Ann.Bot.Lond.,N.S.5, 579
10. 1942. "Heterochromatin in Triton". Proc.roy.Soc. B. 130, 324.
11. 1943. (with H.N.Barber) "The effects of cold and colchicine on mitosis in the newt". Proc. roy. Soc. B. 131, 258.
12. 1943. "Distribution of nucleic acid in the cell" NATURE, 152, 503
13. 1944. (with H.N.Barber) "Distribution of nucleic acid in the cell". NATURE, 153, 109.

14. 1947. (with G. Montalenti) "Chiasma interference in mosquitoes". J. Genet., 48, 119.
15. 1948. "Alcune proprietà fisiche della membrana nucleare". Ricercha Scientifica, 18, 1.
16. 1948. "Ribose nucleic acid in the Drosophila egg". NATURE, 161, 440.
17. 1949. "Cleavage rate, oxygen consumption and ribose nucleic acid content of sea urchin eggs". Biochem. et Biophys. Acta, 3, 92.
18. 1949 (with J.T. Randall, F.R.S., and S.G. Tomlin) "An electron microscope study of the nuclear membrane" NATURE, 163, 280.
19. 1949. "Chiasma interference in diploid, tetraploid and interchange spermatocytes of the earwig". J. Genet. 49, 209.
20. 1950 (with H. Spurway) "Hybrids between some members of the Rassenkreis Triturus cristatus". EXPERIENTIA, in press.
21. 1950 (with H. Spurway) "A study of meiosis in inter-racial hybrids of the newt Triturus cristatus". J. Genet. in press.
22. 1950 (with G.L. Brown and G. Leaf) "The chemical nature of nuclear sap". NATURE, in press.
23. 1950 (with S.G. Tomlin) "Experimental studies on amphibian oocyte nuclei. I. Investigation of the structure of the nuclear membrane by means of the electron microscope". Proc. roy. Soc. B, in press

## Cell Size in Millipedes

THE idea of the constancy of cell size for a particular tissue in different individuals of an animal species has long held sway in zoological literature. This concept has frequently been extended to cover nearly related species<sup>1</sup>. I wish to thank Dr. J. R.

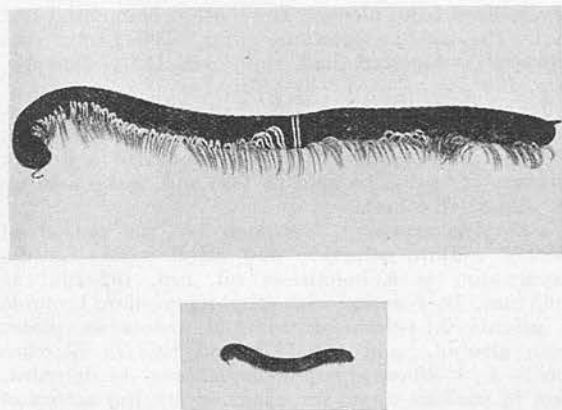


Fig. 1.

ABOVE, *Spirostreptus stenorhynchus*; BELOW, *Cyliandroiulus londinensis*. BOTH ABOUT TWO THIRDS NATURAL SIZE.

Baker, of Oxford, for suggesting this investigation into a case which proved to be at variance with the generally accepted view.

*Spirostreptus stenorhynchus* is a very large millipede from Ceylon (Fig. 1). A specimen examined was 13.8 cm. long. The fore-gut (of stomodæal origin) of this animal was fixed in Flemming's fluid with reduced acetic acid content, and transverse sections cut at  $5\mu$ . Fat was removed from the sections by immersion in turpentine, and they were afterwards stained in iron hæmatoxylin. These sections were very kindly lent me by Dr. Baker. *Cyliandroiulus londinensis*, a British millipede, of average length 2.7 cm., was treated in exactly the same way. It should be noted that these two animals are quite closely related species, both being formerly included in the genus *Iulus*. Flemming's fluid was used since it gives a reasonably good fixation of the nucleus without causing excessive shrinkage in the cytoplasm.

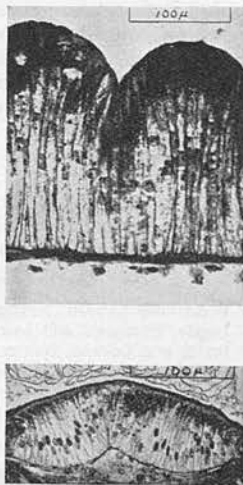


Fig. 2.

FORE-GUT CELLS OF *Spirostreptus* (ABOVE) AND *Cyliandroiulus* (BELOW).

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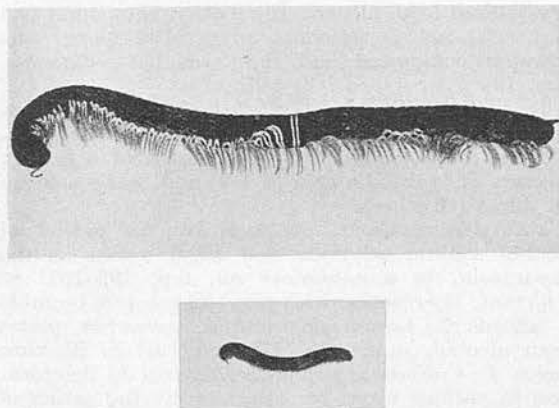


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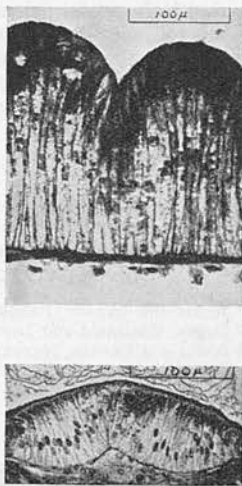


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FORE-GUT CELLS OF *Spirostreptus* (ABOVE) AND *Cylandroiulus* (BELOW).



In the Ceylon millipede, fifty of the epithelial cells of the fore-gut (see Fig. 2) had an average length of  $189\ \mu$ , and an average width of  $7.8\ \mu$ . Corresponding dimensions of the cells of the British millipede were  $54\ \mu$  and  $3.9\ \mu$  respectively. It will therefore be seen that the cells of the Ceylon millipede are about fourteen times as large by volume as those of the British millipede. This really enormous difference in cell size (not, however, comparable with the difference in body volume) may perhaps be typical of single-layered epithelia, where symmetry relations according to the size of the lumen would demand larger cells in the larger species.

It may be pointed out, moreover, that W. F. Abercrombie<sup>2</sup> and W. Trager<sup>3</sup>, working independently on the larva of the flesh-fly, *Lucilia sericata*, found that the entire growth of the larva from the first instar "is accounted for by increase in size of cells".

The photographs were taken at the John Innes Horticultural Institute by kind permission of Sir Daniel Hall.

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Oxford.

<sup>1</sup> See Painter, T. S., *J. Exp. Zoo.*, **50** (1928); also Berrill, N. J. *Phil. Trans.*, B, **225** (1935).

<sup>2</sup> *J. Morph.*, **59** (1936).

<sup>3</sup> *J. Exp. Zoo.*, **71** (1935).

*1939-1940*

**The Absence of a Sex-hormone controlling regeneration  
of the Hectocotylus in *Octopus vulgaris* Lam.**

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*Estratto dalle "Pubblicazioni della Stazione Zoologica di Napoli", - Vol. XVIII, Fasc. 1, 1939  
(licenziato per la stampa il 17 ottobre 1939-XVII)*

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# The Absence of a Sex-hormone controlling regeneration of the Hectocotylus in *Octopus vulgaris* Lam.

H. G. CALLAN <sup>1)</sup>

(From the Stazione Zoologica di Napoli)

(ricevuto il 28 luglio 1939-XVII)

(con 3 figg. nel testo)

## Introduction.

A few years before his untimely death, Professor E. SERENI, of Naples, published a short note on the regeneration of the Hectocotylus in castrated *Octopus*, stating that « I caratteri sessuali secondarii (ectocotile) sono determinati in via umorale, come è dimostrato dall'esito della castrazione, praticata sia nei maschi che nelle femmine ». However between the publication of this statement and the date of his death, SERENI performed many further experiments on the subject. The notes which he left on this work showed that his opinion had changed as a result of these later experiments, in which longer periods were allowed for regeneration. The question is obviously one of great interest since, as SERENI recognised, it provides the only opportunity yet available for investigating directly whether or not the secondary sex characters of Molluscs are controlled in development by sex-hormones. Mr. J. Z. Young of Oxford, who examined SERENI's notes, therefore suggested to me that the experiments ought to be repeated and, if necessary, a further account of the matter published. This has been the object of part of my work while occupying the Oxford University Table at the Zoological Station, Naples during 1938/39. To Dr. Dohrn and the staff of the Zoological Station I wish to express my grateful thanks for continued interest and help. I am also much indebted to Professor E. S. Goodrich and Mr. J. Z. Young for having read and criticised the manuscript of this work.

The arms of *Octopus* are triangular in section, with a broad inner surface bearing two rows of suckers which diminish in size to the tips, and a narrow sharp outer edge. The third right arm of the ♂ is « hectocotylized », i. e. it bears along the ventral surface of the outer edge a longitudinal groove serving for the passage of spermatophores to the female at copulation.

<sup>1)</sup> Adress: Oakfield; 52, Cranleigh Rd; Merton Pk. London SW 19.

The tip, further, differs from those of the other arms in bearing no suckers, being apparently used in the actual transference of spermato-phores to the oviduct of the female (see fig. 1 a and b).

The idea of the experiments was very simple; to castrate male *Octopus vulgaris* and remove the tip of the hectocotylied arm for about a centimetre, thereby including two or three pairs of the suckers near to the suckerless extremity. The condition of the regenerate was subsequently studied to see whether hectocotyliation developed even after castration. In a similar manner female *Octopus* were also castrated, the condition of the regenerate again being observed.

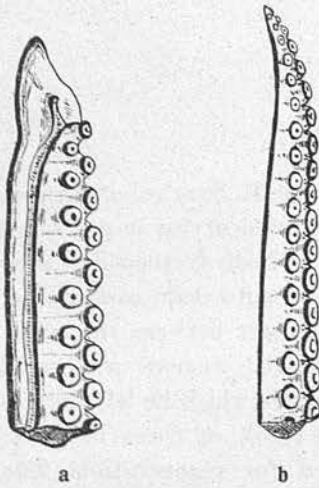


Fig. 1. — Arm-tips of *Octopus*.  
a: hectocotylied, b: normal.

### Method.

In all operations the animals were anaesthetised by immersion in seawater containing an excess of Magnesium Chloride. The strength of the solution was so adjusted that complete anaesthesia occurred after about twenty to thirty minutes (approximately 100 gms. of crystals to 4 litres of sea-water). A more concentrated and therefore quicker acting solution was very toxic to the skin of *Octopus*; in such a solution it became cracked and lost the normal covering of mucus. During the operations the branchial chamber was occasionally bathed in flowing seawater, sufficient to make the end of the operation coincide with the return of the animal to muscular activity. If the mantle did not start of its own accord to make the rhythmical respiratory movements of the normal animal, it was gently massaged until these set in.

Two different castration techniques were used, neither of which avoided a heavy mortality of operated animals. The first consisted simply in making a transverse incision at the ventral posterior surface of the mantle region. This naturally involved a ruthless treatment of the mantle musculature, but had the advantage of being straightforward. In fact, it gave more satisfactory results than the more round-about technique later to be described. The gonad, testis or ovary, immediately came to light as a whitish mass near the heart. After having been carefully separated from the heart and spiral caecum of the gut, in the case of the male the penis, and in the case of the female the two oviducts were ligatured and cut, and the gonad removed. Subsequently the cut edges of the mantle

muscles were sewn up with silk, the whole operation taking about ten minutes to complete.

The second technique was used for the removal of the whole of the reproductive system of the male, penis included. The vertical muscular

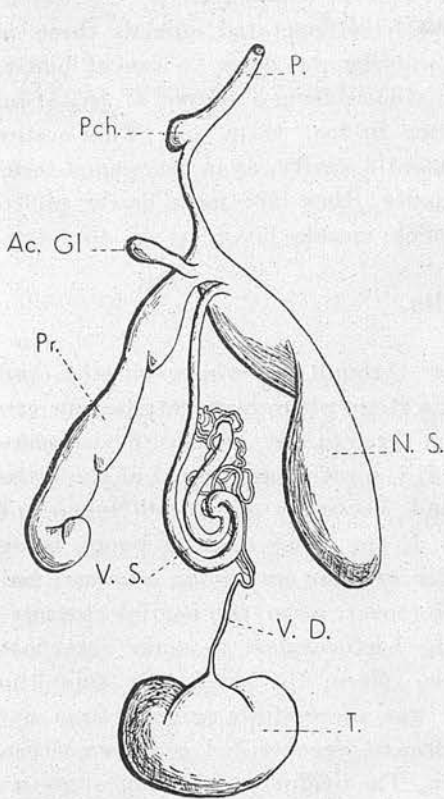


Fig. 2.

Fig. 2. — Diagram of the reproductive system of the male *Octopus*. *Acc. Gl.* accessory gland; *N.S.* Needham's sack; *Pch.* pouch; *Pr.* prostate; *T.* testis; *V. D.* vas deferens; *V. S.* vesicula seminalis.

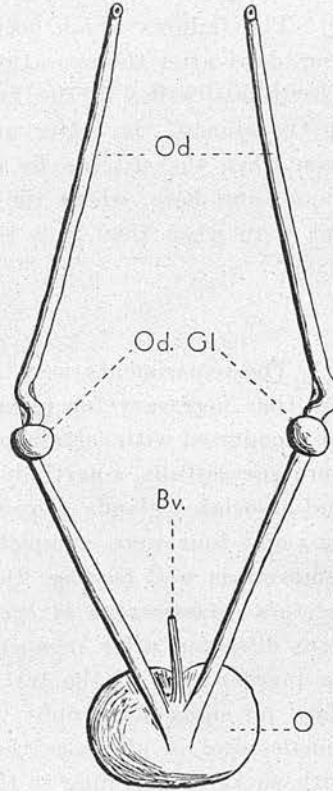


Fig. 3.

Fig. 3. — Diagram of the reproductive system of the female *Octopus*. *Bv.* blood vessels; *O.* ovary; *Od.* oviduct; *Od. Gl.* oviducal gland.

septum, which runs from the ventral part of the mantle to join the visceral mass, was cut through, and the mantle completely turned inside-out as one can do with the finger of a glove. The whole of the male reproductive system can then be seen through the thin skin of the visceral mass. A short transverse cut was made through the skin above the testis, the penis ligatured and cut, and the testis and associated glands removed. Then the skin-fold which subtends the distal end of the penis was cut through, and the cut continued posteriorly as far as the left efferent branchial vein which runs medianwards to join the left auricle. Thus the

penis can be lifted out without danger to this most important blood vessel. Considerable care has to be taken to avoid damage to the small ganglion at the base of the gill. The two skin cuts were then sewn up, the mantle reversed to its normal position and the vertical septum also sewn together. This technique takes about twenty minutes to complete.

The failures which occurred by deaths of operated animals three or four days after the operation can certainly be put down to loss of blood. The difficulty lies in the fact that the stiches form a source of irritation to the animal, the latter using its arms to tear them out. This occurs even when the stitches lie inside the mantle cavity, as in the second technique, and here, where the skin is thinner, they are more easily pulled out than when they pass through a thick muscle layer, as in the first.

### Results.

The experiments were carried out through the winter months, and the time necessary for regeneration to a stage where hectocotylisation can be recognised with certainty varied from three to five months. Seven males were successfully « partially » castrated, i. e. with the removal of the testis and associated glands (« prostate » gland, accessory gland and NEEDHAM'S sac) and four were completely castrated, the penis and its pouch being removed as well (see fig. 2). *After either of these operations a normal hectocotylus regenerated in every case.* Moreover, when the partial castrates were dissected after regeneration of the hectocotylus, to make sure that no regeneration of the testis had taken place, the penis was found to show no signs of atrophy. The ovary was successfully removed from five females and in all cases the third right arm regenerated as a normal tip, with suckers extending to the extremity. The oviducts and oviducal glands (see fig. 3) which were left after operation showed no signs of atrophy.

It is thus clear that, as SERENI'S unpublished notes suggest, his original statement was premature. If there are any internal secretions which control the development of the hectocotylus, these certainly do not proceed from the testis, « prostate » gland, accessory gland or NEEDHAM'S sac in the male. It is also clear that the non-development of the hectocotylus in the female is not the result of any inhibitory secretions from the ovary.

### Summary.

1. After castration of male *Octopus* the tip of the third right arm regenerates as a normal hectocotylus.

2. After castration of female *Octopus* the third right arm regenerates a normal tip.

3. There is therefore no evidence that the gonads of these animals produce a sex-hormone for the control of their secondary sexual characters.

#### Literature recited :

1929 Sereni, E. — Correlazioni umorali nei Cefalopodi: *Amer. J. Physiol.*,  
*Vol. 90, p. 512.*

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THE EFFECTS OF CASTRATION BY PARASITES AND  
X-RAYS ON THE SECONDARY SEX CHARACTERS  
OF PRAWNS (*LEANDER* SPP.)

By H. G. CALLAN, B.A.

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*Reprinted from the Journal of Experimental Biology,*  
Vol. XVII. No. 2, pp. 168-179, APRIL, 1940.

# THE EFFECTS OF CASTRATION BY PARASITES AND X-RAYS ON THE SECONDARY SEX CHARACTERS OF PRAWNS (*LEANDER* SPP.)

BY H. G. CALLAN, B.A.

St John's College, Oxford

(Received 14 January 1940)

(With One Plate and Five Text-figures)

## I. THE EFFECTS OF CASTRATION ON THE PERMANENT DIFFERENCES BETWEEN THE SEXES

### *Introduction*

IN 1837 Rathke made the observation that, in prawns of the genus *Palaemon* (*Leander*), only the females were parasitized by *Bopyrus*. Subsequently Giard, in 1888, pointed out that there is an alternative explanation for this remarkable observation: that any males which are parasitized show the phenomenon of sex reversal ("castration parasitaire") and take on the form of females. Giard himself examined some parasitized specimens of *Leander* (unfortunately he is not explicit as to which species) and found that his presumption had been correct. He notes however, as an exception, that one male of *L. serratus* showed no modification of the secondary sex characters. Giard also cites the observation of Hansen that in the genus *Hippolyte* only females are parasitized, and this he explains in the same way.

That the Epicarids can cause sex reversal of their male hosts is known from two other examples. Bonnier, in his monograph on the Bopyrids (1900), gives an account of the case of *Galathea intermedia* parasitized by *Pleurocrypta galathea*, illustrating the modifications of the abdominal appendages of the male towards the female type. More recently we have the excellent paper of Tucker (1930), which describes the effects of *Gyge branchialis* on *Upogebia littoralis*, and this paper goes deeply into the general relations of the Epicarids with their hosts and discusses in all its aspects the problem of sex reversal due to the action of parasites.

During my tenure of the Oxford Biological Scholarship at the Zoological Station, Naples, 1938-9, I noticed that two species of *Leander* occurring there, *L. xiphias* Risso and *L. squilla* (L.), were very frequently parasitized by two species of *Bopyrus*, *B. xiphias* Giard & Bonnier and *B. Helleri* Giard & Bonnier respectively, and I thought that with such abundance of material it would be of value to make a detailed investigation of the conditions obtaining in these species. I would like here to express my great indebtedness to Dr R. Dohrn and the staff of the Zoological Station for their kindness and help at all times during my stay in Naples.

## Observations

The general relations existing between *Leander* and its parasite *Bopyrus* resemble those described by Tucker (1930) for *Upogebia* and its parasite *Gyge*. Thus, *Bopyrus* is a gill-chamber parasite sucking blood from the branchiostegite, taking up a position on the right or left side and developing a bilateral asymmetry accordingly. The dwarf male is almost always to be found with his larger female partner, and it is only in the earliest stages of parasitic life that the female is to be found alone. About 5% of the host species are infested and, in upwards of 500 examined, four specimens were carrying a *Bopyrus* in both gill chambers.

The presence of *Bopyrus* does not prevent *Leander* from moulting, in fact parasitized females at the breeding season moult much more frequently than do unparasitized individuals of the same sex at this time. The parasite has, furthermore, little or no effect on the final size attained by the host. Parasitism begins when the host is very small (when the overall length from the tip of the rostrum to the tail is approximately 15 mm.) and, as Tucker found in *Upogebia*, so close is the correlation between host size and parasite size that one must assume that the initial attachment is confined to this stage.

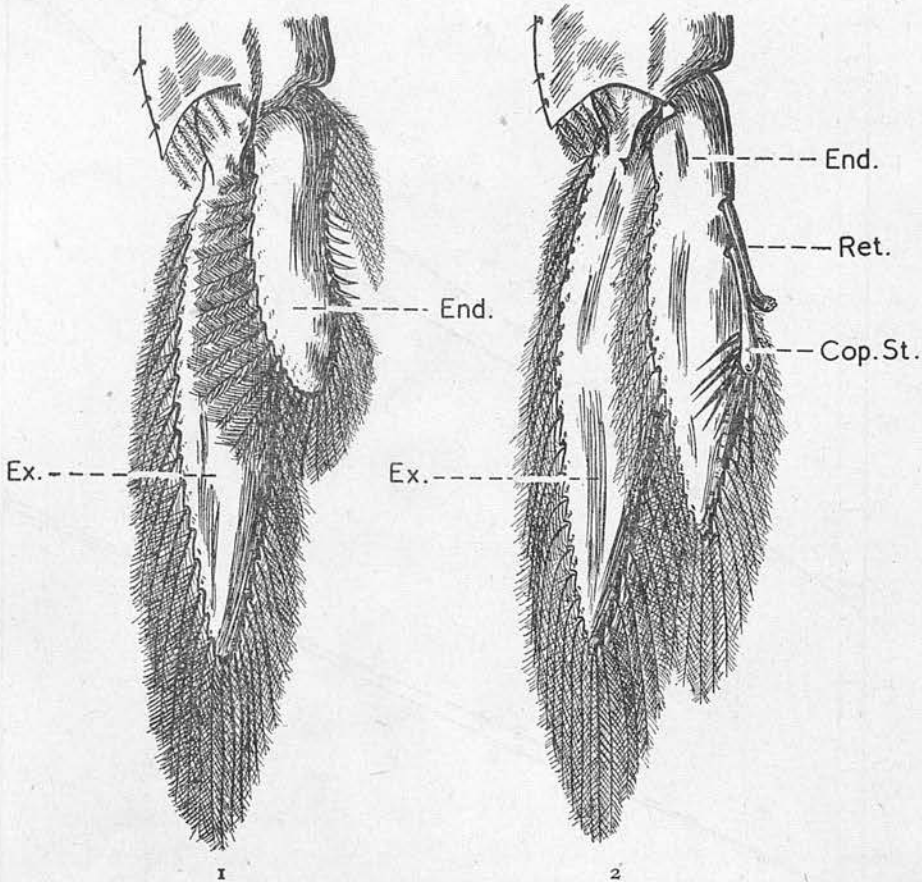
Giard has summarized the features which distinguish the sexes externally in *Leander*:

Male	Female
1. The endopodite of the first pleopod is relatively longer	Relatively shorter
2. For the middle third of its length the internal border of the endopodite of the first pleopod bears a row of small, often recurved and heavily chitinized hairs	Throughout its length, this endopodite bears long, unspecialized, feathered hairs
3. The endopodite of the second pleopod bears on its internal border a retinaculum and a copulatory style armed with stiff spines	This endopodite, as all the remainder, bears only a retinaculum
4. The internal branch of the external ramus of the first antenna, which bears the olfactory hairs, is relatively longer and bears these hairs in greater number.	Relatively shorter, and bearing fewer hairs
5. The chelae are relatively longer	Relatively shorter
6. The free space between the bases of the fifth pair of pereopods is relatively narrower	Relatively wider
7. The maximum size reached is absolutely smaller	Absolutely greater

Giard notes that the characters which show definite modification in the female direction in parasitized male *Leander* are: endopodite of the first pleopod (reduced in size, and becoming of female shape without specialized hooks), copulatory style (reduced in size), free space between the bases of the fifth pair of pereopods (sometimes widens). I have dealt mainly with the first two of these characters.

At first I made a point of determining the sex of a parasitized *Leander* from examination of its gonad. However, I soon found that the sexes could be readily distinguished, whether parasitized or not, by the length and shape of the endopodite of the first pleopod (see Text-figs. 1 and 4). Out of about 300 parasitized specimens scored as females by this character, there was not a single individual

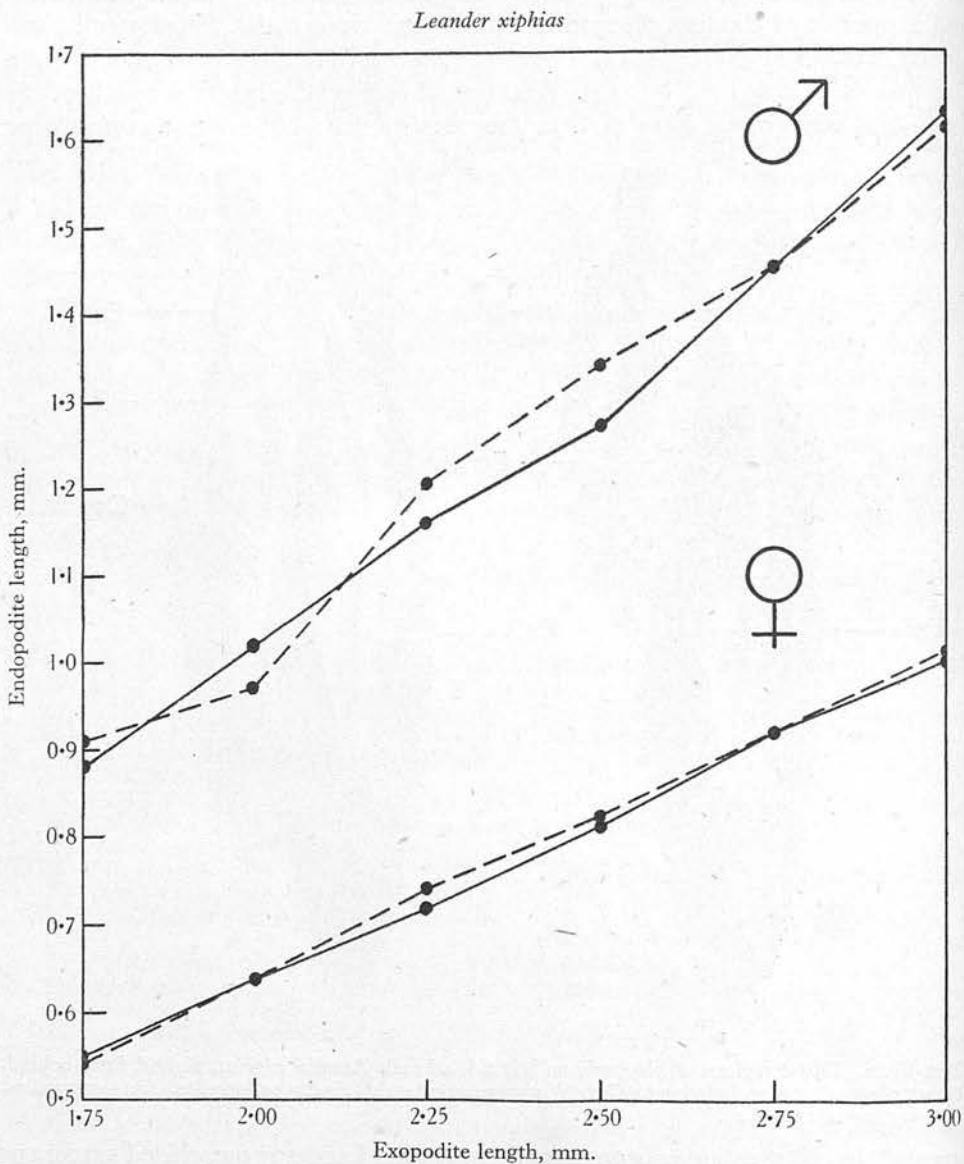
which showed signs of a copulatory style on the second pleopod (see Text-fig. 1, 2). Subsequently I made a series of relative length measurements for the endopodite and exopodite of the first pleopod in males, parasitized males, females and parasitized females of both species. The exopodite measurements were lumped into size groups of 0.25 mm. interval. Averages of endopodite length for each of the exopodite size groups were used in the construction of the two accompanying



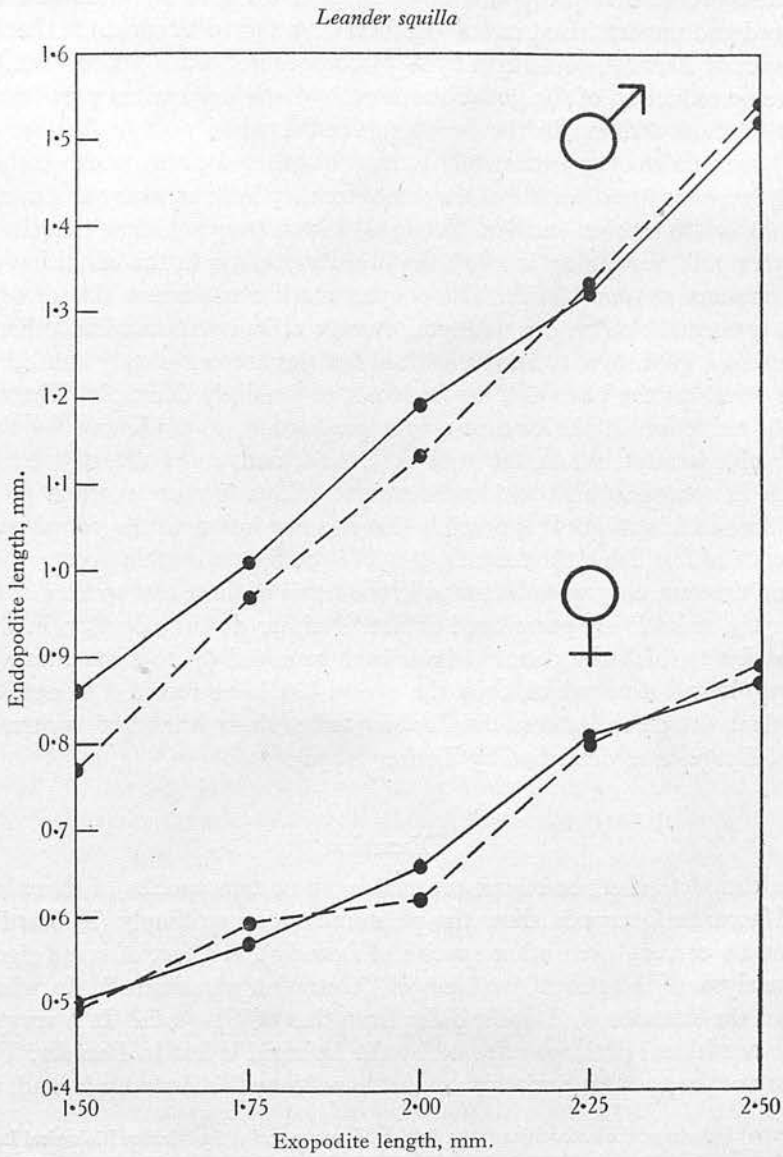
Text-fig. 1. Distal regions of pleopods on left side of male *Leander xiphias*, viewed from behind. 1 = 1st pleopod; 2 = 2nd pleopod. *Cop. St.* = copulatory style. *End.* = endopodite. *Ex.* = exopodite. *Ret.* = retinaculum.  $\times 25$ .

graphs. In all, 1057 animals were used: 270 normal and 297 parasitized specimens of *L. xiphias*, and 245 normal and 245 parasitized specimens of *L. squilla* (see Text-figs. 2, 3).

The measurements indicated that there was a considerable degree of variability between individuals, but none the less, as is shown by the graphs, no significant difference between parasitized and unparasitized males. The same conclusion emerges from a study of the relative sizes of the copulatory style and endopodite of the second pleopod: parasitized males show no reduction of this organ. There is a



Text-fig. 2. Graph showing endopodite length plotted against exopodite length for the first pleopod of *Leander xiphias*. Full lines = normal animals. Broken lines = parasitized animals. Each point represents mean of many individuals, grouped into exopodite size groups of 0.25 mm. interval. (Total number of animals examined = 567.)



Text-fig. 3. As Text-fig. 2, but for *Leander squilla*. (Total number of animals examined = 490.)<sup>1</sup>

<sup>1</sup> The correlation tables used in the construction of the graphs have been deposited at the British Museum.

great deal of variability in the length and degree of curvature of the hooks on the endopodite of the first pleopod, but here again there is no difference between parasitized and unparasitized males. In fact, I am forced to conclude that in these two species of *Leander*, parasitism by *Bopyrus* does not cause sex reversal.<sup>1</sup>

Extreme reduction of the gonad is shown by both sexes when parasitized. The contrast is most striking in the breeding season, when mature females of both species have ovaries of approximately 12 mm. length and 5 mm. width at the widest part, while a parasitized female of the same size may have an ovary of 4 mm. length and 2 mm. width or even smaller. Sections of such an ovary show that the oocytes never store yolk, remaining through the breeding season in the condition of those of an immature normal female. The oocytes reach a maximum size of 0.12 mm., whereas a normal ovarian egg with yolk storage at its maximum has a diameter of 0.45 mm. (see Pl. I, figs. 1, 2). Parasitized females are completely sterile.<sup>2</sup>

The effect on the testis of *Leander* is not so precisely definable. There is considerable reduction in the rate of sperm production, as evidenced by the small number of sperm stored in the vasa deferentia, and, more directly, by the few divisions in spermatogonia and spermatocytes. This is more marked in *Leander xiphias* than in *L. squilla*. It is possible that in some instances the parasitized males are capable of fertilizing the females, but I have no data on this point.

At no time are oocytes to be found in the testes of these two species of *Leander*, whether the animals are parasitized or not. Neither do they develop in the testes of males from which the parasites have been removed up to a maximum time of 137 days, long before which time the gonad has been restored to active sperm production. In these features the *Leander* testes show a marked contrast to the testes of *Upogebia* as described by Tucker (1930).

### Discussion

The complete absence of sex reversal in these two species of *Leander*, when other Macruran Decapods show the phenomenon so strikingly (if Giard's interpretation be correct, even other species of *Leander*), is a fact of some significance in the analysis of the general problem of "castration parasitaire". In what causal way does the situation in *Leander* differ from that of *Upogebia*? It is very unlikely that the nutritional drainage effected by the Epicarid is less in *Leander*. The great reduction of the gonad is a criterion of just how heavy this drainage is, and, were the

<sup>1</sup> By the kindness of the authorities at the British Museum and at the Marine Biological Laboratory, Plymouth, I have been able to examine a few further species of prawns carrying Epicarid parasites. (a) *Leander serratus* (Pennant), Epicarid *Bopyrus squillarum* Latreille. Out of about eighty specimens, thirty were males and in no case was there any sign of sex reversal. (b) *Pandalina brevis* (Rathke), Epicarid *Pleurocrypta cluthae* T. Scott. Out of seven specimens, two were males showing no signs of sex reversal. (c) Nineteen specimens of *Processa canaliculata* Leach = *Nika edulis* Risso, *Hippolyte* spp. and *Spirontocaris* spp. parasitized by *Phryxus* spp. or *Bopyrus* spp., were all females. In no case was there any trace of the copulatory style, and thus it seems unlikely that any were sex-reversed males. However, the fact of not finding a single male in this material is puzzling.

<sup>2</sup> This is not the case in *Pandalina brevis*, where I have seen a parasitized female carrying a few eggs in the incubatory chamber. If the parasite dies, females of *Leander serratus* can recover completely and bear a full complement of eggs in the incubatory chamber.



difference to originate from this cause, one would have expected that at least some effect would be shown by the secondary sex characters. Furthermore, it must be remembered that in those cases where sex reversal occurs, the authors have frequently remarked how little correlation there is between the state of the gonad and that of the secondary sex characters (Courrier, 1921; Tucker, 1930).

The fundamental difference between the two host-parasite relationships would appear to lie, therefore, not in any variable factors brought in by the parasite, but in those peculiar to the host species. Tucker has pointed out that there is a graded series of types showing sex reversal: (a) as in *Inachus* (Smith, 1906) where oocytes develop in the testes of males from which the parasite *Sacculina* has been removed; (b) as in *Eupagurus* (Potts, 1906) where oocytes develop in the testes while the parasite *Peltogaster* is *in situ*; and (c) as in *Upogebia* (Tucker, 1930) where oocytes develop in the testes of many males quite independent of parasitic attack, their number, however, augmenting as a result of parasitism by *Gyge*. This series may well be considered to show an increasing tendency towards intersexuality in the normal sex determination, a situation which can arise, as Goldschmidt (1931) has shown in the interracial crosses of *Lymantria*, from a greater or lesser degree of unbalance in the relative strengths of the sex-determining genes. The parasite then merely accentuates the latent possibility of sex reversal.

The fact that oocytes develop in the testes of males which are parasitized indicates the level at which the parasite acts. Its effects on the secondary sex characters are not due to an alteration in the production of some specific hormone by the testes; they are due to alteration in the production of the fundamental sex-determining substance or substances, as Smith emphasized. In an animal where sex is but weakly determined, the parasite can produce sex reversal, but in *Leander xiphias* and *L. squilla*, where there is apparently precise and complete "sex separation", *Bopyrus* cannot succeed in tipping the scales from male to female.

## II. THE EFFECTS OF CASTRATION ON THE CYCLICAL BREEDING CHARACTERS OF THE FEMALES

Sollaud (1923), in a work on the embryology of *Palaemon* (*Leander*), has described a series of modifications which arise in the abdominal region of breeding females and result in the formation of an incubatory chamber for carrying the eggs during the early developmental stages. He has suggested that the differentiation of these breeding characters is directly dependent on the state of the ovary at maturity.

### *Observations*

I will first describe the nature of these modifications. To form the roof of the incubatory chamber, the first three abdominal sternites become greatly expanded laterally, thereby separating the bases of the first three pleopods. The sides are formed from the enlarged pleura of the first three abdominal segments, the second pleuron overlapping both the one in front and the one behind. The precoxae of the first three pairs of pleopods become elongated, while the external margins of the

basipodites of these pleopods become expanded posteriorly forming the "lames externes" of Sollaud. These modifications Sollaud describes as the "caractères secondaires tardifs" and he states that they are definitive structures appearing at the first breeding moult and persisting afterwards. However, I have kept some females of *Leander squilla* from the breeding period (in Naples this extends from February to May) into the out-of-season condition and can thus say that these characters are really cyclical in their development. It would be surprising if this were not so, for during the winter one can only find unmodified females, and this species is certainly capable of breeding for more than one season.

In addition to these characters there are specially developed hair tracts concerned in the formation of the incubatory chamber. First, there are the hairs which bear the eggs when these are laid. These are the "soies basales internes" of Sollaud, stout and unbranched, arising from the internal ridge of the basipodites of the first three pairs of pleopods, one each from the coxopodites of the first four pairs of pleopods, and a small tuft springing from the base of the basipodite of the fourth pair of pleopods. The other hair tracts have a protective function. On the first three pairs of pleopods a row of long barbed hairs arise from the internal surfaces of the "lames externes", pointing inwards and posteriorly ("soies basales postéro-externes" of Sollaud). From the anterior borders of the basipodites of the first four pairs of pleopods spring a row of hairs, much better developed in the first pair of pleopods than in the others ("soies basales antérieures" of Sollaud). Finally, there is a pair of hair rows, also long and barbed, borne by the internal margins of the fourth pair of pleopods, which point inwards and mat together in the mid-line thus forming the posterior limit of the incubatory chamber (see Text-figs. 4, 5). These hair tracts are the "caractères sexuels périodiques" of Sollaud, though, as we have seen, there is really no justification for this distinction.<sup>1</sup>

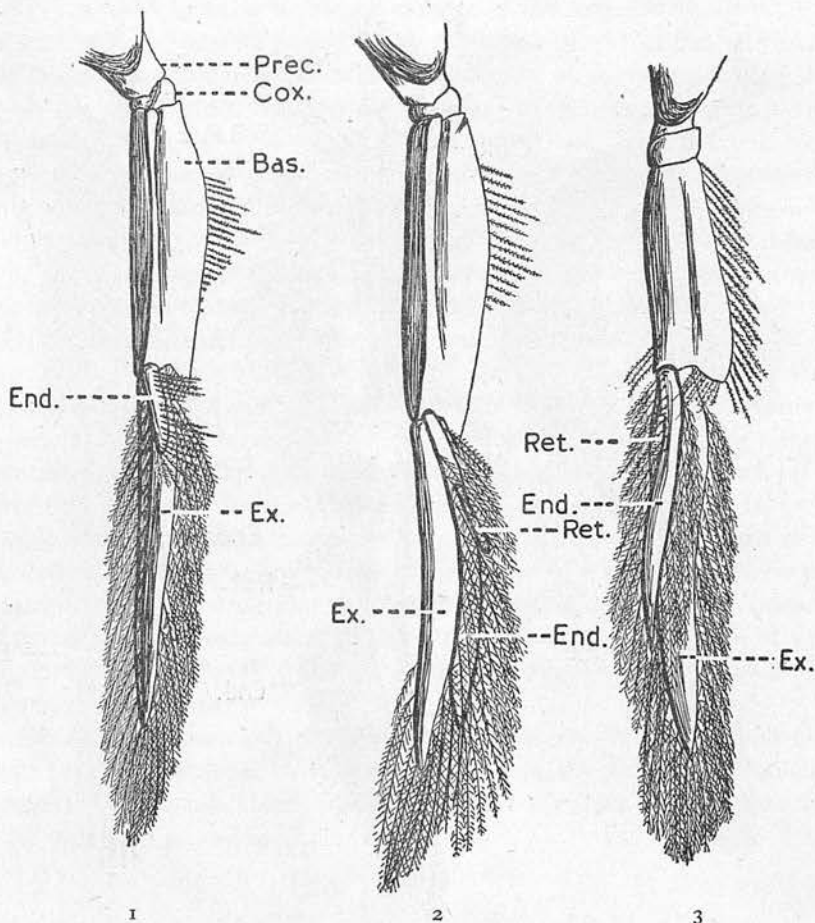
These breeding characters all arise at the moult before the deposition of the eggs, and, since it is very rare to find a female with these characters developed, yet with the eggs still in the ovary, we must suppose that egg deposition occurs immediately after the moult. If there is no further egg-laying after the first batch of young has hatched, at the next moult these breeding characters are lost; sometimes, however, there is a further egg-laying, and then they are retained at the moult.

Large parasitized females of *L. xiphias* and *L. squilla* at the breeding season form none of these characters, neither do they carry any eggs. This is not a result of an inhibition of moulting; as pointed out on p. 169, the moulting of parasitized females occurs more frequently than that of normal females. It implies, as Sollaud has suggested, a correlation between the state of the gonad and the formation of the breeding characters.

In an attempt to find out whether this was really the case, some large females were castrated with X-rays. Females of *L. xiphias* were chosen measuring 6 cm. or more from the tip of the rostrum to the tail, and of *L. squilla* measuring 4.5 cm. In the breeding season all unparasitized females of these sizes develop the breeding

<sup>1</sup> A further character developed by the breeding females of *Leander* consists of large numbers of white chromatophores around the incubatory chamber (see Knowles & Callan, 1940).

characters and carry eggs. I found that the maturation of the oocytes could be entirely or almost entirely inhibited by a single dose of 6800 r. units. This was obtained by an exposure of 20 min. at 12 cm. distance from the anti-cathode, using 180 kV. and 10 mA. with the instrument at my disposal. Pl. I, figs. 3, 4, shows sections of ovaries of irradiated animals after a lapse of 6 weeks. Mortality of



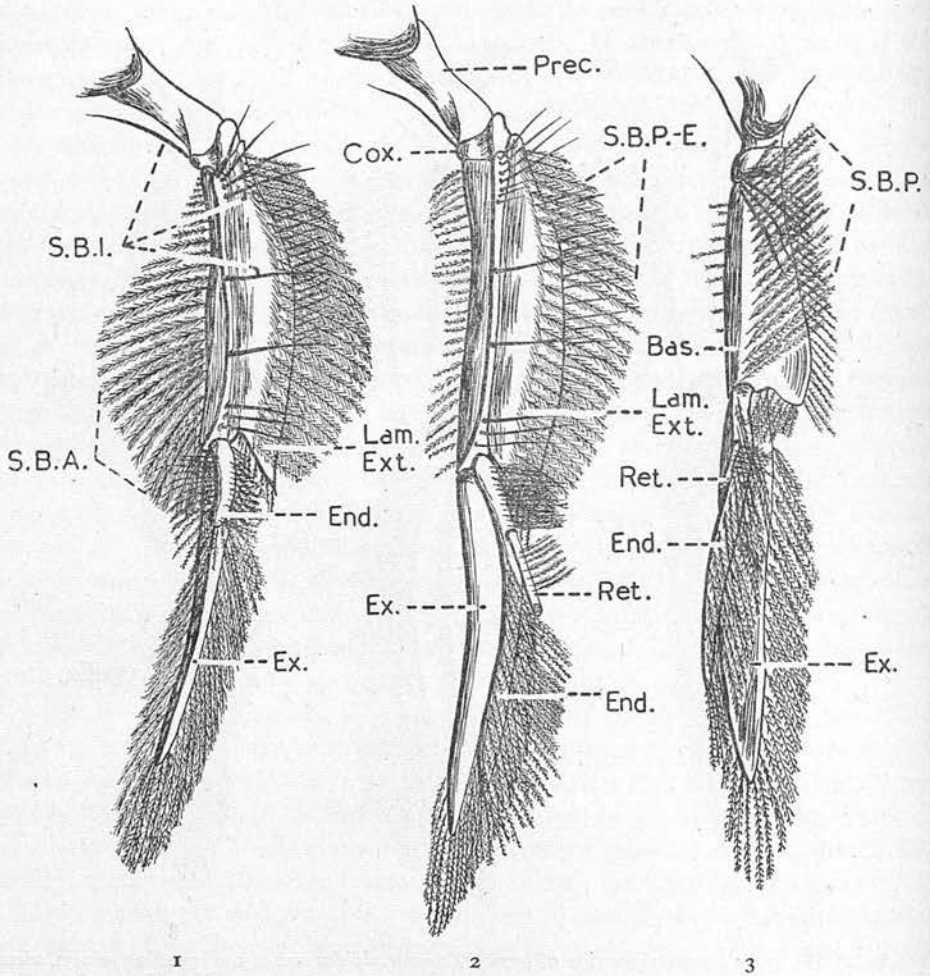
Text-fig. 4. Pleopods on the right side of female *Leander xiphias* out of the breeding season, viewed from the left. 1=1st pleopod; 2=2nd or 3rd pleopod; 3=4th pleopod. Bas.=basipodite. Cox.=coxopodite. End.=endopodite. Ex.=exopodite. Prec.=precoxa. Ret.=retinaculum.  $\times 10$ .

animals subsequent to irradiation was heavy, occurring mainly at moults, but in spite of this about thirty significant results were obtained for each species. The experimental animals were kept with males through the breeding season, during which time they moulted, but formed none of the breeding characters, which were developed by the controls.

I wish here to express my thanks to Prof. F. Pentimalli and the staff of the Cancer Institute in Naples for allowing me to use their X-ray apparatus, and for much personal assistance.

## Discussion

These observations on the formation of cyclical breeding characters parallel those of two other authors. Haemmerli-Boveri (1926) has described the development of the brood sac in *Asellus*, showing that it appears in a cyclical manner in



Text-fig. 5. Pleopods on the right side of female *Leander xiphias* in the breeding season, viewed from the left. 1 = 1st pleopod; 2 = 2nd or 3rd pleopod; 3 = 4th pleopod. Bas. = basipodite. Cox. = coxopodite. End. = endopodite. Ex. = exopodite. Lam. Ext. = "lame externe". Prec. = precoxa. Ret. = retinaculum. S.B.A. = "soies basales antérieures". S.B.I. = "soies basales internes". S.B.P. = "soies basales postérieures". S.B.P.-E. = "soies basales postéro-externes".  $\times 10$ .

adult females in correlation with the state of the ovary at the moult. On castration with radium radiation, the brood-sac regressed through the subsequent moults and remained so for the rest of the animal's life.

The experiments of Le Roux (1931a, b) are also completely in agreement. She found that in the normal development of *Gammarus*, at the ninth moult oostegites

appear in the females and at the fourteenth moult these bear ovigerous hairs. Vitellogenesis and the formation of the ovigerous hairs coincides in time. However, when the females are parasitized by the Acanthocephalan larva *Polymorphus minutus*, although the animals moult the ovigerous hairs do not appear. Using radium bromide needles, she was able to check the maturation of the ovaries for a time. A corresponding delay in the appearance of the ovigerous hairs occurred, in spite of moults in the interim period, but when vitellogenesis of the oocytes recommenced there was a precisely correlated formation of the ovigerous hairs. She found, on the other hand, that the development of the oostegites went on quite independently.

The natural deduction to be drawn from this work is that the differentiation of such breeding characters in the females studied is directly dependent on stimulation from substances elaborated in the ovary and liberated in the blood. The close nature of the correlation is obvious, but unfortunately the experimental conditions of X-ray and radium castration are not critical. It may always be argued that the effects on the somatic tissues is a direct one, and the lowered viability of the experimental animals gives weight to this criticism. Further, there is the possibility that gonad and secondary sex characters are together reacting to some common third factor, general metabolic level, for example, or nutrition, as Avel (1929) found to be the governing factor in the development of the clitellum of *Lumbricus*. It is unfortunate that surgical castration of a crustacean is impracticable. In any case, however, castration experiments must be supplemented by injections of gonad extracts or gonad implantations before the presence of a hormone can be proved. The analogy between the development of the breeding characters of *Leander* and the preparation of a female mammal by luteal hormone for the birth of young is none the less striking, even though the mechanism of co-ordination may be entirely different in the two cases.

Finally, a point to notice is that the effect of *Bopyrus* on the metabolism of the host does not balance the inactivity of the ovary in the parasitized animal. This host-parasite relationship clearly does not follow the lines of the more familiar cases of "castration parasitaire".

#### SUMMARY

1. Contrary to the assertion of Giard that *Leander* males when parasitized by *Bopyrus* undergo sex reversal, *Leander xiphias* and *L. squilla* show no modifications of the secondary sex characters, while the testes, although reduced in size, never produce oocytes. This is probably true also of *L. serratus*.

2. The power of a parasite to induce sex reversal is probably limited by the degree of sex separation of the host species. The species which show sex reversal ("castration parasitaire") are thus considered to possess a less complete sex-separating mechanism than those, such as *Leander*, which are not reversed by parasites.

3. Breeding females of *Leander* develop an incubatory chamber under the abdomen, involving many structural changes.
4. These "breeding characters" are not formed by parasitized females, nor by females castrated by X-rays.
5. It is possible that there is a female sex hormone in Crustacea, but the evidence is not yet conclusive.

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## EXPLANATION OF PLATE I

Sections of ovaries of *Leander xiphias*, Bouin-Sea water, Iron Haematoxylin. (All  $\times 60$  diameters.)  
 (1) Small region of section of ovary fixed in the breeding season before egg deposition, showing oocytes filled with yolk, and a reserve of immature oocytes. (2) Section of whole ovary of parasitized female in the breeding season, showing entire absence of yolk in the oocytes. (3) Half of section of ovary fixed in the breeding season, six weeks after X-ray treatment. One oocyte has escaped destruction and accumulated yolk. A few residual immature oocytes appear unharmed. The remainder of the oocytes were destroyed during vitellogenesis. (4) As (3), but castration by X-rays complete. No oocytes present.

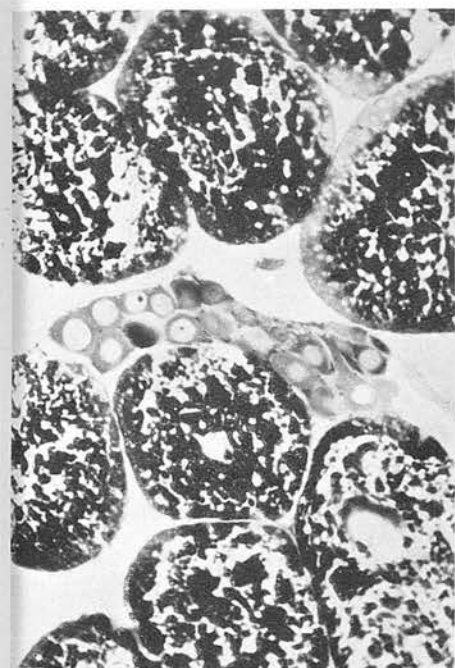


Fig. 1.



Fig. 3.

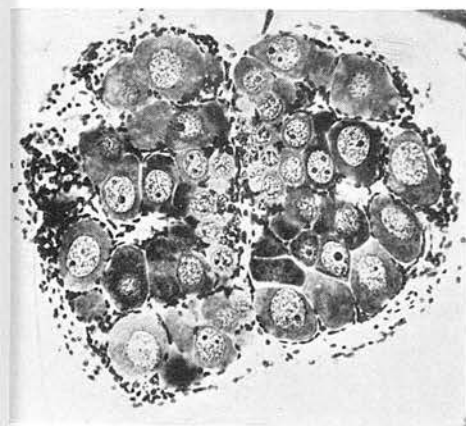


Fig. 2.

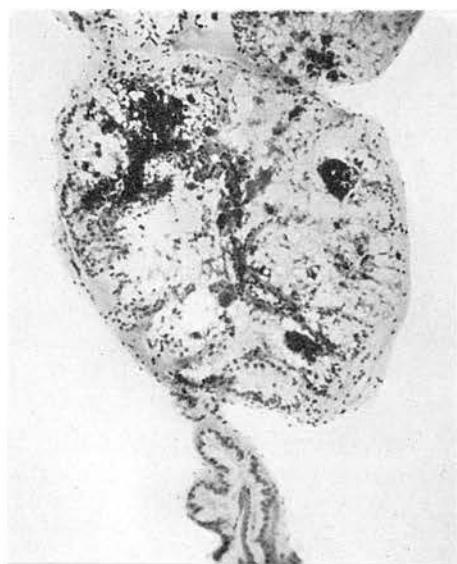


Fig. 4.

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## A CHANGE IN THE CHROMATOPHORE PATTERN OF CRUSTACEA AT SEXUAL MATURITY

BY F. G. W. KNOWLES, D.PHIL.<sup>1</sup> AND H. G. CALLAN, B.A.<sup>2</sup>

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Stazione Zoologica, Naples, Italy

[Received 16 November 1939]

(With One Text-figure)

### I. INTRODUCTION

WHILE studying the chromatophores of the prawn, *Leander serratus*, it was noticed that the ovigerous females possessed certain groups of white chromatophores which were absent in the males or immature females of this species. These chromatophores appeared on the egg-bearing segments after the "breeding-moult", when the pleopods of the females became structurally modified. It has not been previously suggested that the chromatophore pattern of Crustacea becomes modified at sexual maturity, and an investigation was therefore undertaken to determine the occurrence and behaviour of these chromatophores. *Leander serratus*, *L. squilla*, *L. adspersus* and *L. xiphias* were examined at Naples; *Palaemonetes varians* from Plymouth was also examined. In all these species the additional white chromatophores were found in ovigerous females.

### II. STRUCTURE AND DISTRIBUTION OF THE WHITE CHROMATOPHORES IN THE PALAEMONIDAE

(a) *Immature males and females.* The structure and behaviour of the normal white chromatophores of shrimps and prawns has already been described (Knowles, 1939). Two main types were observed: The first type is polychromatic and contains red and yellow pigments in addition to the white pigment. This type responds as an independent effector to a change of illumination and is not affected by an injection of eye-stalk extract. It is found distributed at regular intervals along the posterior edges of the body segments of *Leander serratus* and *L. squilla*, and assists in the production of the bars of colour found in these species. The second type responds to an injection of eye-stalk extract by contracting. It contains only a white pigment and is found irregularly scattered in the epidermis of *L. adspersus*, *L. xiphias* and *Palaemonetes varians*, influencing the shade of the body colour, but not assisting in the formation of a body pattern.

<sup>1</sup> Oxford University Naples Scholar, 1937-8.

<sup>2</sup> Oxford University Naples Scholar, 1938-9.

(b) *Ovigerous females.* The additional white chromatophores which are found in ovigerous shrimps and prawns appear gradually during the early spring as the female approaches the breeding condition. They are situated on the sides of the pleura, at the basal joints of the first four pleopods, and on the expanded external posterior margins of the basipodites of the first three pleopods. The full development of these chromatophores occurs after the "breeding-moult" when the female acquires a number of structural modifications of the pleopods. Thus the precoxae of

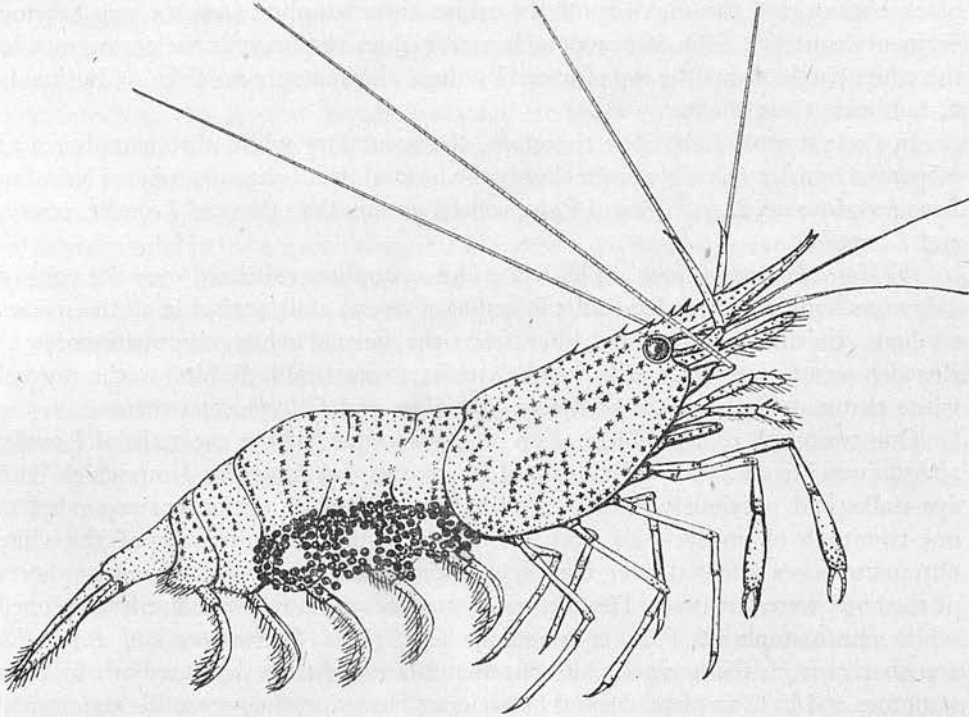


Fig. 1. An ovigerous *Leander serratus*, showing the position of the patches of white chromatophores which appear on the egg-bearing segments during the breeding season. These chromatophores restore the protective pattern which is upset by the presence of eggs.

the first three pairs of pleopods become elongated, the external posterior margins of the basipodites become expanded posteriorly, and a number of new setae tracts, for the protection of the eggs, appear on the first four pleopods (Sollaud, 1923).

Only a white pigment can be seen in these expanded white chromatophores of *Leander serratus*, *L. squilla*, *L. adspersus* and *Palaemonetes varians* although traces of a red pigment can be seen when they are contracted. The white chromatophores of *Leander xiphias*, however, all possess a central patch of red pigment.

## III. FACTORS INFLUENCING THE DEVELOPMENT AND CONDITION OF THE ADDITIONAL WHITE CHROMATOPHORES OF OVIGEROUS FEMALES

(a) *Normal conditions.* The secondarily developed white chromatophores of all the species studied are more sensitive to changes of illumination than to changes of background. They contract sharply in darkness and expand in light, but behave in a more erratic manner on illuminated black or white backgrounds. On an illuminated white background they are always maximally expanded, whereas on an illuminated black background the majority of the white chromatophores on the egg-bearing segments contract. This contraction, however, does not prevent the appearance of the white patches over the eggs formed by these chromatophores (Fig. 1), but tends to minimize their effect.

In their normal behaviour therefore, the secondary white chromatophores of ovigerous females resemble more closely the normal white chromatophores found in *Leander adpersus*, *L. xiphias* and *Palaemonetes varians* than those of *Leander serratus* and *L. squilla*.

(b) *Injection experiments.* The white chromatophores situated over the eggs of ovigerous females responded to the injection of an eye-stalk extract in all the species studied. In this respect they differ from the normal white chromatophores of *Leander serratus* and *L. squilla*, but behave in a comparable fashion to the normal white chromatophores of *L. adpersus*, *L. xiphias* and *Palaemonetes varians*.

One-twentieth of a millilitre of an aqueous extract of the eye-stalk of *Leander serratus* was injected into individuals of *L. serratus* and *L. squilla*, from which both eye-stalks had previously been removed. Each injection, which corresponded to one-twentieth of an eye-stalk, was followed by a marked contraction of the white chromatophores situated over the eggs, although the main white chromatophores of the body were not affected by the injection. Therefore, the secondarily developed white chromatophores seen in ovigerous females of *L. serratus* and *L. squilla* are distinct from the normal white chromatophores of these species both in their structure and in their physiological behaviour. However, they resemble the normal white chromatophores of *L. xiphias* and *L. adpersus* and *Palaemonetes varians*, although they are more sensitive to the injection of the eye-stalk extract than are the normal white chromatophores of these species.

(c) *Castration experiments.* Females of *Leander xiphias* and *L. squilla* were examined after their ovaries had been prevented from maturing by X-rays or parasites. The largest available individuals were chosen as experimental animals, i.e. in the case of *L. xiphias*, those which had an overall length from the tip of the rostrum to the extremity of the uropods of approximately 6 cm. In *L. squilla* the largest animals obtainable had an overall length of approximately four and a half centimetres.

The ovarian oocytes of a number of these animals were destroyed by X-ray irradiation, some 1-3 months before the breeding season. It was found that one dose of 6800 r. units was sufficient to inhibit vitellogenesis. Post-mortem examination by sections of the gonads of females which had been treated in this way showed that

the oocytes had almost without exception been destroyed, and that the gonads consisted simply of a connective tissue stroma. The mortality of experimental animals was high, but about thirty significant results were obtained for each species. These irradiated animals moulted normally, and survived during the breeding season. The irradiated animals and controls were kept living until the first week in May, by which time the controls had all developed the white chromatophores, passed the "breeding-moult", and laid their eggs. In the irradiated animals, however, no ova were laid, and the white chromatophores did not appear.

Females, the maturation of whose gonads had been inhibited by parasites, were also examined. *Leander xiphias* is parasitized by *Bopyrus xiphias* (Giard & Bonnier) and *Leander squilla* by *Bopyrus Hellier* (Giard & Bonnier). These Bopyrid parasites interfere with the general metabolism of their hosts by withdrawing blood from the circulation. Very large parasitized females are rare. However, twenty individuals of each species of, or exceeding the sizes given above for the average egg-laying females, were examined. These females did not develop the white chromatophores which are found in the normal ovigerous females; a postmortem examination of their gonads showed that the ovaries of parasitized animals are similar to those found in immature females, consisting entirely of small oocytes without any deposition of yolk.

It was noticed that in females castrated, whether by X-rays or parasites, the suppression of the development of the white chromatophores was not due to a prevention of moulting. The parasitized animals moult much more frequently than normal individuals, while in the case of X-rayed females only individuals which had moulted were considered.

#### IV. DISCUSSION

It is clear that the white chromatophores which appear on the egg-bearing segments of the female prawn's body are developed in immediate relation to the presence of maturing ovaries, since the suppression of the normal development of the ovaries will prevent the appearance of these chromatophores. This dependence of the appearance of a secondary sexual character on the presence of a normal gonad is a striking and unusual phenomenon for a Crustacean. The change cannot, however, be taken as demonstrating the existence of a "female sex hormone" in these animals, since it is very probable that it is related simply to the metabolic conditions which obtain during yolk deposition. It is not surprising to find that nuptial colorations due to pigment modifications should appear during the internal modifications which accompany sexual maturity. It is probable that the white pigment contained in the chromatophores of Crustacea is guanin, a purine derivative which is found widely distributed in the integument of vertebrates and invertebrates. This substance is known to augment at maturity, and it plays an important part in the nuptial colorations of many fishes, batrachians and reptiles (Verne, 1930). The male of *Triton alpestris* develops a nuptial pattern at maturity due to an accumulation of guanin on the back. *Lacerta viridis* and the teleostean fishes *Gasterosteus aculeatus*, *Perca fluviatilis*, and *Phoxinus laevis* all develop at sexual maturity nuptial colorations of

which guanin is an important constituent. It is probable that elaboration of guanin in these forms is due to the violent nucleo-protein movements which occur at gametogenesis.

It is not clear, however, why certain regions of the body in prawns and shrimps are particularly sensitive to the disturbances in metabolism which lead to guanin accumulation, as indicated by the constancy in position of the white chromatophores. The white chromatophores nearest to the eggs are the most affected, and only a small increase is observed in the number of white chromatophores in other parts of the body. Probably sexual maturity leads to a general disturbance of the body metabolism in female shrimps and prawns, but the chromatophores on the egg-bearing segments have developed a greater sensitivity to guanin augmentation because of the special protective coloration which they give. Such groups of white chromatophores situated directly over the gonads serve to mask the eggs and to render males and females less easily distinguishable, besides breaking the continuous line of shadow under the body due to the presence of the eggs (Fig. 1). Since the egg-bearing females are less agile than the males, the development of these white chromatophores at maturity may thus help to protect the females from possible predators.

It is difficult to determine the origin of the white chromatophores on the egg-bearing segments. Possibly they may replace red chromatophores by a substitution or transformation of the red pigment. This suggestion is supported by the behaviour of these chromatophores, which in *Leander serratus* and *L. squilla* function in a manner different from the normal white chromatophores of these species.

#### SUMMARY

1. White chromatophores appear on the egg-bearing segments of female prawns and shrimps at sexual maturity.
2. Such chromatophores do not appear in the females whose gonads have been destroyed by X-rays or by parasites.
3. It is probable that this appearance of white chromatophores at sexual maturity is correlated with the metabolic changes occurring at yolk deposition in the ovaries.

We should like to take this opportunity to thank Prof. R. Dohrn for the facilities granted us at the Stazione Zoologica at Naples and to thank J. Z. Young, M.A., for his helpful criticism of this paper.

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H. G. CALLAN

**The Chromosomes of the Cymothoid Isopod  
Anilocra**

# The Chromosomes of the Cymothoid Isopod *Anilocra*.

By

H. G. Callan

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With 3 Text-figures.

---

## MATERIAL AND METHOD.

*Anilocra mediterranea* M. Edw. is a fish-louse belonging to the Cymothoid group of Isopods, found abundantly at Naples. In common with other fish-lice, *Anilocra* is a protandric hermaphrodite, descriptions of its sexual organization having been given by Bullar (1876) and Mayer (1879). For a cytological study of this animal I took individuals while in the predominantly male phase, removing the paired testes and ovaries, and fixed them in La Cour's 2BD (1937) made up in sea-water. The gonads were fixed for one hour, hardened in 1 per cent. chromic acid for twenty-four hours, washed in running tap-water for half an hour, and then dehydrated and imbedded in wax in the usual manner. Sections were cut at  $15\mu$  and stained by the gentian violet-iodine method (see La Cour, 1937).

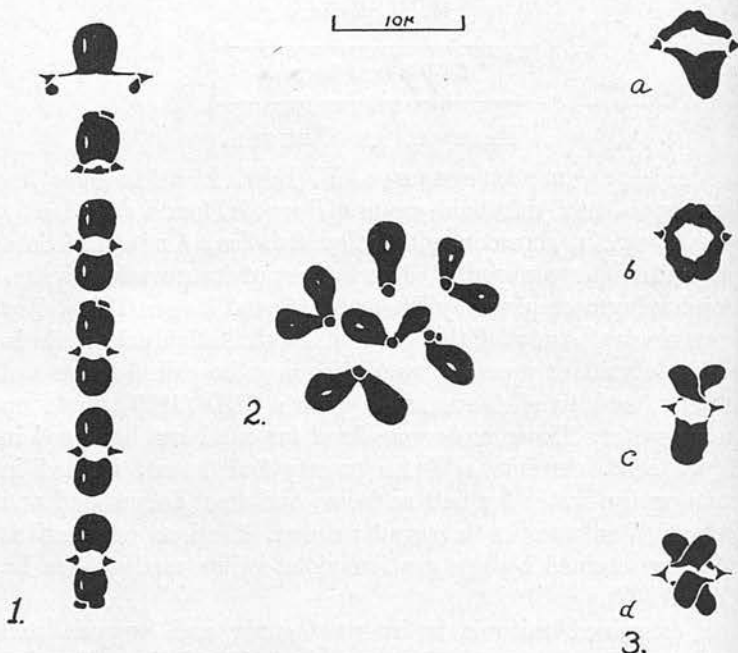
I examined mitoses in spermatogonia and oogonia and meioses, first and second, in the male germ-cell line. Fixation of the material was satisfactory for general purposes, although not for great detail, the fault lying not in the technique employed but probably in the high degree of hydration of the chromosomes, for other fixatives tried, such as strong Flemming and Bouin in sea-water, all gave indifferent results. In general, mitoses fixed rather better than meioses, especially the prophase stages.

## OBSERVATIONS AND DISCUSSION.

The diploid chromosome number is twelve, eight of the chromosomes having median (M) and four subterminal (S) centromeres. This is a low chromosome number for a crustacean. No particular interest attaches to the mitoses except

the variability of the staining reaction, a point to which reference will be made later.

The configurations of the bivalents at first meiotic metaphase show that there is extreme localization of chiasmata in this












TEXT-FIG. 1, somewhat diagrammatic.

1. Side view, and 2. polar view, of first meiotic metaphase, normal type.  
3. Side view of exceptional configurations of M-chromosomes at first meiotic metaphase.

species. The M-chromosomes regularly form two chiasmata in each arm, one immediately adjacent to the centromere, and one at or near to the end of the arm (see Text-figs. 1 and 2). Four exceptional conditions have been observed: (a) where in one arm a proximal chiasma has failed, (b) where both proximal chiasmata have failed, (c) where in one arm a distal chiasma has failed, and (d) where both distal chiasmata have failed (see Text-figs. 1, 3).

Out of 365 M-bivalents examined at metaphase, 350 were of

Association of M-chromosomes			
Type	Number		Xta
Normal	350		4
a	4		3
c	5		3
b	4		2
d	2		2
	0		2
	0		2
	0		1
	0		1

TEXT-FIG. 2. Diagram showing association of M-chromosomes.  
Xta, chiasmata.

the normal type, four of type *a*, four of type *b*, five of type *c*, and two of type *d* (see Text-fig. 2). The mean chiasma frequency in these bivalents is thus 3.94. The variance from the mean can be calculated from the formula  $V = \frac{1}{n-1} \left[ \sum (x^2) - \frac{(\sum x)^2}{n} \right]$ , where

$V$  = variance,  $n$  = the number of M-bivalents, and  $x$  = the chiasma frequency per bivalent. This has a value of 0.087, which represents 0.022 of the mean (see Table I). Values approaching this latter figure have only been found in organisms with extreme localization of chiasmata (*Fritillaria ruthenica*, Darlington (1936), *Mecostethus grossus* and *Metrioptera brachyptera*, Callan, unpub., from data of White (1936)). Haldane (1931) has shown that, where pairing

Number of M-bivalents	0	6	9	350	Mean	Variance
Number of chiasmata realized	1	2	3	4	3.94	0.087

TABLE I.

and chiasma-formation are normal, the value of variance approaches the mean, though never reaching this value on account of interference. The variance in this particular case is thus extremely low.

In order to analyse the factors responsible for producing such invariability I have made a study of the proportions in which the various types of association of the M-bivalents occur, with a view to establishing possible correlations. A table is appended (see Table II) showing the number of chiasma failures observed as against the numbers to be expected, assuming absence of correlation between the failures.

The calculation proceeds as follows:

In the case of the proximal chiasmata, out of 730 possible,  $2 \times 4 + 4 = 12$  failed.

Thus the chance of a chiasma failing =  $\frac{12}{730} = 0.0164 = q$ .

Thus the chance of a chiasma being realized =  $1 - 0.0164 = 0.9836 = p$ .

The chances of none, one, or two chiasmata failing are, therefore, as  $p^2 : 2pq : q^2$  respectively, i.e. as 0.9674 : 0.03234 : 0.0002702.

The corresponding chances for the distal chiasmata failing are 0.9755 : 0.02435 : 0.0001520.

In the table the chances of the various possible combinations of chiasma failure are worked out by multiplication together

of their respective components, and then expressed as absolute numbers out of the observed total of 365. The significant squares are those where both proximal chiasmata have failed, the expected number being 0.10 and the observed 4; and where both distal chiasmata have failed, the expected number being 0.05 and the observed 2. There is thus evidence of strong positive correlation in the failing of the two proximal chiasmata,

<i>Expected</i>		<i>Failure of proximal chiasmata in M-bivalents</i>		
		<i>0</i>	<i>1</i>	<i>2</i>
<i>Failure of distal chias- mata in M-bivalents</i>	<i>0</i>	344.45	11.51	0.10
		350	4	4
	<i>1</i>	8.60	0.29	0.00
		5	0	0
	<i>2</i>	0.05	0.00	0.00
		2	0	0

TABLE II.

M-bivalents, chromosomes having median centromeres.

and in the failing of the two distal chiasmata. An idea of the relative strengths of these correlations can be obtained from the formula  $\frac{n_0 n_2}{(\frac{1}{2} n_1)^2}$ , where  $n_0$  is the number of bivalents where none,  $n_1$  where one, and  $n_2$  where two have failed. When there is complete positive correlation this has a value of  $\infty$ ; when there is no correlation, of 1; and when there is complete negative correlation, of 0. For the proximal chiasmata the value is 357 and for the distal chiasmata 115, showing that the former correlation is the stronger.

In the case of the correlation between the failing of the two proximal chiasmata, it would appear that proximity on the chromosome of the two chiasma-forming regions when pairing takes place is the factor of prime importance. When one region

has successfully paired there is an increased likelihood for the nearby regions to pair up also, in accordance with the 'zip' principle. That there should be, however, a correlation between the failing of the two distal chiasmata cannot be accounted for in this way. One may presume that the two free ends of the zygotene chromosome lie close together, so that if pairing is effected with one of them, the probability is that the other will manage to pair also. Clearly this second correlation indicates that interference plays no part in the restriction of chiasmata distribution down the length of the chromosome: limitation of the initiation of pachytene pairing to the proximal and distal regions and the positions taken up by the zygotene threads within the nucleus are the major factors which secure such invariability and localization of chiasmata as are found in *Anilocra*.

The S-chromosomes form two chiasmata in the long arm, one proximal and one distal, and no exceptions have been observed; while in the short arm a chiasma may or may not occur. Often in the same metaphase one of the S-bivalents has the centromeres close together and retained by a chiasma in the short arms, while the other has the centromeres much wider apart and the short arms retracted and not in contact. Such is the position taken up later by the S-bivalents at early anaphase, whether or not a chiasma has occurred in the short arms. In the S-chromosomes there is therefore a corresponding invariability about the association of the long arms such as occurs in the M-chromosomes.

The conclusions drawn from the metaphase configurations cannot be substantiated from a study of diplotene, for the fixation of this stage is very poor. However, the fact that a 'bouquet' is formed at pairing stages probably indicates loop formation by the M-chromosomes, an orientation which was inferred from the consideration of the correlation of chiasma failures.

At anaphase the separation of the S-chromosomes precedes that of the M-chromosomes, as is to be expected, since the short arms offer less resistance to separation than do the long arms. The M-chromosomes separate quite regularly and in time with

one another, thus providing circumstantial evidence that identical conditions obtain in each one.

The extreme form of chiasma localization found in *Anilocra* implies a rigidity in the recombination mechanism which is possibly to be correlated with this animal's parasitic mode of life, and it may be taken to indicate that an evolutionary standstill has been reached.

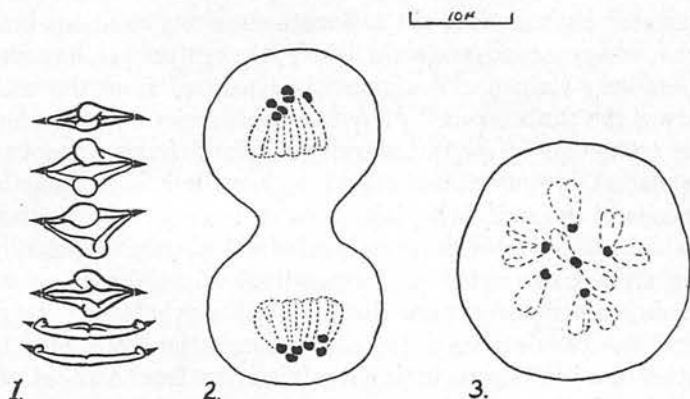
A further interesting feature of the meiotic chromosomes is their staining reaction. At full metaphase the bivalents have a bloated appearance and stain lightly; the centromere, however, is intensely stained and apparently separated from the main body of the chromosome by a non-staining region. At this stage it is triangular in outline, showing tension. During anaphase the stainability of the non-centric regions diminishes, irregular deposits of the stain being laid down on the surface. The stage at which this occurs varies greatly in individual cells. Eventually, soon after the completion of separation, only the centromeres remain stained and six are visible at either pole (see Text-figs. 2 and 3). The outlines of the chromosomes then show only by virtue of a difference in their refractivity from that of the cytoplasm. The centromeres are now rounded off, showing no tension; they are slightly under  $1\mu$  in size. For comparison, it may be noted that the M-chromosomes at metaphase have a length of  $7\mu$  and width of  $2\mu$ .

The stainability of the mitotic chromosomes and of the second meiotic chromosomes is also variable. In the majority of mitoses the anaphase chromosomes remain stained, though sometimes the centromeres only. In the majority of second meioses, even at metaphase, the centromeres only are stained, and in fact the chromosome material remains invisible until late in the maturation of the sperm head. It can thus be seen that there is a progressive tendency through the chromosome cycle, from the somatic divisions to the secondary spermatocyte, for the chromosomes to be unstainable.

Variation in staining reaction given by the same stages of division cannot be the result of differences in fixation through the material, for the unstainable divisions are not distributed through the tissue with any reference to the boundaries of the



latter. Furthermore, when there is a certain staining reaction given by one chromosome, the same reaction is given by all the remaining chromosomes in the division. The differential staining reaction is probably the consequence of labile differences in the character of the chromosome surface (gentian violet being a surface stain). This is perhaps dependent on local differences in pH, which would account for the bad fixation of



TEXT-FIG. 3, somewhat diagrammatic.

1. Side view of early anaphase of first meiosis.
2. Side view, and 3. polar view, of late anaphase of first meiosis, outlines of unstained chromosomes indicated by dotted lines.

the chromosomes, related as it is to their degree of hydration. It is not the result of a differential distribution of nucleic acid between centromere and non-centric regions. I destained a gentian violet preparation clearly showing the differential staining, and then restained, using the Feulgen technique: no differential staining resulted.

What relation the centromeres, as seen fixed and stained, bear to their size and shape in life is a question of interpretation. It might be argued that what can be stained is really the region of the chromosome near to and including the centromere, not the latter alone. However, its discreteness in stainability and its similar size and shape in all the chromosomes suggests that the artefact is at least an artefact of the centromere itself, and

it is of interest to find that in *Anilocera* there is this clear-cut evidence of a distinction between the centric and non-centric regions of the chromosome.

The fixations were made at Naples during my tenure of the Oxford Biological Scholarship at the Stazione Zoologica for the year 1938/9. The further work was carried out at the John Innes Horticultural Institution, Merton Park. I am much indebted to Dr. Mather for help with the statistical treatment of chiasma-frequency, and to Dr. Darlington for many criticisms and suggestions in the preparation of the argument.

#### SUMMARY.

*Anilocera mediterranea* has a diploid chromosome number of twelve, eight of the chromosomes having median, and four subterminal centromeres.

The localization of chiasmata is extreme and is of a previously undescribed type, combining the terminal and centric systems.

The centromeres stain differentially from the non-centric regions of the chromosomes; they are the only parts stained at anaphase of first meiosis, and at metaphase and anaphase of second meiosis.

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THE SEX-DETERMINING MECHANISM OF  
THE EARWIG, *FORFICULA AURICULARIA*

BY

H. G. CALLAN

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# THE SEX-DETERMINING MECHANISM OF THE EARWIG, *FORFICULA AURICULARIA*

BY H. G. CALLAN

*John Innes Horticultural Institution*

(With Plates 11-13 and Twelve Text-figures)

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## PREVIOUS WORK ON SEX DETERMINATION IN DERMAPTERA

THE order Dermaptera has attracted the attention of many cytologists. The chief interest of their work lies in the interpretation of the variability of chromosome number in the male germ-cell line and the complex sex-determining mechanism which exists in some species.

As early as 1885, Carnoy noted that the first and second spermatocytes of *Forficula auricularia* Linn. contained from 10 to 14 "chromosomes". La Valette St George (1887) found 12 "chromosomes" in first and 12-14 in second spermatocytes. Sinéty (1901), on the other hand, gave the male diploid number as 24, haploid 12, without any numerical variability. However, the problem was reopened by Zweiger (1906, 1907) who found 12, 13 or 14 "chromosomes" in first and second spermatocytes. He interpreted the numerical variations as due to varying numbers of sex chromosomes, and hence came to the remarkable conclusion that the sex chromosomes could have no significance in sex determination. Zweiger described occasional lagging chromosomes at the meiotic anaphases, which in his opinion were related in some way to these inconstantly distributed "sex chromosomes".

From 1908 the observations become of importance. They have been summarized in Table 1. The simplest situation is found in the two genera

Table 1. Summary of work on *Dermaptera* since 1908

Species	Author	Mitosis		Meiosis ( $\sigma^7$ )		Interpretation of sex chromosomes	
		$\delta$	$\text{♀}$	1st	2nd	$\delta$	$\text{♀}$
<i>Labidura bidens</i>	Morgan (1928)	12	12	6 <sup>II</sup>	6	XY	XY
<i>Labidura riparia</i>	Asana & Makino (1934)	14	14	7 <sup>II</sup>	7	XY	XX
<i>Labia minor</i>	Morgan (1928)	14	14	7 <sup>II</sup>	7	XY	XX
<i>Anisolabis maritima</i>	Morgan (1928)	25 (24)	26	11 <sup>II</sup> + 1 <sup>III</sup>	12, 13	$x_1 x_2 Y$	$x_1 x_1 x_2 x_2$
	Kornhauser (1921)	25	26	11 <sup>II</sup> + 1 <sup>III</sup>	12, 13	$x_1 x_2 Y$	$x_1 x_1 x_2 x_2$
	Morgan (1928)	24	24	12 <sup>II</sup> ("11, 13, 16, 19")	12	$x_1 x_2 Y$	$x_1 x_1 x_2 x_2$
	Randolph (1908)	25	26	11 <sup>II</sup> + 1 <sup>III</sup> , 12 <sup>II</sup> + 1 <sup>I</sup>	12, 13	$x_1 x_2 Y$	$x_1 x_1 x_2 x_2$
<i>Anisolabis marginalis</i>	Sugiyama (1933)	25	26	11 <sup>II</sup> + 1 <sup>III</sup> , 12 <sup>II</sup> + 1 <sup>I</sup>	12, 13	$x_1 x_2 Y$	$x_1 x_1 x_2 x_2$
<i>Anisolabis annulipes</i>	Morgan (1928)	25	26	11 <sup>II</sup> + 1 <sup>III</sup> , 12 <sup>II</sup> + 1 <sup>I</sup>	12, 13	$x_1 x_2 Y$	$x_1 x_1 x_2 x_2$
<i>Forficula auricularia</i>	Stevens (1910)	24	—	12 <sup>II</sup>	12 (11, 13)	XY	XX
	Brauns (1912)	—	26	—	—	—	—
	Payne (1914)	24	25 ?	12 <sup>II</sup> , 11 <sup>II</sup> + 2 <sup>I</sup> , 10 <sup>II</sup> + 4 <sup>I</sup>	12 (11, 13, 14)	XY ?	XX ?
	Morgan (1928)	25 (24, 26, 27)	24, 25	12 <sup>II</sup> + 1 <sup>I</sup> , 11 <sup>II</sup> + 3 <sup>I</sup>	12, 13	$XY (X = x_1 + x_2)$	XX, $Xx_1 x_2$
	Morgan (1928)	24 (23)	24, 25	12 <sup>II</sup>	12	$x_1 x_2 Y$	XX
	Morgan (1928)	25 (26)	—	11 <sup>II</sup> + 1 <sup>III</sup> , 12 <sup>II</sup> + 1 <sup>I</sup> ("11")	12, 13	XY	XX
<i>Forficula scudderi</i>	Misra (1937)	24	—	12 <sup>II</sup>	12	XY	XX

I = univalent; II = bivalent; III = trivalent.

Numbers in brackets are counts from exceptional cells: those in inverted commas are of unspecified associations at first metaphase.

*Labidura* and *Labia*. *Labidura bidens* (Morgan, 1928),  $2n=12$ , has five pairs of autosomes and an unequal sex pair in the male. *Labidura riparia* (Asana & Makino, 1934) and *Labia minor* (Morgan, 1928) both have six pairs of autosomes and an unequal sex pair in the male. In all three species the maturation divisions are regular, chromosome numbers are constant and there are no laggards. Since the females have the same diploid numbers as the males, the sex-determining mechanism is clearly of the XY-XX type, the male being heterogametic.

Species of *Anisolabis* form a second group. Three have been examined, and they all possess a complex sex mechanism. The male diploid number is 25, the female 26. At meiotic prophase in the male three chromosomes condense precociously and may all lie in intimate association. *A. maritima* (Kornhauser, 1921; Morgan, 1928) has 12 "chromosomes" at first meiotic metaphase (eleven bivalents and a sex trivalent). The trivalent may be "triangular" or linear in form. The largest member disjoins from the other two at first anaphase and so must be the Y chromosome. At first anaphase the two X chromosomes become detached from each other although passing to the same pole, with the consequence that equal numbers of 12- and 13-type second metaphases are produced.

*A. marginalis* (Sugiyama, 1933) closely resembles the former species, with the exception that in 1% of the first meiotic metaphases the sex trivalent is replaced by a bivalent and univalent. In *A. annulipes* (Morgan, 1928) the process is carried further, 8% of the first metaphases possessing bivalent and univalent sex chromosomes. When a univalent is present there is nothing to ensure the correct disjunction of the sex chromosomes, but the constancy of the male and female diploid numbers indicates that abnormal complements are eliminated. Morgan notes that in *A. annulipes* disjunction of the trivalent frequently lags behind that of the autosomes. An observation of Kornhauser, the significance of which will be discussed later, is that in certain cysts of the testes of *A. maritima* the spermatogonial divisions show 24 instead of 25 chromosomes.

The results of Randolph's work on *A. maritima* (Randolph, 1908) are at variance with those of the above authors. She found the diploid number of male and female to be 24, and that there were 12 bivalents at first meiotic metaphase in the male, none of which could be recognized with certainty as the sex pair. Occasional cells contained 11, 13, 16 or 19 "chromosomes" at first metaphase. Randolph states that these were abnormal spermatocytes, frequently possessing multipolar spindles, and that second meiotic metaphase was constant in showing 12 chromosomes.

The third group is formed by *Forficula* and here the most extreme complexity exists. The early work has already been summarized. Stevens (1910) has described unequal *XY* pairs in male material of *F. auricularia* from Heligoland and Eisenach, the diploid number being 24. There were regularly 12 bivalents at first metaphase and this was the normal number for second metaphase. 11- and 13-type second metaphases were found exceptionally, however. Stevens accounts for 11-type second metaphases by assuming that *X* and *Y* sometimes pass to the same pole at first anaphase. This would also account for 13-type second metaphases but the latter are sometimes produced in another manner when one of the chromosomes divides equationally at first anaphase. Stevens gives a figure showing such a division. She also figures the remarkable behaviour of a lagging chromosome which sometimes is seen at second anaphase. This chromosome does not divide, but becomes stretched between the daughter telophase nuclei and transected by the newly formed cell boundary.

Payne (1914) worked with *F. auricularia* from Würzburg. His account describes much more variable conditions than does that of Stevens. All but one of his specimens had a spermatogonial number of 24. 12-, 13- and 14-type first metaphases were found, twelve being predominant, and Payne interprets the higher counts as due to unpaired chromosomes, two in 13- and four in 14-type metaphases. Payne hesitates to identify a sex bivalent in all divisions, but he notes one bivalent, one member of which is remarkable in being bilobed at its centric end. He shows that meiotic univalents lag at first anaphase and may divide equationally and he describes and figures a lagging chromosome sometimes found in second anaphases which is clearly of the same type as that noticed by Stevens. One of Payne's specimens had a diploid number of 25, and in this individual first meiotic metaphases showed 12 bivalents and a univalent. In this testis there were abnormal spermatogonial counts, 24-, 26- and 27-type plates being found.

The most complete account of the cytology of male *F. auricularia* is that of Morgan (1928). His material came from Zürich, Switzerland. Many males were examined; 50% had a spermatogonial number of 24, the remainder 25. The meiotic process was very much less variable in 24- than in 25-type males. First metaphase in the former showed 11 autosomal bivalents and an unequal *XY* pair. Morgan notes, with Payne, that one of these sex chromosomes, the larger, was frequently bilobed. First anaphase was regular, with lagging chromosomes very rare, and the subsequent second metaphase showed constantly 12 chromosomes.

25-type males fell into three classes according to the constitution of first metaphase. In the first class 11 bivalents and a trivalent occurred more frequently than 12 bivalents and a univalent. In the second class there were equal numbers of both, while in the third class 12 bivalents and a univalent were in excess. In most 25-type males there were equal numbers of 12- and 13-type second metaphases, but a few specimens showed a preponderance of 13-type plates, on account of the equational division of a univalent sex chromosome at first anaphase. Morgan notes that there were many lagging chromosomes at first and second anaphases in the 25-type males.

In females of *F. auricularia* mitoses show 24 or 25 chromosomes according to Payne and Morgan, and Brauns has reported 26-types as occurring also. On Morgan's interpretation of the sex-determining mechanism in *Forficula*, the 25-type males are of the constitution  $x_1x_2Y$ , similar in this respect to *Anisolabis*. 24-type differ from 25-type males in that  $x_1$  and  $x_2$  are fused in the former to produce the larger bilobed X member of the XY pair. 24-type females are thus XX, 25-type  $x_1x_2X$  and 26-type  $x_1x_1x_2x_2$ .

*F. scudderi* has been investigated by Misra (1937). This species has  $2n=24$  in the male, and the sex chromosomes form an unequal bivalent at first meiotic metaphase as in the 24-type males of *F. auricularia*. The meiotic process follows the same lines as in the latter, and Misra adopts Morgan's interpretation: that the larger sex chromosome represents a permanent fusion of  $x_1$  and  $x_2$ .

#### MATERIAL AND METHODS

I have worked with specimens of *F. auricularia* Linn. from four different localities: Merton (S.W. London), Wallington (Surrey), Marcham (Berkshire) and Gillingham (Kent). Smears and sections of testes were fixed in La Cour's 2 BD (La Cour, 1937) and stained by Newton's Gentian Violet-Iodine method or by the Feulgen technique. A few sectioned ovaries were also examined.

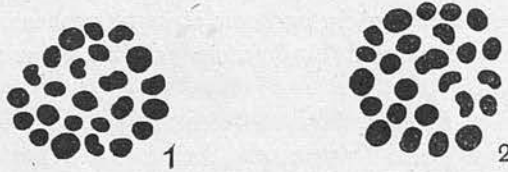
The testes of *Forficula* are paired organs lying dorsal to the gut. Each consists of two separate follicles which open posteriorly into a common vas deferens. To obtain complete series of meiotic stages, testes of adults were fixed in August. The free end of the follicle at this period contains spermatogonia, with plentiful mitoses. The bulk of the follicle contains first spermatocytes in various stages of meiotic prophase. Posterior to these follows a zone where first and second meiotic divisions



are taking place and then follows a zone of spermatids and finally spermatozoa.

#### THE TWO TYPES OF MALE

It is clear from the account given by Morgan that there are two cytologically distinguishable types of male *F. auricularia*,  $2n=24$  and 25 respectively. I am able to confirm this (Text-fig. 1). My material shows



Text-fig. 1. Spermatogonial mitoses in *F. auricularia*. 1, 24-type; 2, 25-type. ( $\times 3000$ .)

the significant fact that in different populations the proportions of the two types vary (Table 2). The proportion of 25-type males in my material falls far short of the 50% found by Morgan in earwigs from Switzerland.

Table 2. *Proportions of male types in four populations*

Locality	Number of 24-type males	Number of 25-type males	Percentage of 25-type males
Merton (S.W. London)	60	0	0
Marcham (Berkshire)	71	7	9
Gillingham (Kent)	52	13	20
Wallington (Surrey)	15	5	25

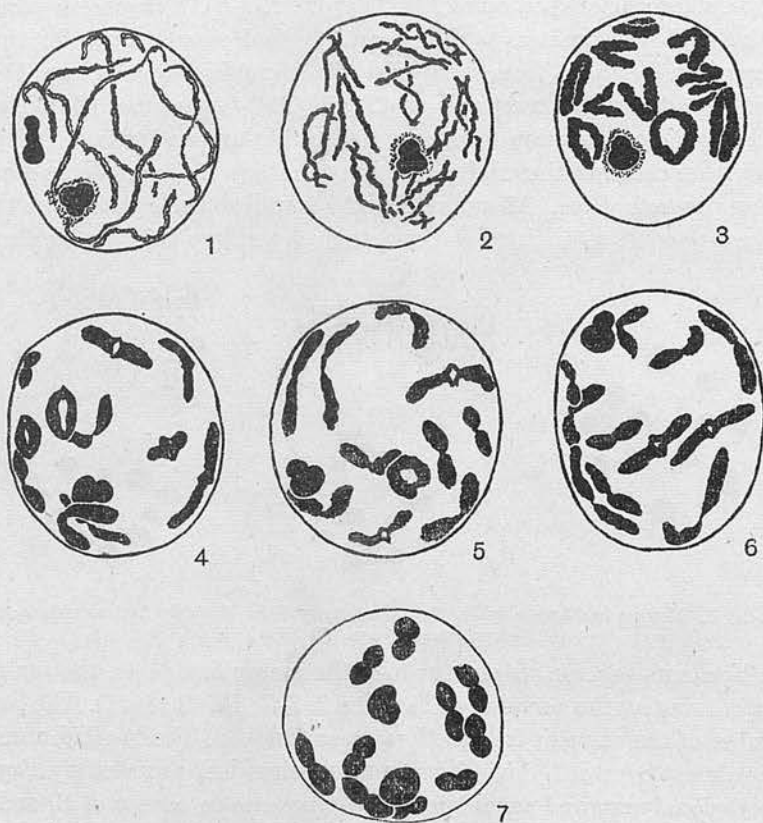
I find occasional follicles of spermatogonial mitoses in which the chromosome number does not agree with the "zygotic" diploid number found generally in other follicles of the testis. Thus 25 counts sometimes occur in 24-type males and 24-, 26- and 27-counts in 25-type males. Such irregularity is a characteristic of *Forficula* (as noted by Morgan and Payne), and its significance will be discussed at a later stage.

24- and 25-type males are externally indistinguishable, and undoubtedly belong to the same species of *Forficula*. With Morgan, I can confirm that chromosome number is in no way correlated with the high and low dimorphism of males which exists in this species (Bateson, 1894).

#### MEIOSIS IN THE 24-TYPE MALE

Prophases of meiosis in *Forficula* fix very well (Text-fig. 2). The leptotene nucleus contains, in addition to the single autosomal chromosome strands, two bodies which stain intensely with Gentian Violet. One of these is irregular in outline, the other of firm outline and shaped like an indian club. The former is Feulgen-positive, and consists of the

sex chromosome pair with a covering of nucleolar material which is Feulgen-negative. The latter is Feulgen-negative and represents the nucleolus. The sex pair come together at the telophase of the last spermatogonial mitosis. They are fully condensed when they associate, and it is highly improbable that their association leads to chiasma formation. In

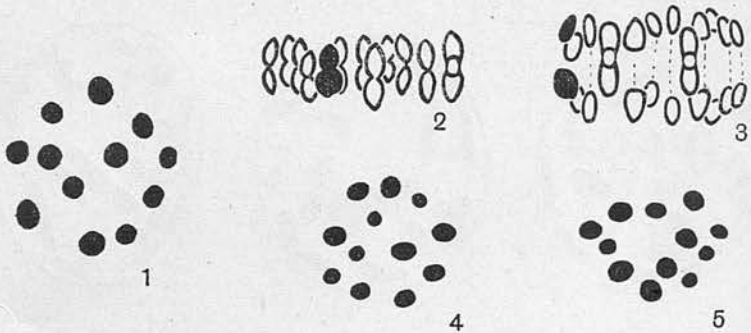


Text-fig. 2. Meiotic prophase in 24-type male. 1, zygotene (note nucleolus and sex pair); 2, diplotene (note chiasmata and relational coiling); 3, later diplotene; 4-6, early diakinesis, 13, 12 and 11 chiasmata respectively; 7, late diakinesis. ( $\times 3000$ .)

fact it seems likely that their association is conditioned by some non-specific quality which they hold in common, rather than by any precise structural homology since, as will be seen later, associations exist which cannot have arisen by particulate pairing. Autosome pairing at zygotene is unpolarized. Pachytene is a long stage, the diplotene which follows being of much shorter duration. The nucleolus disappears at this stage. Chiasmata can be made out clearly at diplotene: one or two are formed

by each bivalent, but in any one nucleus there are never more than two bivalents which form two chiasmata apiece. A high degree of relational coiling exists between the paired chromosomes at diplotene (Text-fig. 2, 2). As the nucleus approaches diakinesis, this relational coiling is undone, the chromosomes thicken and the chiasmata terminalize to give rise to dumb-bell or ring-shaped bivalents (Text-fig. 2, 4-6). Chiasma frequency can be readily estimated at diakinesis. Typical mean values for three different individuals from analyses of 20 nuclei in each are 11.05 (variance = 0.05), 11.3 (variance = 0.43) and 11.7 (variance = 0.64). There is no significant variation from cyst to cyst within one testis.

Further condensation and smoothing of outline of the bivalents occurs during prometaphase. At metaphase the spiralization is so intense that it is not possible to distinguish between one- and two-chiasma bivalents.



Text-fig. 3. Meiosis in 24-type male. 1, 2, first metaphase; 3, early first anaphase; 4, 5, second metaphase. ( $\times 3000$ .)

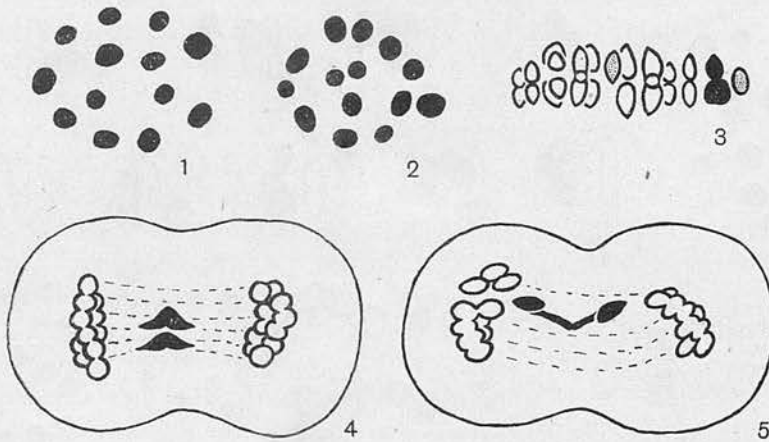
The bivalents become spread out over the metaphase plate, the sex pair always lying at the periphery (Text-fig. 3, 1-3; Pl. 11, fig. 1). The larger member of the sex pair frequently appears bilobed. The haploid number 12 is almost constant, deviations from it being due, as noted by Payne, to occasional unpaired autosomes. This gives rise to counts of 13 and 14 (Text-fig. 4, 1, 2). At first meiotic anaphase the sex pair disjoins in time with the autosomes. Sometimes one or two of the latter lag slightly; it is probable that these are bivalents which have formed two chiasmata. In a few anaphases I have seen sticking between one pair of arms of such bivalents (Text-fig. 4, 5). Univalent autosomes lag at anaphase. They generally divide equationally (Text-fig. 4, 4).

Second metaphases (Text-fig. 3, 4, 5) show regularly 12 chromosomes, and the deviations from this number which occur are the results of the equational division of univalents at first anaphase. Second anaphase may show no laggards, daughter univalents passing late and undivided

to one pole or, in some testes not infrequently, a chromosome which becomes stretched between the two telophase groups and subsequently transected by the cell boundary.

The second telophase nuclei subsequently undergo processes leading to the formation of the mature sperm nucleus. The autosomes pass into a diffuse state, the sex chromosomes remaining for some time condensed. Then the nucleus gradually diminishes in volume and increases in stainability, until the highly condensed sperm nucleus is produced.

In a number of 24-type testes I have found cysts with spermatogonial counts of 25 and at first meiotic metaphase an accessory centric sex



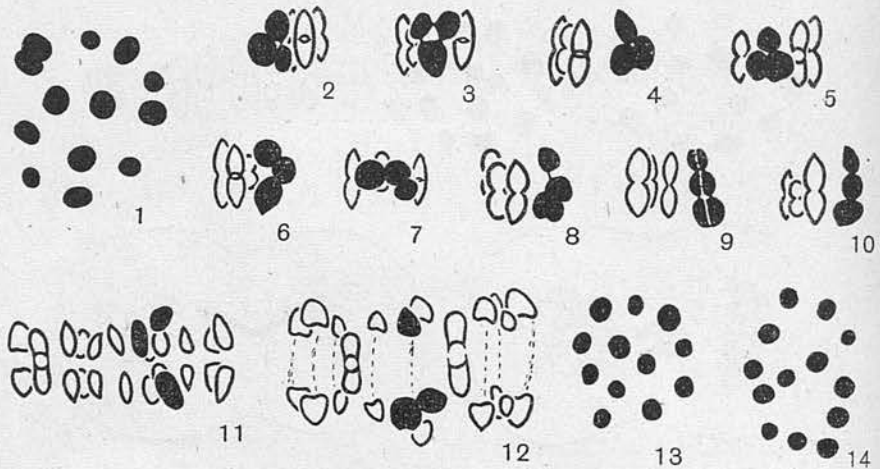
Text-fig. 4. Meiosis in 24-type male. 1-3, univalents at first metaphase; 1, 13-type plate; 2, 14-type plate; 3, side view, two univalents; 4, two univalents at first anaphase; 5, "sticking" of arms of a bivalent at first anaphase. ( $\times 3000$ .)

fragment (Text-fig. 9, 1, 2). This fragment must have arisen by an anomalous process at some early spermatogonial division. At first metaphase the fragment is generally seen associated with the sex bivalent. It disjoins with either member of the latter, and at second anaphase divides equationally like any other normal chromosome.

#### MEIOSIS IN THE 25-TYPE MALE

The behaviour of the autosomes in the 25-type male follows the same lines as in the 24-type male. In the following account the sex chromosomes only will be considered. Any anomalous behaviour of these latter will be superimposed on occasional autosomal abnormalities consequent on univalency at first meiosis, hence making the interpretation of chromosome number in the spermatocytes more complex than in the 24-type.

The leptotene nucleus contains one or two Feulgen-positive bodies and a nucleolus negative to Feulgen. The Feulgen-positive bodies are the sex chromosomes: when there is only one such body, this is the sex trivalent: when there are two, one is bivalent and the other univalent. No further fusion of the sex chromosomes occurs after the telophase of the last spermatogonial division, i.e. a bivalent and univalent condition of the sex chromosomes at leptotene persists until the anaphase of the first meiosis. The form of the trivalent is first clearly recognizable at late diakinesis. It may be "triangular" or linear. That the trivalent may be "triangular" at diakinesis indicates that there is active pairing between



Text-fig. 5. Meiosis in 25-type male. 1, first metaphase, 12-type plate, trivalent at top left; 2-10, trivalent at first metaphase in side view; 2-5, "triangular"; 6-10, linear; 11, 12, early first anaphase; 11, correct disjunction; 12, mal-disjunction; 13, 14, second metaphase, 12- and 13-type plates. ( $\times 3000$ .)

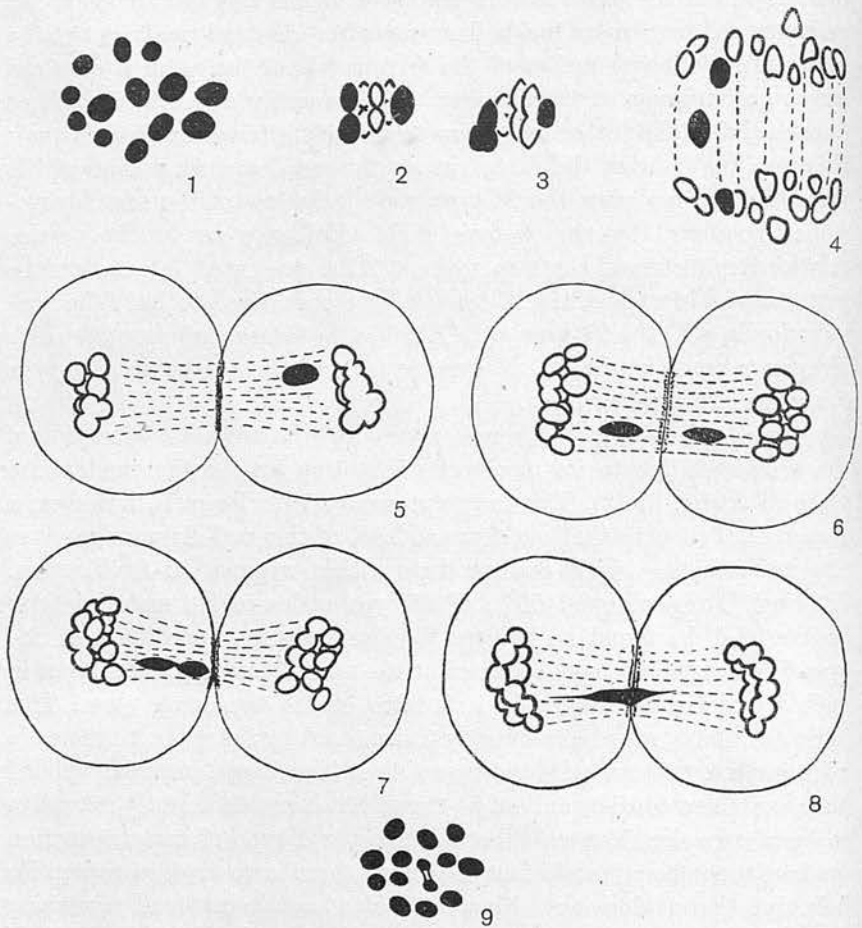
all three component chromosomes: the "triangular" configuration is not merely the result of convergent co-orientation on the first metaphase plate. Lorbeer (1934) has found in one of the liverworts, *Frullania dilatata*, a sex-chromosome trivalent remarkably similar to that of *Forficula* with this same property of pairing between all three members.

At metaphase the sex chromosomes lie at the periphery of the plate (Text-fig. 5, 1-10; Pl. 11, figs. 2-9). A "triangular" trivalent is usually so orientated that the largest member subtends the other two. This is the disjunction type in *Forficula* as in *Anisolabis* (Text-fig. 5, 11): it is therefore probable that the largest chromosome is the Y. When the three sex chromosomes of the 25-type male are compared with the two of the 24-type, it can be seen that Morgan's "fusion" theory of the

origin of the  $X$  in the latter is untenable. This theory supposes that the total volume of the sex trivalent is equal to that of the bivalent in the 24-type male. It is, however, manifestly greater. The size of the individual chromosomes making up the bivalent and the trivalent can be compared by camera lucida drawings after identical treatment of the testes. The largest member of the trivalent is of the same size as the larger chromosome of the bivalent. It frequently appears bilobed, as does the latter. The other two chromosomes of the trivalent are subequal; they are both about the same size as the smaller chromosome of the bivalent. On my view the 24-type male lacks one of the sex chromosomes possessed by the 25-type male. Evidence as to the precise relationship between the two types will be presented later. For the purpose of description, the 25-type will be considered to have the constitution  $x_1x_2Y$ , the 24-type  $x_1Y$  ( $Y$  being the larger chromosome which Morgan takes to be the  $X$ ). 24-type females would thus be  $x_1x_1$ , 25-type  $x_1x_1x_2$  and 26-type  $x_1x_1x_2x_2$ .

In some 25-type males there is a very high rate of mal-disjunction of the trivalent, due to its incorrect orientation on the first metaphase plate (Text-fig. 5, 12). This may approach 30%. There is, however, a reason for believing that one form at least of this mal-disjunction gives rise to gametes which can produce viable zygotes at fertilization. Although Morgan found 50% of 25-type males in his material from Switzerland, he found no 26-type females, only 24- and 25-types. 26-type females should have occurred at the same frequency as 24-types in such a population to give a 1:1 ratio of the two male types. This indicates that  $x_2$  may be frequently transmitted by the sperm to the male offspring, i.e. that an  $x_2Y$  gamete is viable. The female material which I have examined consists only of 24-types, but is not sufficiently extensive to be of any significance. Whether the  $x_2-x_1Y$  type of mal-disjunction occurs cannot be certainly stated, though there is no *a priori* reason for believing that it does not. Since no males have been found possessing from the zygote more than one  $Y$  chromosome (duplication of the  $Y$  when it occurs is not due to an abnormal zygotic complement), the presumption is that the female cannot carry a  $Y$  chromosome, and thus  $x_1Y$  gametes, if they occur, fail to produce viable, or at least sexually functional zygotes. In this connexion it may be pointed out that externally normal males without testes are sometimes found. These may perhaps be individuals with anomalous sex chromosome complements. I have also found one specimen, externally female, possessing testes. This proved to be an apparently normal 24-type  $x_1Y$  individual.

The linear trivalent takes on a variety of forms (Text-fig. 5, 6-10; Pl. 11, figs. 7-9). The three chromosomes may be arranged in a straight line, or bent. This apparently depends on the points of contact between



Text-fig. 6. Sex univalent at meiosis in 25-type male. 1, first metaphase, 13-type plate; 2, 3, first metaphase in side view; 2, univalent *Y*; 3, univalent *x*; 4, early first anaphase, univalent *Y* at equator; 5-8, behaviour of univalent *Y* at first anaphase; 5, passing undivided to one pole; 6, equational division; 7, "partial equational division"; 8, "dicentric stretching"; 9, 14-type second metaphase derived from anaphase of "partial equational division" type. ( $\times 3000$ .)

the sex chromosomes, though it is possible that the bent linear trivalent may sometimes result from the breakage of one contact point between the chromosomes of a "triangular" trivalent. The *Y* chromosome generally lies to one end, but it may rarely occupy the middle position.

It is unlikely that this has any significant bearing on the lengths of possible homologous regions, for true pairing does not occur. As with the "triangular" trivalent, the linear trivalent may disjoin correctly or incorrectly.

When the sex chromosomes fail to form a trivalent, a bivalent and univalent being formed instead, it is almost invariable for the *Y* chromosome to be left as the univalent (Text-fig. 6, 1-4; Pl. 12, fig. 2). It is thus rare to find a markedly unequal sex bivalent as exists in the 24-type male. However, when such a bivalent is found, it is of the same form as that found in the latter.

The behaviour of the univalent *Y* at the meiotic anaphases leads me to suppose that it is dicentric. At first anaphase it may do one of four different things:

(1) Pass undivided to one pole (Text-fig. 6, 5; Pl. 12, fig. 4). This must in any case lead to an illegitimate disjunction type, since  $x_1$  separates from  $x_2$ . Further, there is nothing to ensure that *Y* should pass to the same pole as  $x_2$  rather than  $x_1$ .

(2) Divide equationally (Text-fig. 6, 6; Pl. 12, fig. 3). This results in  $x_1Y$  and  $x_2Y$  telophase nuclei. A univalent  $x_1$  or  $x_2$  always behaves in this way.

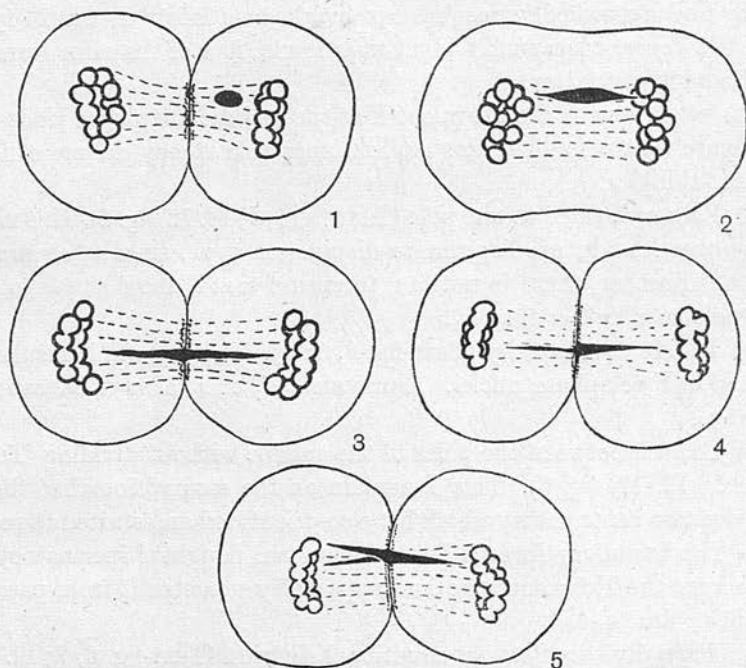
(3) Stretch between the poles of the spindle without dividing (Text-fig. 6, 8; Pl. 12, fig. 6). This I explain on the assumption that the *Y* possesses two centromeres which have co-orientated and started to move apart. The boundary formed between the two daughter spermatocytes cuts across the *Y* chromosome and apparently prevents it from passing to either pole.

(4) Partially complete an equational division (Text-fig. 6, 7; Pl. 12, fig. 5). *Y* lingers for a long time at the equator of the spindle. One of its centromeres divides and the two chromatids spring apart. The other undivided centromere, however, prevents the division from being completed. The cell boundary may form and cut across the *Y*, or the latter may be pulled entire into one or other of the daughter spermatocytes. It may pass to one pole still incompletely divided, or complete the division late.

Counts of second metaphase chromosomes show that 12- and 13-plates predominate (Text-fig. 5, 13, 14; Pl. 12, figs. 7, 8). These are to be expected to occur in equal numbers as a result of disjunction of the trivalent. Such is sometimes the case, but more generally 13-types outnumber 12-types, and this is clearly the result of equational division of univalent *Y* chromosomes at first anaphase in a proportion of cells.



11-type second metaphases also occur, but rarely. They may result from the sex chromosomes failing to disjoin at first anaphase. 14-type second metaphases (Text-fig. 6, 9; Pl. 12, fig. 9), which occur more frequently, are the result of both products of the delayed equational division of *Y* passing to the same pole. In some of these the daughter *Y* univalents remain connected at one point (the undivided centromere) clearly demonstrating their mode of origin.



Text-fig. 7. Second anaphase in 25-type male. 1, daughter univalent passing undivided to one pole; 2-5, "dicentric stretching" of *Y*. In 5 one of the centromeres has divided equationally. ( $\times 3000$ .)

Second meiotic anaphases may be quite regular, without lagging chromosomes, or a daughter univalent may pass late and undivided to one pole (Text-fig. 7, 1; Pl. 13, fig. 1). A more frequent abnormality, however, is for one chromosome to stretch along the spindle between the two poles. This behaviour also occurs (as has been mentioned) in the 24-type male. It is due to "dicentric separation" of the *Y* chromosome. In 24-type males the latter consists of two chromatids. In the 25-type male it may be similarly constituted, or alternatively of only one chromatid, the latter condition being that of a daughter univalent. At early anaphase this chromosome stretches evenly (Text-fig. 7, 2; Pl. 13, fig. 2).

Later it is transected by the cell boundary formed between the spermatids. The transection may occur about the middle of the chromosome, when the stretching remains even (Text-fig. 7, 3; Pl. 13, fig. 5). More often, however, the *Y* was nearer one pole than the other when the cell boundary was formed. The result of this is that the smaller portion stretches out into a long thin filament, and the appearance is not unlike the centric misdivision of an almost telocentric chromosome (Text-fig. 7, 4; Pl. 13, figs. 3, 4). Sometimes, when the stretching *Y* consists of two chromatids, one limb may split owing to the delayed equational division of its centromere. The cell boundary prevents the split from spreading into the other limb (Text-fig. 7, 5). Rarely, second anaphase may show two such stretching chromosomes. These are presumably the undisjoined daughter univalents of a *Y* chromosome. It is unlikely that a dicentrically stretched *Y* chromosome is ever included in the spermatid nuclei. The cell boundary prevents it from reaching the poles of the spindle, and fragments do not appear to break loose. Presumably this chromosome is shed when the cytoplasm of the sperm is sloughed at the formation of the mature sperm. Were viable zygotes to be produced from gametes carrying fragments of a sex chromosome, they would be noticed in testis fixations. But sex fragments when they appear in the spermatocytes are not general to the whole testis, and they have a different origin.

The hypothesis that *Y* is dicentric cannot, unfortunately, be tested by examination of the mitotic chromosomes, since these are as much condensed as the meiotic chromosomes even in embryo divisions. The bilobed appearance of the *Y* at meiosis, however, provides some evidence as to the validity of the hypothesis.

Table 3 *a, b* shows the frequency of the different types of behaviour of the sex chromosomes in eight 25-type males taken at random. It is important to realize, however, that conditions obtaining in one cyst may differ markedly from those in other cysts of the same testis. The significance of this is obscure. It is thus not possible, for example, to compare accurately the relative frequency of equational divisions of univalent *Y* chromosomes at first anaphase with the excess of 13-type second metaphases. Specimen no. 5 showed in one cyst of second metaphases equal numbers of 12- and 13-counts, in another a large excess of 13-counts. However approximate agreement exists. The relative frequency of linear trivalents to those of the "triangular" type bears an approximate proportionality to sex univalent frequency. In this connexion it may be noted that *Anisolabis* spp. with low univalent frequency also show comparatively few linear trivalents. This indicates that the linear trivalent

Table 3

(a) Sex-chromosome behaviour at first meiosis in 25-type males of *Forficula auricularia*

Eight individuals arranged in order of decreasing sex-univalent frequency

G = Gillingham; M = Marcham

Specimen	First metaphase				First anaphase				
	Polar views		Side views		Univalent pole to one (Y)	Equational division of univalent (x or Y)	"Partial equational division" of univalent (Y)	"Dicentric stretching" (Y)	Frequency of equational division %
	11 bi-valents + tri-valent	12 bi-valents + uni-valent	Frequency of sex-univalent %	"Tri-angular" trivalent					
1 (G)	22	13	37	43	7	7	2	2	18
2 (M)	44	24	35	60	23	6	1	0	9
3 (M)	54	22	29	58	11	6	1	0	18
4 (G)	29	7	19	55	8	2	0	3	4
5 (G)	26	5	16	42	10	6	2	0	7
6 (M)	35	2	5	53	3	0	1	1	0
7 (M)	47	0	0	40	8	1	0	0	2
8 (G)	29	0	0	27	2	4	0	5	9

(b) Sex-chromosome behaviour at second meiosis in 25-type males of *Forficula auricularia*

Eight individuals arranged in the order of (a) above

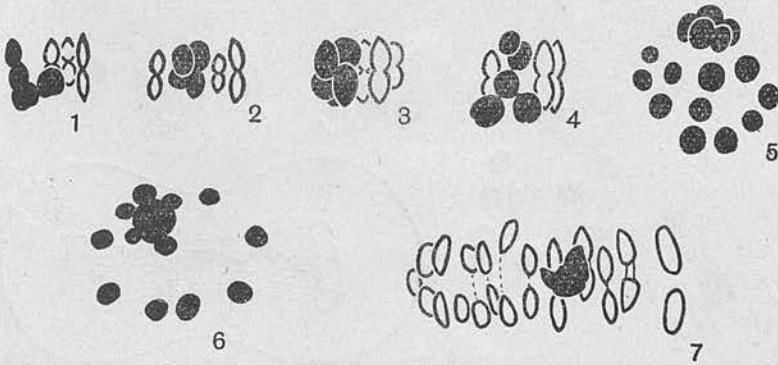
Specimen	Second metaphase				Second anaphase				
	11-type		12-type		13-type		14-type		Frequency of excess 13-types %
	0	13	22	65	105	26	42		
1 (G)	0	13	22	65	105	26	42	0	26
2 (M)	2	24	40	35	40	0	25	16	0
3 (M)	1	27	35	28	39	1	73	4	0
4 (G)	0	10	23	0	28	0	39	10	0
5 (G) { Cyst 1	0	29	42	0	28	0	0	0	38
6 (M) { Cyst 2	0	39	37	0	42	0	4	27	2
7 (M)	0	29	37	0	37	0	12	2	0
8 (G)	0	20	25	0	25	0	11	2	5

No. laggards	Daughter univalent pole to one (x or Y)		"Dicentric stretching" (Y)	Uncertain types	Frequency of "dicentric stretching" %
	0	1			
57	0	0	20	5	26
60	16	0	0	0	0
26	4	0	0	1	0
49	10	0	0	1	0
44	0	0	27	0	38
47	0	0	1	0	2
73	2	0	0	0	0
52	2	2	2	3	4
42	0	0	4	0	5

and the sex univalent are both manifestations of reduced "pairing power". A further point of interest is that dicentric stretching of the Y at first and second anaphases does not occur in all testes, indicating that the Y chromosome is not identical in all males of *Forficula*.

#### LOSS, DUPLICATION AND FRAGMENTATION OF THE Y CHROMOSOME

The instability of mitotic chromosome number in *Forficula* is explicable, on the supposition that Y is dicentric, in the following way. Intercentric relational coiling would account for abnormal mitotic behaviour leading to loss, duplication or fragmentation of the Y chromosome.



Text-fig. 8. Reduplicated Y at first metaphase in 25-type male. 1, 2, sex quadrivalent; 3-5, sex quinquevalent; 6, 7, anomalous reduplication. ( $\times 3000$ .)

In 25-type males I have noticed three testes where in one or two cysts the Y chromosome was missing. In these cysts at first meiotic metaphase  $x_1$  and  $x_2$  form an almost equal sex bivalent. In two of these testes nearby cysts contain cells of the complementary type with two Y chromosomes, which associate with  $x_1$  and  $x_2$  to form a sex quadrivalent at first metaphase (Text-fig. 7, 1, 2). It would thus appear that at an early spermatogonial division the daughter Y chromatids failed to separate and passed together to the same pole.

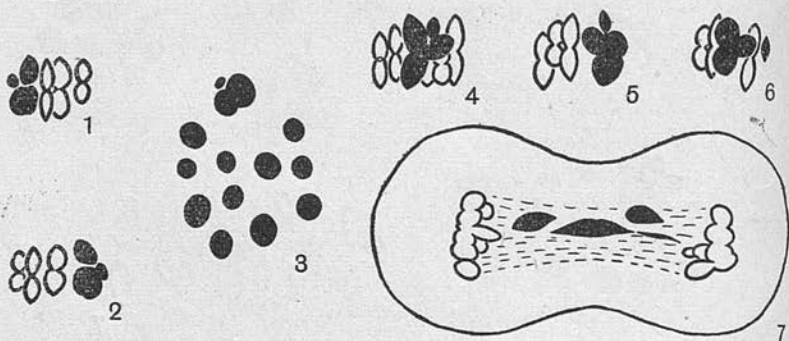
I have also seen two cysts where there were five sex chromosomes in association in all the cells at first metaphase (Text-fig. 8, 3-5; Pl. 13, fig. 8). Fixation was poor in both cases and it is not possible to state whether fragmentation of one of the Y chromosomes, or alternatively successive reduplication due to failure of anaphase separation, was responsible. The latter supposition is more plausible.

The sex chromosomes in such quadrivalents and quinquevalents associate in indiscriminate conformations. Centric repulsions are not

adjusted, consequently at first anaphase they lag behind the autosomes and disjoin in a highly irregular manner (Pl. 13, fig. 9).

In the third testis where the Y chromosome was missing in one cyst, a nearby cyst contained spermatocytes at first metaphase with a structure which I was unable to interpret. The chromosomes were arranged in a hollow spindle, 12 bivalents and a large body of irregular shape to which some of the bivalents were attached (Text-fig. 8, 6, 7; Pl. 13, fig. 10). At anaphase disjunction was very irregular due to the lagging of this body and its attached bivalents.

In 24-type males (as has been mentioned) and in 25-type males more frequently, cysts of spermatocytes containing a sex fragment as well as the normal complement may be found side by side with normal cysts.



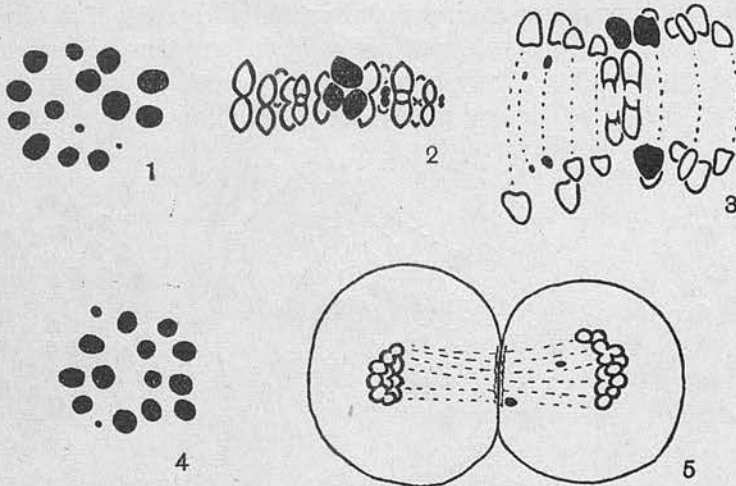
Text-fig. 9. Centric Y fragment in 24- and 25-type males. 1, 2, associated fragment at first metaphase in 24-type male; 3-6, associated and non-associated fragment in the 25-type male; 7, associated fragment at first anaphase in 25-type male. ( $\times 3000$ .)

I interpret these fragments as being parts of the Y chromosome broken at a spermatogonial mitosis owing to intercentric coiling. They are generally seen associated with the sex chromosomes at first metaphase, though occasionally they may lie separate (Text-fig. 9, 1-6; Pl. 13, fig. 6). An associated fragment may or may not interfere with the disjunction of the sex chromosomes (Text-fig. 9, 7; Pl. 13, fig. 7). A non-associated fragment divides equatorially at first anaphase and passes to one pole at second anaphase.

It is important to realize that these are not "zygotic" abnormalities, i.e. the result of fertilizations where the gametes possessed abnormal complements. Only one case of the latter has been found. In this  $x_1x_2Y$  male there were two autosomal fragments in all the cells of both testes (Text-fig. 10; Pl. 13, figs. 11, 12). These fragments remained unpaired at first metaphase. They divided equatorially at first anaphase without lagging, so that all the second metaphases also showed both fragments.

At second anaphase they lagged behind the other chromosomes and passed undivided to one or other pole. They may have originated from autosomal misdivisions at the maturation divisions of the parental sperm or egg.

A striking feature of the testes of *Forficula* is the large number of cysts of necrotic spermatogonia. It is possible that these spermatogonia possess abnormal complements and are unable to compete successfully with their balanced neighbours.



Text-fig. 10. Two autosomal fragments in 25-type male. 1, 2, first metaphase; 3, first anaphase; 4, second metaphase; 5, second anaphase. ( $\times 3000$ .)

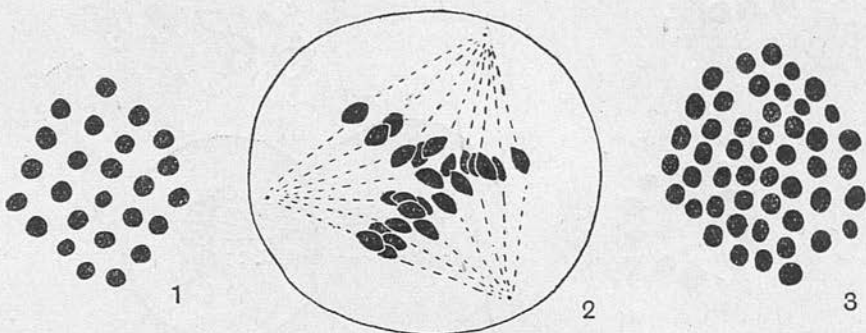
#### POLYPLOID CELLS

The testes of *Forficula* contain polyploid cells of various kinds. These may be classified as follows:

(1) *True tetraploid first spermatocytes*. These cells are the products of division of a "doubled-up" spermatogonium. The "doubling-up" may occur early or late in the spermatogonial lineage, for various numbers of such tetraploid spermatocytes may be found together. These tetraploid cells are characterized by the presence of multivalent chromosome associations at first metaphase: chiasma formation will be described in a later paper. They give rise to clumps of diploid second spermatocytes.

(2) *Syndiploid first spermatocytes* (Makino, 1939). These cells are produced when two diploid diakinetive nuclei break down for metaphase orientation, all the chromosomes passing on to a common spindle. They occur singly in an otherwise diploid cyst, and possess no multivalent associations. They give rise to pairs of diploid second spermatocytes.

(3) *Restitution diploid second spermatocytes*. This type of polyploid cell occurs extremely commonly in testes of *Forficula* (Text-fig. 11, 1; Pl. 2, fig. 10). It is produced when first meiotic anaphase has failed to separate the telophase nuclei completely, and no cell boundary has been formed. This may occur when the first metaphase complement was normal, when there were univalents or when some abnormal first meiotic association was present which prevented disjunction (e.g. a duplicate Y chromosome). Restitution diploid second spermatocytes occur singly or in groups. Chromosome number may be slightly in excess of the diploid number if, at the previous anaphase, certain univalent chromosomes divided equationally. Many of these cells possess tri- or tetra-polar spindles: both centrosomes of the preceding division have persisted,



Text-fig. 11. Restitution second spermatocytes. 1, diploid second metaphase; 2, diploid second metaphase with tripolar spindle; 3, tetraploid second metaphase. ( $\times 3000$ .)

with their capacities to organize spindles, and one or both have divided (Text-fig. 11, 2; Pl. 12, fig. 11). When a normal dipolar spindle is formed in these second spermatocytes, it is too small to accommodate all the chromosomes present in a flat plate: the plate is almost always curved. This is in contrast to the flat plates of tetraploid first spermatocytes. Restitution second spermatocytes may also occur after the division of a tetraploid first spermatocyte, and thus tetraploid second spermatocytes are occasionally produced (Text-fig. 11, 3).

#### THE SEX MECHANISMS OF *ANISOLABIS* AND *FORFICULA*

The  $x_1x_2Y$  sex mechanism of *Anisolabis* appears to have been the original sex mechanism of *Forficula*. That the number of  $x$  chromosomes may be one or two but not more, and that *Anisolabis* spp. generally possess two, indicates that the two  $x$  chromosomes are not identical. We are not dealing here with a reduplication of like chromosomes such

as occurs in *Cimex* (Darlington, 1939*b*) but rather with a multiple  $x$  mechanism similar to that which exists in some Mantids (Oguma, 1921; White, 1940). *Forficula* differs from these, where both  $x$  chromosomes are essential, since in the zygote only one  $x$  is necessary to determine a viable and fertile male. The other  $x$  is superfluous in sex-determination, but is not necessarily inert.

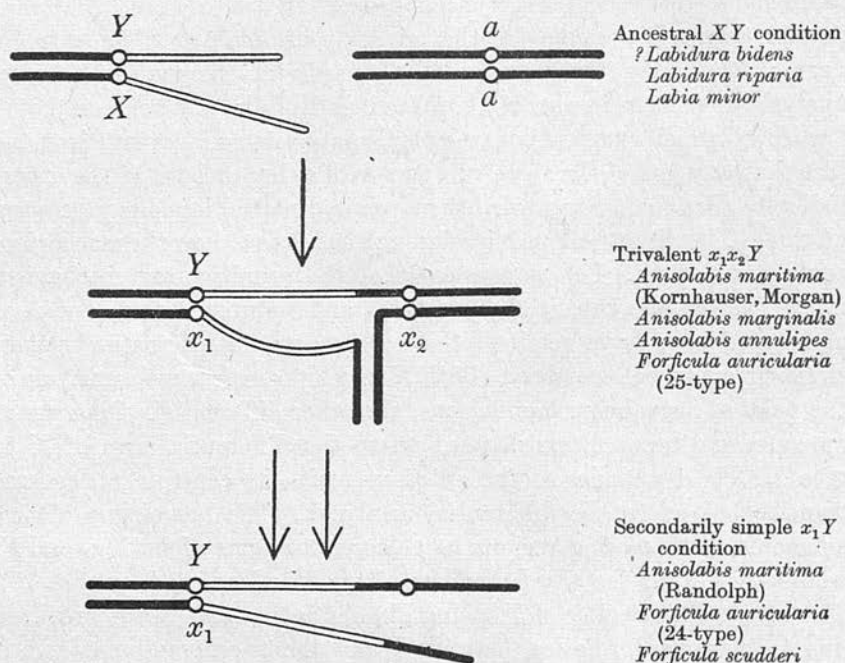
The sex trivalents of *Paratylotropidia* (King & Beams, 1938) and of certain Mantids (White, 1940) are thought to have been the results of interchanges between the sex chromosomes and autosomes. A similar interpretation is possible for the trivalent of *Forficula*. The extreme degree of positive heteropycnosis at meiosis in the male makes an analysis of pairing impossible. In fact, as has already been seen, the "pairing" which exists is not true particulate pairing. Nevertheless, the fact that any one of the three can pair with either or both of the others indicates that there was probably an original partial homology between all three. The dicentric  $Y$  provides us with a clue as to how the mechanism could have arisen. Let us suppose that the primitive sex mechanism in the Dermaptera consisted of simple  $X$  and  $Y$  chromosomes. *Labidura* and *Labia* may have retained this mechanism, or alternatively their simplicity may be secondary. Both  $X$  and  $Y$  possessed two arms, one of the pairs of arms being homologous, the other differential. One arm of an autosome became translocated on to the differential arm of  $X$  to give  $x_1$ . The remainder of the autosome, with its centromere, became translocated on to the differential arm of  $Y$ . The homologue of this autosome, with pairing regions homologous to parts of both  $x_1$  and  $Y$ , would then form the third member of the trivalent,  $x_2$  (Text-fig. 12). The intercentric region of  $Y$ , being differential, would escape breakage due to chiasma formation between  $Y$  and the  $x$  chromosomes and it would merely have to withstand the possibility of discordant behaviour during mitoses in the male. Reduction of intercentric coiling would be necessary to achieve this, but both *Forficula* and *Anisolabis* show that  $Y$  is not always successful in passing through its mitotic divisions without non-disjunction or fragmentation. In this connexion it is interesting to note that Tanaka (1940) has shown that two types of *Scirpus lacustris* possess dicentric chromosomes. These dicentrics behave irregularly at meiotic anaphase: they are associated by chiasmata and frequently break. However, reproduction is doubtless largely vegetative and the permanence of the dicentric within a clone indicates that its behaviour at mitosis is regular.

The sex trivalent as we now see it is probably very different from its



ancestral condition. Strict pairing homology has been lost, and to compensate for this a new mechanism of pairing has arisen involving precocious condensation of the whole of the sex chromosomes, original pairing regions and differential regions alike.

In a trivalent where all three chromosomes are "paired" there must be a special centric mechanism to ensure that orientation and disjunction at meiosis are regular. The dicentric Y is perhaps adapted to perform this function. There are four centromeres in the trivalent which



Text-fig. 12. Diagram illustrating the possible evolution of sex chromosomes in the Dermaptera. Primitive pairing regions are represented by full lines.  $a$  = autosome.

can effectively repel one another in pairs. Two of these, since they lie on the same chromosome, will behave as a unit at meiosis if their nearness to one another bears a certain relation to the proximity of their respective mates. The irregular disjunction of the trivalent in *Forficula auricularia* is probably secondary; strict accompaniment of  $x_1$  by  $x_2$  is no longer essential and misdivision of one of the Y centromeres may have unbalanced the delicately adjusted mechanism. We can assume that the centromeres of the Y chromosome are not invariably of the same strength (Darlington, 1939a) since the frequency with which dicentric separation of the Y occurs varies considerably from one testis to another.

When the sex chromosomes fail to pair completely and one is left as a univalent (generally the Y) the latter chromosome is placed in a new mechanical situation to which its dicentric nature is not directly adapted. Hence the Y may behave in an anomalous manner at first or second anaphase. Incomplete pairing permits the separation of  $x_1$  from  $x_2$  and this was no doubt the way in which types lacking  $x_2$  came into being. Thus the 24-type male *Forficula auricularia* has reverted to a simple sex mechanism (Text-fig. 12, 3). On my interpretation in *F. scudderi* the reversion has been completed. The same evolutionary step would explain the account of meiosis in *Anisolabis maritima* given by Randolph, which so flatly contradicts that of Kornhauser and of Morgan. She was presumably dealing with a population where  $x_2$  had been lost.

If in *Forficula* types can be produced which lack the second  $x$  chromosome, and these types perform the meiotic process in a regular manner, it is important to understand the selective advantage possessed by the 25-type male whose meiosis is remarkable for its extreme eccentricity. If the 25-type male possessed no such advantage, it would presumably have become extinct, owing to inferior sperm production. Perhaps, as suggested by Darlington to explain the presence of the supernumerary  $x$  chromosomes of *Cimex*,  $x_2$  is not inert, and the 25-type male is more viable than the 24-type. The situation in *Cimex* is to some extent analogous, for here selection seems to favour a high supernumerary  $x$  complement, yet when there are too many of these the meiotic process cannot deal with them efficiently. On the other hand there is another explanation which appears to me more plausible. Brindley (1912) has made an exhaustive study of the sex ratio in wild populations of *Forficula auricularia*. There is almost without exception a male deficiency, which varies in magnitude between different populations. When his counts are summated, the male:female ratio is approximately 45:55 (total counted=31820), while in one locality, Round Island, Scilly Isles, the males form only 16% of the total population (total counted=3655). A low proportion of males may be of considerable selective advantage to an organism such as *Forficula*; populations are generally dense and the animals are active, so that fertilization is easily accomplished and one male may serve many females. This selective advantage would be roughly proportional to the density of the population. In less dense populations the selective advantage would be less and vice versa. The Y chromosome is lost at a high rate in these 25-type males, hence there is a deficiency of male-determining sperm. On this view the 25-type male would be somewhat analogous to the male of *Drosophila* spp. carrying the sex-

ratio gene (Sturtevant & Dobzhansky, 1936). It will be of great interest to find out the proportions of 24- and 25-type males in populations with extreme male deficiency.

The 25-type male of *Forficula* must produce a high proportion of unbalanced gametes. An estimate of this proportion cannot unfortunately be made, since  $x_1$  cannot be distinguished from  $x_2$  cytologically. Whit has found a similar situation in certain Mantids (White, 1940) where incorrect disjunction of a sex trivalent is again the cause. Gametic selection in animals has never been demonstrated; zygotic selection, though far more wasteful for the species, has always been proved or presumed when the parents produce unbalanced gametes. On this supposition a mechanically inefficient and wasteful sex-determining system such as exists in the 25-type male of *Forficula auricularia* must have strong compensatory advantages of which sex-ratio adjustment is probably the most important. The dicentric Y is, I suggest, a primitive endowment which has survived as a "long-range" adaptation to the species.

#### SUMMARY

1. *Forficula auricularia* has eleven pairs of autosomes. There are two types of male:  $x_1Y$  and  $x_1x_2Y$ , and three types of female:  $x_1x_1$ ,  $x_1x_1x_2$  and  $x_1x_1x_2x_2$ .

2. The sex chromosomes in the male pair at the telophase of the final spermatogonial mitosis. They associate by mass, not particulate, pairing. In the  $x_1x_2Y$  male there may be pairing between all three to give a "triangular" or linear trivalent, or pairing may be incomplete, when a bivalent and univalent result.

3. The Y chromosome is dicentric, judged by its shape at first metaphase and behaviour at first and second anaphases. It may fragment and non-disjoin at the spermatogonial mitoses. The dicentric Y is adapted to orientate the "triangular" trivalent.

4. Owing to incomplete pairing of the sex chromosomes in the  $x_1x_2Y$  male meiosis is irregular. The Y chromosome may be lost with high frequency. Different relative numbers of  $x_1Y$  and  $x_1x_2Y$  males may thus adaptively modify the male proportion to suit the population density.

5. Of the two types within this species, the  $x_1x_2Y$  male represents the primitive condition. The nearly related genus *Anisolabis*, with possibly one exception, possesses only  $x_1x_2Y$  males. *Anisolabis* females are of the constitution  $x_1x_1x_2x_2$ .

6. In *Forficula auricularia*  $x_2$  has become superfluous as regards sex determination. When the Y fails to pair with  $x_1$  and  $x_2$  these form a bivalent and disjoin from one another at first anaphase. This accounts for the origin of males lacking  $x_2$ . In *Forficula scudderi*  $x_2$  has been lost entirely.

## ACKNOWLEDGEMENT

I wish to thank Dr C. D. Darlington for advice and criticism during the preparation of this paper.

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## EXPLANATION OF PLATES 11-13

Microphotographs. All  $\times ca.$  3500.

## PLATE 11

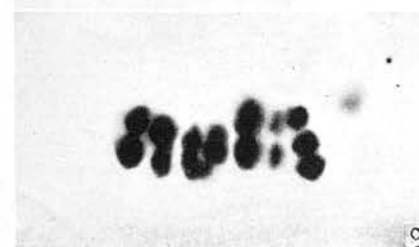
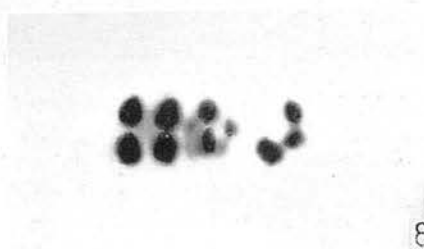
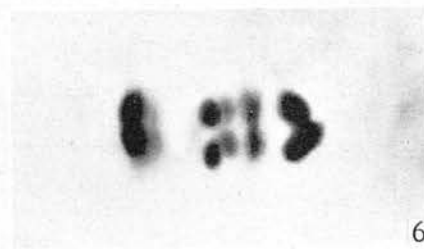
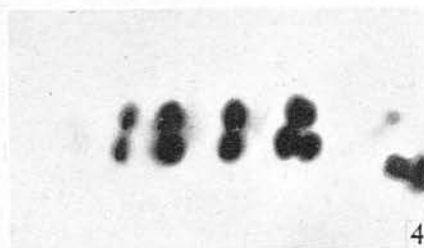
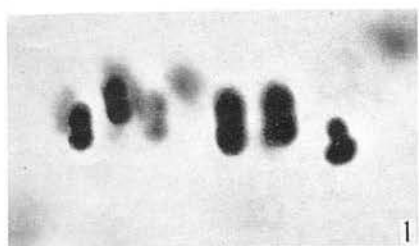
- Fig. 1. First metaphase of meiosis in 24-type male: side view showing sex bivalent.
- Figs. 2-10. First metaphase of meiosis in 25-type male. Figs. 2-6. "Triangular" trivalent in side view. Figs. 7-9. Linear trivalent in side view. Fig. 10. Polar view of 12-type plate with trivalent at left.

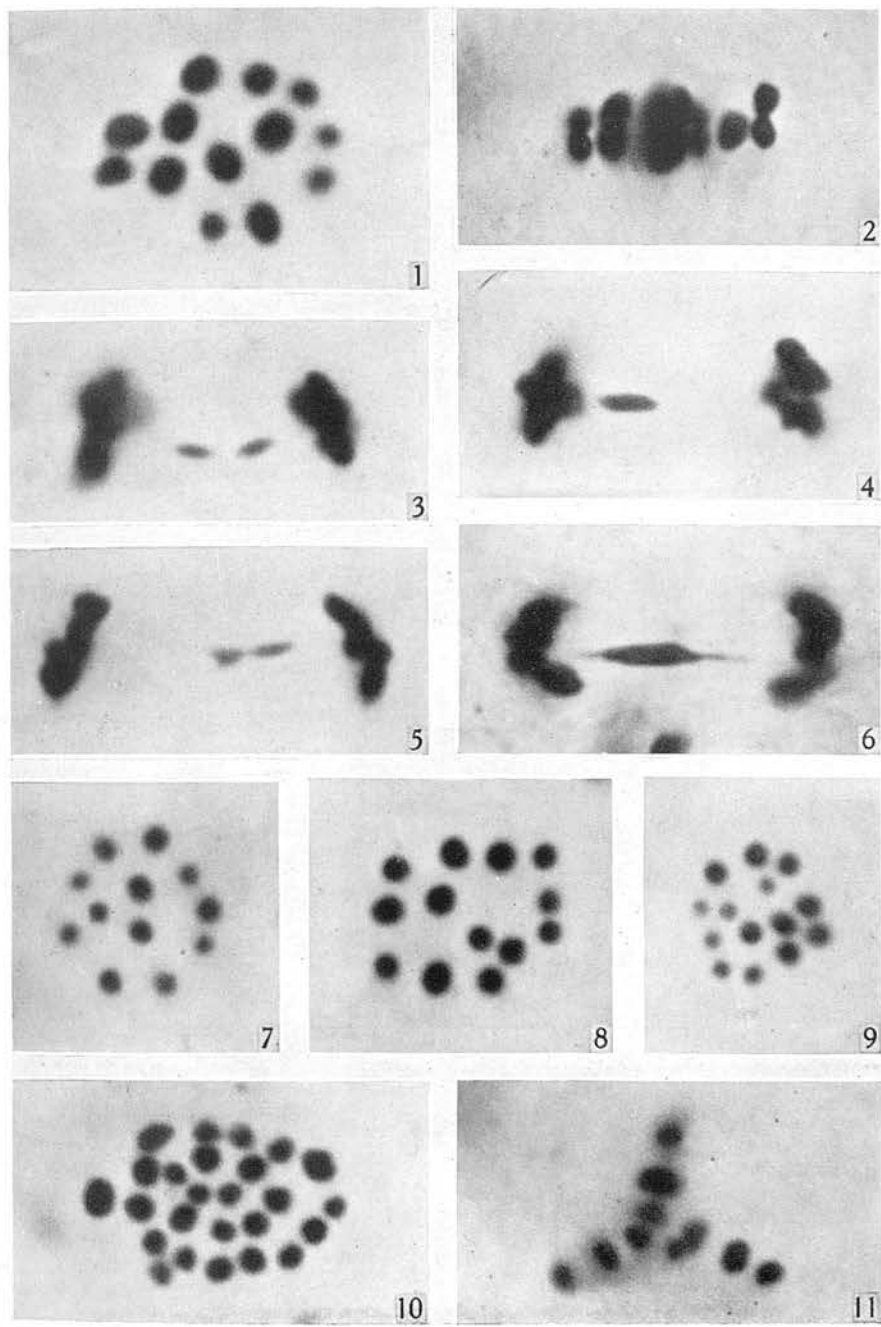
## PLATE 12

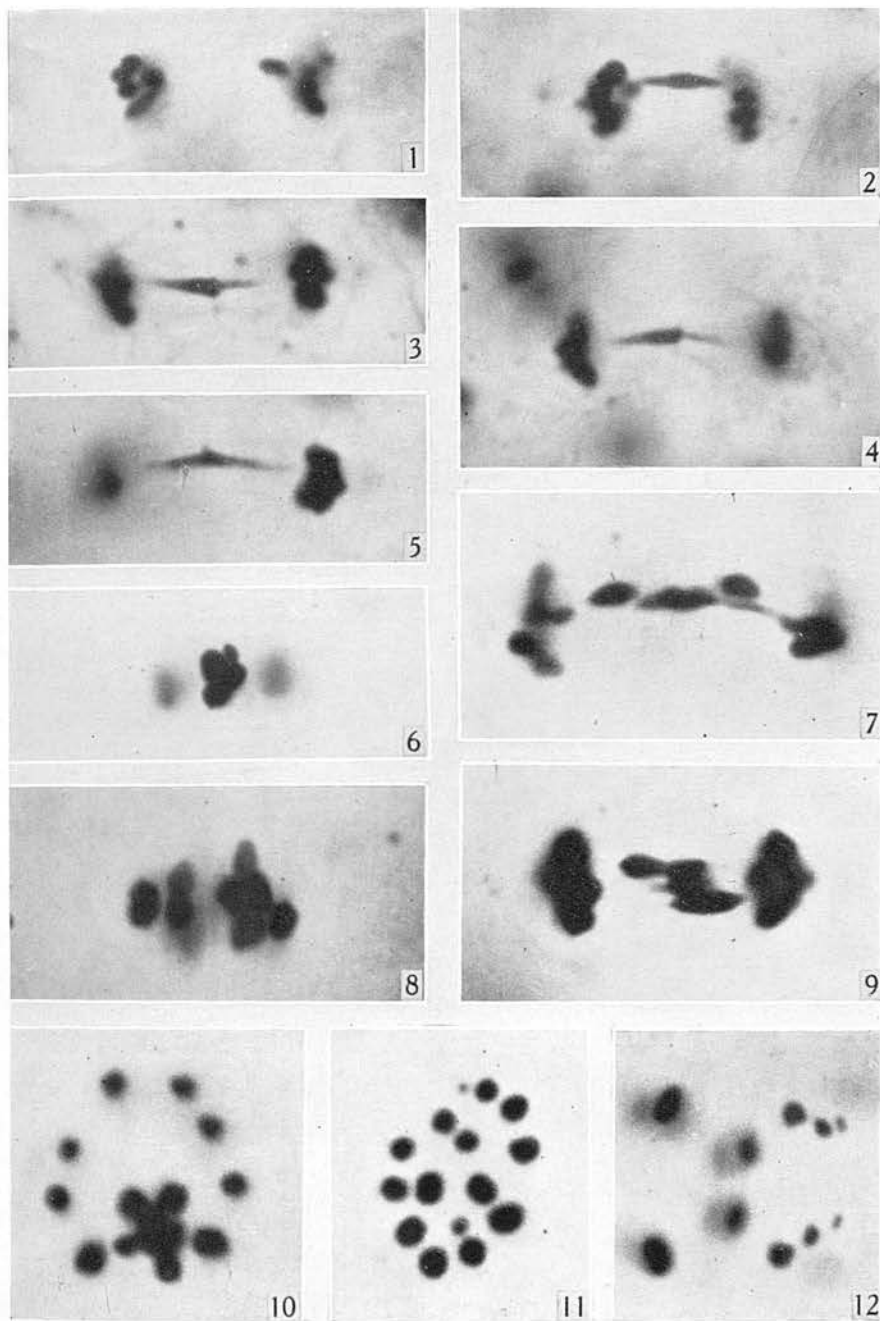
- Figs. 1, 2. First metaphase in 25-type male. Fig. 1. Polar view of 13-type plate. Fig. 2. Side view showing univalent Y.
- Figs. 3-6. Behaviour of univalent Y at first anaphase in 25-type male. Fig. 3. Equational division. Fig. 4. Passing undivided to one pole. Fig. 5. "Partial equational division." Fig. 6. "Dicentric stretching".
- Figs. 7-9. Second metaphase in 25-type male. Fig. 7. 12-type. Fig. 8. 13-type. Fig. 9. 14-type.
- Figs. 10, 11. Restitution second metaphases in 25-type male. Fig. 10. Polar view. Fig. 11. Side view showing tripolar spindle.

## PLATE 13

- Figs. 1-5. Behaviour of daughter univalent Y at second anaphase in 25-type male. Fig. 1. Passing undivided to one pole. Fig. 2. "Dicentric stretching", early. Figs. 3-5. "Dicentric stretching", later. Cell boundary has formed.
- Figs. 6, 7. Centric Y fragment in 25-type male. Fig. 6. Side view of first metaphase. Fig. 7. First anaphase.
- Figs. 8, 9. Reduplicated Y in 25-type male. Fig. 8. Side view of quinquevalent at first metaphase. Fig. 9. First anaphase. Irregular disjunction.
- Fig. 10. Anomalous reduplication of Y in 25-type male. "Sticking" of bivalents and hollow spindle.
- Figs. 11, 12. Two autosomal fragments in 25-type male. Fig. 11. First metaphase. Fig. 12. First anaphase.









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### Determination of Sex in Scalpellum

SEXUAL conditions in the Cirripedia are very varied<sup>1</sup>. They comprise such species as:

(1) Hermaphrodite forms with 'complemental' males, for example, *Scalpellum scalpellum*, *Ibla cumingi*;

(2) Hermaphrodites with sterile complemental larvæ, for example, *Rhizocephala*;

(3) Hermaphrodites only, for example, most *Pedunculata* (*Lepas*, *Pollicipes*, etc.), *Operculata* (*Balanus*, *Coronula*, etc.), *Ascothoracica*;

(4) Females with complemental males, for example, *Scalpellum velutinum*, *S. ornatum*, *Ibla quadrivalvis*, *Acrothoracica*.

The chromosomes of *Lepas anatifera*, which falls into group 3, were examined by Witschi<sup>2</sup>. He found the diploid number to be 26 and showed that no distinguishable sex chromosomes were present.



GROUP OF COMPLEMENTALS ATTACHED TO THE MANTLE OF A HERMAPHRODITE *Scalpellum scalpellum*, INCLUDING FOUR CYPRIS LARVÆ, NINE DWARF MALES AND ONE QUASI-HERMAPHRODITE. ( $\times 12$ .)

were typical degenerate dwarf males, and one excluded from the male-containing pocket of the hermaphrodite, had the structure of a small hermaphrodite (see accompanying illustration).

The absence of sex chromosomes, presence of oogonia in the complemental males and this odd hermaphrodite-like complemental point to an environmental control of sex determination such as

I have examined the chromosomes of *Scalpellum scalpellum*, which falls into group 1. The diploid number of 32 was found in spermatogonia of complemental dwarf males and of hermaphrodites. No sex chromosomes can be distinguished at meiosis, the haploid number being 16.

Gravel<sup>3</sup> observed oogonia in the complemental males of *S. peronii*, which also falls into group 1. I have seen the same in *S. scalpellum*. Moreover, one hermaphrodite of *S. scalpellum* which I received from Plymouth had an unusually large number of complementals. Of these four were unmetamorphosed cypris larvæ, nine

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Trisomic Grasshopper

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## A Trisomic Grasshopper

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H. G. CALLAN

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# A TRISOMIC GRASSHOPPER

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WHEN the chromosome complement of an organism is not an exact multiple of the haploid set, the organism is termed aneuploid. This condition has been found quite frequently in plants, but among animals, apart from the unusual and specialized sex chromosomes of some of the Hemiptera<sup>5</sup> and certain cases of inert chromosomes, *Drosophila melanogaster* is the only species in which the same state of affairs has been observed.<sup>8</sup> Probably the main reason for this anomaly is that the sexual reproduction of triploids, which is the source of most aneuploids in plants, is rare in animals.

Among thirty males of the acridioid Grasshopper *Mecostethus grossus* Linn. collected in the New Forest, England, in 1940, one individual was found in which, in all mitoses examined, the third largest autosome was represented three times instead of twice. Normally the diploid complement of the male *Mecostethus* consists of 22 nearly telocentric autosomes and a single sex chromosome. This individual possessed 23 autosomes and a sex chromosome and was therefore a trisomic aneuploid.

*Mecostethus grossus* is an organism showing a very special distribution of crossing-over as seen at first meiotic metaphase. This was illustrated by McClung<sup>10</sup> (cf. White<sup>11</sup>). In many individuals each bivalent is held together by only one chiasma, which is placed very close to the centromere. A consequence of this proximal localisation is that in spite of the fact that in the trisomic there are three homologous 3rd chromosomes, these never associate to form a bivalent. They are always present as bivalent and univalent; clearly it is a matter of chance which one of the three, and they are cytologically indistinguishable, is left as univalent (Figure 4A).

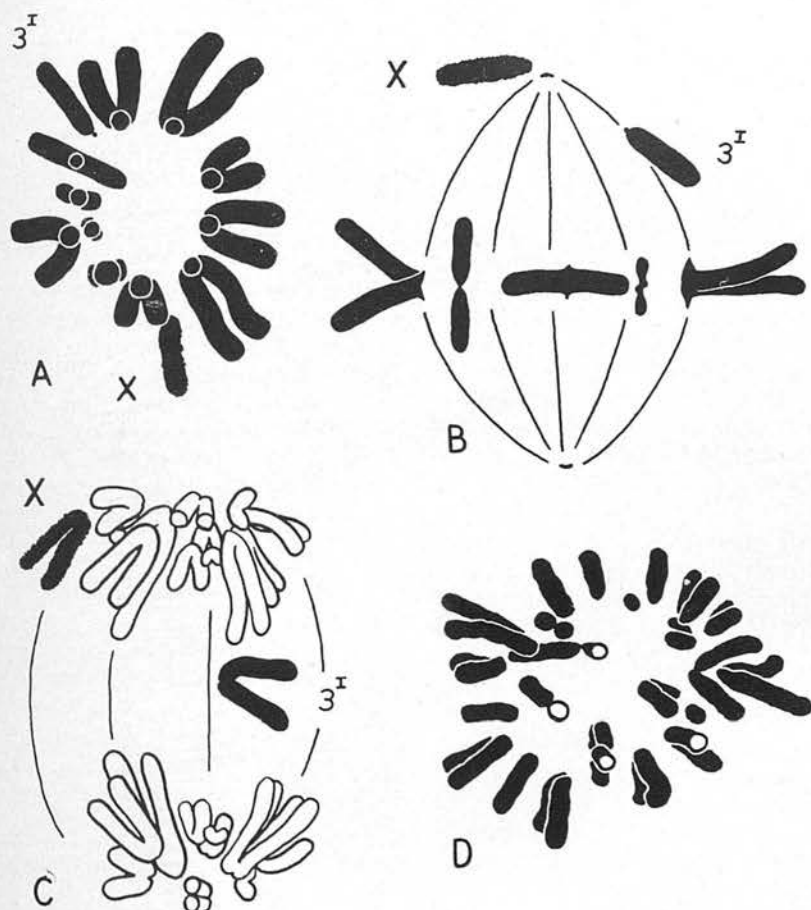
The univalent third autosome is readily distinguishable from the heterochromatic X-chromosome at first meiotic metaphase. It shows more clearly than the bivalents that the centromere is not

quite terminally placed (Figure 4A and 4B). Darlington<sup>4</sup> has pointed out that in the grasshoppers *Chortippus* and *Stauroderus* what appear to be single-armed chromosomes in reality are not telocentric since they form chiasmata in their short arms.

Now in trisomic *Mecostethus* we have two kinds of univalent. The behavior of the univalent autosome is therefore to be contrasted with that of the univalent sex chromosome at first meiotic metaphase. The centromere of the sex chromosome appears to be almost inactive at this division; the sex chromosome does not congregate with the bivalents at the equator, but lies passively at one pole and seems never to attach itself to the main spindle.

The univalent autosome lags behind the bivalents in congression on the metaphase plate. It may not get on to the plate at all, in which case it behaves like the sex chromosome and is passively included in one telophase group (Figure 4B). If the univalent autosome succeeds in reaching the plate, it remains there after the anaphase movement of the autosome bivalents has begun (Figure 4C). Lapse of chromatid attraction in the univalent autosome coincides with the onset of this condition in the bivalents. This is not always true of the X. The chromatids are carried far apart as the spindle stretches, but they remain attached to one another at the undivided centromere. Unlike univalents in Kihara's<sup>7</sup> *Triticum-Aegilops* hybrids or the sex univalent in *Odonata*<sup>1</sup> the centromere of the *Mecostethus* univalent never divides at the first meiotic division.

When the autosomal univalent is left at the equator the anaphase spindle fails to elongate in normal fashion. Whenever the univalent has become associated with the spindle, the telophase groups are apparently not free to move apart, with the result that during despiralisation and nuclear membrane formation a diploid restitution nucleus is formed. Thus the univalent can never be lost.



## MEIOSIS IN TRIPLOID

Figure 4

A-C. 1st meiosis. A—Polar view of metaphase in trisomic *Mecostethus*, (X=sex univalent,  $3^I$  = autosomal univalent). B—Side view of metaphase. Autosomal univalent not on the plate. C—Side view of anaphase with univalent lagging at the equator. D—Polar view of diploid second meiotic metaphase with 23 autosomes + X. ( $\times 2000$ .)

This is in complete contrast to the effect of undivided univalents in *Triticum* reported by Matsumoto,<sup>9</sup> where the spindle undergoes super-elongation.

As a consequence of this behavior 50 per cent of the second meiotic metaphases are found to be diploid, with 24 chromosomes (Figure 4D). This is an estimate of the frequency with which the autosomal univalent reaches the equatorial plate at the first meiotic division. The haploid divisions contain 11, 12 or 13

chromosomes according to the independent segregation of the sex and autosomal univalents. Second anaphase proceeds regularly in all types of second spermatocyte, and leads to the formation of second spermatids and sperm of the following constitutions:

Complement	Proportion
$23A + X$	4
$12A + X$	1
$11A + X$	1
$12A$	1
$11A$	1

It is possible to recognize spermatids of two sizes, the larger kind being diploid. There is no evidence of degeneration of spermatids or sperm.

The trisomic *Mecostethus* was externally a normal male, with nothing to suggest a subnormal viability. This affords an interesting comparison with *Drosophila melanogaster*, where Li<sup>8</sup> found trisomy of the large autosomes was always lethal at an early stage, whereas trisomy of the minute fourth autosome led to slight reduction in viability. It must be remembered that *Mecostethus* possesses many more chromosomes than does *Drosophila melanogaster*. In fact the third autosome, though large, constitutes only 1/16th of the chromatin bulk at meiosis.

The origin of the trisomic can be almost certainly ascribed to "non-disjunction" occurring in its male or female parent. White<sup>11</sup> has described failure of pairing at meiosis in a normal male *Mecostethus* and this can readily lead to non-disjunction.

Assuming that the unbalanced sperm of this trisomic were fully functional, and we have no evidence in animals that selection occurs during the male gamete stage, it could have given rise to a proportion of trisomic progeny whose survival would depend on their relative viability during development.

Diploid 2nd spermatocytes and sperm have frequently been reported in insect testes (Darlington,<sup>4</sup> Callan,<sup>3</sup> et alii). They can be produced in a variety of ways. They would give rise to triploids. Triploid individuals in a normally diploid animal species, however, have been rarely found. (*Triturus viridescens*,<sup>6</sup> *Triton taeniatus*,<sup>2</sup> and *Triton vulgaris* [Barber and Callan, unpublished] are known examples). Apart from unbalancing the sex determining mechanism in some organisms, the main reason for this appears to be that since far more male than female animals are subject to cytological investigation, triploids would only be found in organisms with male homogamety. Though interesting as an individual, it is therefore unlikely that

this *Mecostethus* trisomic would have provided suitable material for any evolutionary step.

### Summary

A male *Mecostethus grossus* has been found which was trisomic for the third largest autosome. Owing to chiasma localisation in this species, trivalents do not occur. In 50 per cent of the 1st spermatocytes the univalent third chromosome behaves like the X, and is included undivided in one telophase group. In the remaining 50 per cent the univalent congresses on the metaphase plate later than the bivalents. It never divides, and by interfering with the elongation of the spindle causes diploid restitution second spermatocyte nuclei to be formed.

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THE CYTOLOGY OF GAULTHETTYA  
WISLEYENSIS (MARCHANT) REHDER  
A NEW MODE OF SPECIES FORMATION

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# The Cytology of *Gaulthetia wisleyensis* (Marchant) Rehder A New Mode of Species Formation

BY

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With four Figures in the Text

THE ericaceous plant *Gaulthetia wisleyensis* (Marchant) Rehder was described recently by Mulligan\* (1939). Produced at Wisley, it was thought on morphological grounds to be a natural hybrid between the North American species *Gaultheria Shallon* Pursh. and the South American species *Pernettya mucronata* (Linn. f.) Sprengel. At the suggestion of Sir Arthur Hill, Director of the Royal Botanic Gardens, Kew, I examined the chromosomes of *Gaulthetia*, *Gaultheria Shallon*, and *Pernettya mucronata* in order to check the conclusions reached on morphological grounds. Chromosome counts of certain other species of Ericaceae were also made, and these have been included in the list of chromosome numbers of the Ericaceae given in the table (p. 5).

Mitotic counts were taken from sections of root-tips fixed in La Cour's 2 BE and stained by Newton's gentian violet-iodine method (La Cour, 1937). The maturation divisions of pollen mother cells of *Gaulthetia*, *Gaultheria Shallon*, and *Pernettya mucronata* were examined by means of the same technique.

With one exception, the *Gaultheria-Pernettya* complex has a basic chromosome number of 11. *Gaultheria Itoana* is the exception in having a diploid number of 26 (Fig. 4 d). This may represent an ancestral condition in this group, since 13 is the basic number of the Ledeaee, Rhododendreae, and Arbuteae groups of the Ericaceae.

*Gaultheria Shallon* is octoploid with 88 chromosomes. I cannot confirm Hagerup's count of 96 (Hagerup 1928). *Pernettya mucronata* is hexaploid with 66 chromosomes. *Gaulthetia* is heptaploid, and this tallies with the view that the two former plants are its parents ( $44+33=77$ ) (Fig. 1). *Pernettya furiens*, with which *Gaulthetia* has sometimes been confused, is hexaploid.

Both *Gaultheria Shallon* and *Pernettya mucronata* seem to be autopolyploids, since high multivalent associations occur in both species (Fig. 2, a and b). Thus associations as high as octavalents are found in *Gaultheria Shallon* and hexavalents in *Pernettya mucronata*.

\* The plant was named *Gaulnettya* × Wisley Pearl by Mulligan, but this was a *nomen nudum*. Rehder (Manual of Cultivated Trees and Shrubs, 2nd edition, 1940) gave it the name *Gaulthetia wisleyensis* and provided a brief English description.

[Annals of Botany, N.S. Vol. V, No. 20, October, 1941.]

In *Gaulthettya* there is a great deal of physiological sterility on the male side: in many anthers the pollen mother cells reach the first meiotic metaphase, but

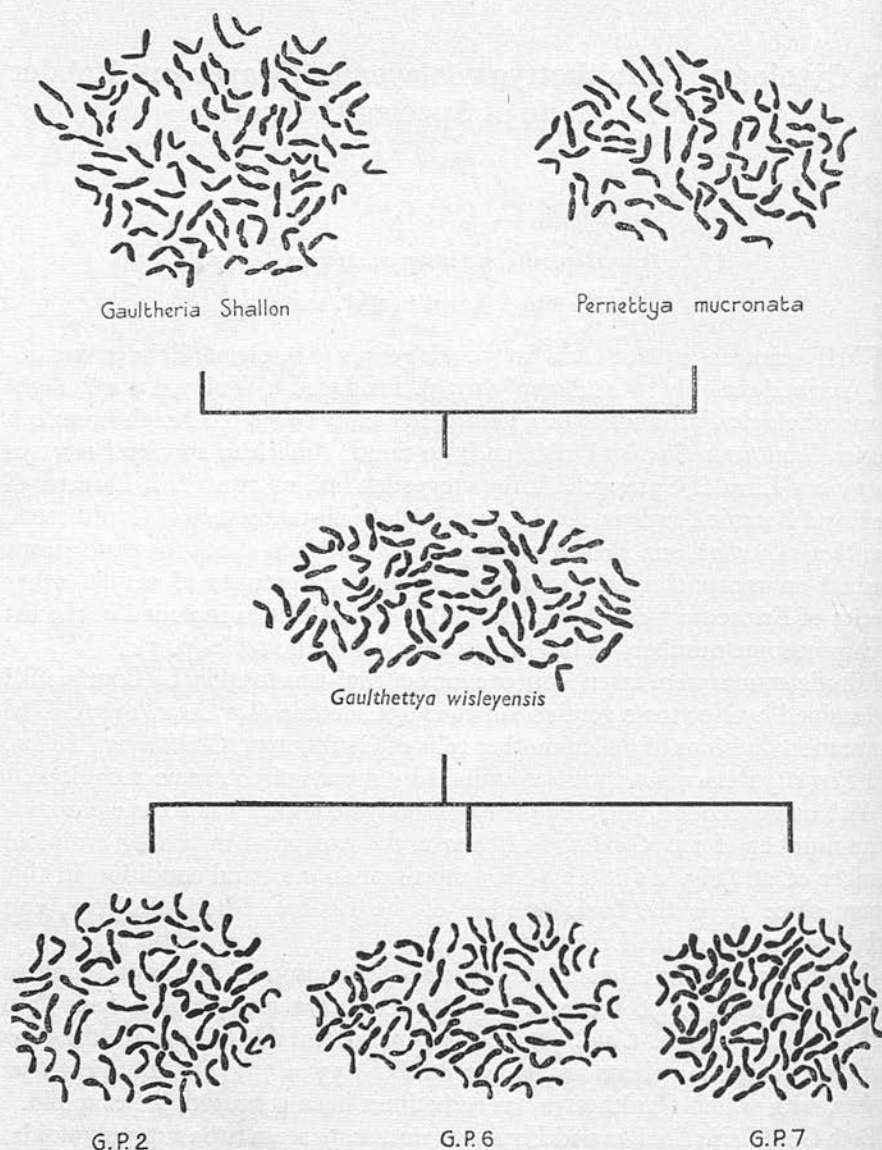


FIG. 1. Metaphase plates from root-tips illustrating relationships of *Gaultheria Shallon* ( $2n = 88$ ), *Pernettya mucronata* ( $2n = 66$ ), and *Gaulthettya wisleyensis* ( $2n = 77$ ). G.P. 2,  $2n = 71$ . G.P. 6,  $2n = 79$ . G.P. 7,  $2n = 70$ . ( $\times 3,500$ .)

anaphase does not follow and they degenerate at this stage. In the degenerating cells the spindle is intensely stained in gentian violet preparations; the chromosomes appear to liquefy and coalesce. This is a condition of the

anther as a whole; other anthers show no degeneration of the pollen mother cells at first metaphase.

In general between five and ten univalents are found at each first metaphase (Fig. 2c). This stage therefore shows far less failure of pairing than is typical

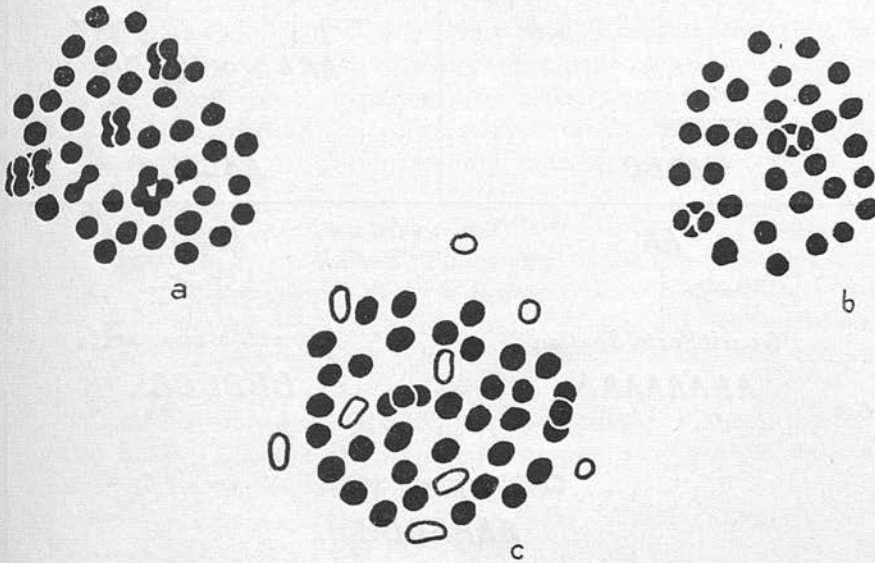


FIG. 2 a-c. Polar views, first meiotic metaphase. a. *Gaultheria Shallon*, 32II+6IV. b. *Pernettya mucronata*, 29II+2IV. c. *Gaulthetia wisleyensis*. 9I+3III+2III. ( $\times 7,000$ .)

of a newly arisen hybrid springing directly from diploid parents. Pairing in such a hybrid is mainly conditioned by the degree of homology existing between the haploid complement of one parent and that of the other. In the case of *Gaulthetia*, however, we must bear in mind that its probable parents are autopolyploids. Such pairing as takes place is therefore probably pairing within the reduced complements of each parent. *Gaulthetia* effectively consists of a tetraploid *Gaultheria* component added to a triploid *Pernettya* component. The failure of pairing which does exist in *Gaulthetia* probably depends on its *Pernettya* component; trivalents do not form regularly because of a low chiasma frequency per chromosome.

The constitution of *Gaulthetia* approximates to that of an allopolyploid hybrid; its relatively regular pairing is a parallel function. Its origin, however, is to be contrasted with that of an allopolyploid. In an allopolyploid, identity of chromosome partners arises by the doubling of the diploid hybrid, e.g. *Primula kewensis* and *Raphano-Brassica*. In *Gaulthetia*, on the other hand, the doubling preceded hybridization (Fig. 3).

Although less fertile than either of the parent species, about 10 per cent. of the open-pollinated seeds of *Gaulthetia* are full. Both Mulligan and I have raised seedlings showing genetic segregation. Mine were from isolated plants

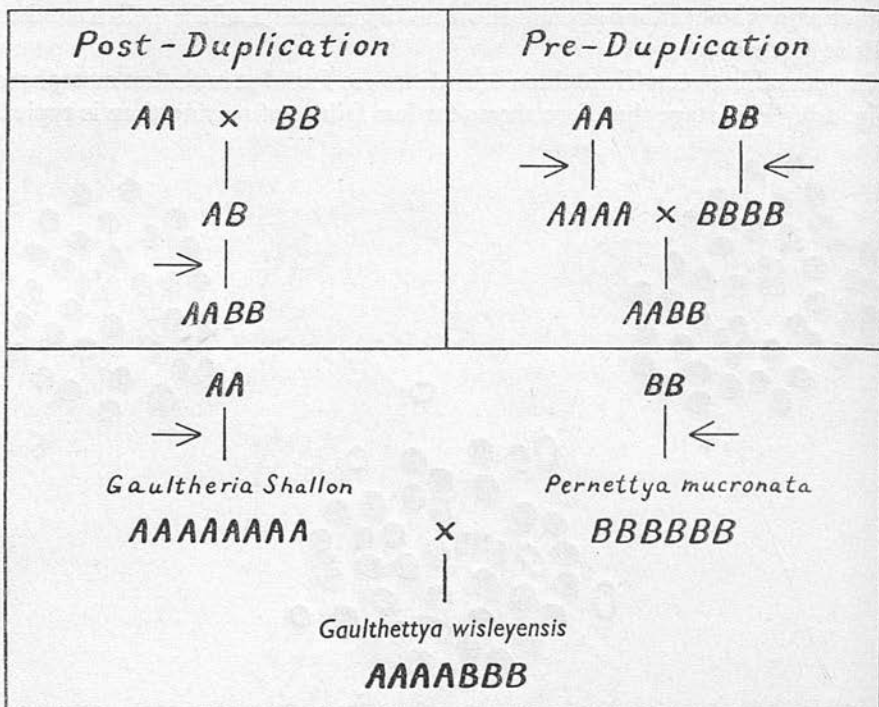


FIG. 3. Diagram to illustrate the relationship between allopolyploidy (post-duplication) and the polyploidy of *Gaulthettya* which has arisen by pre-duplication.

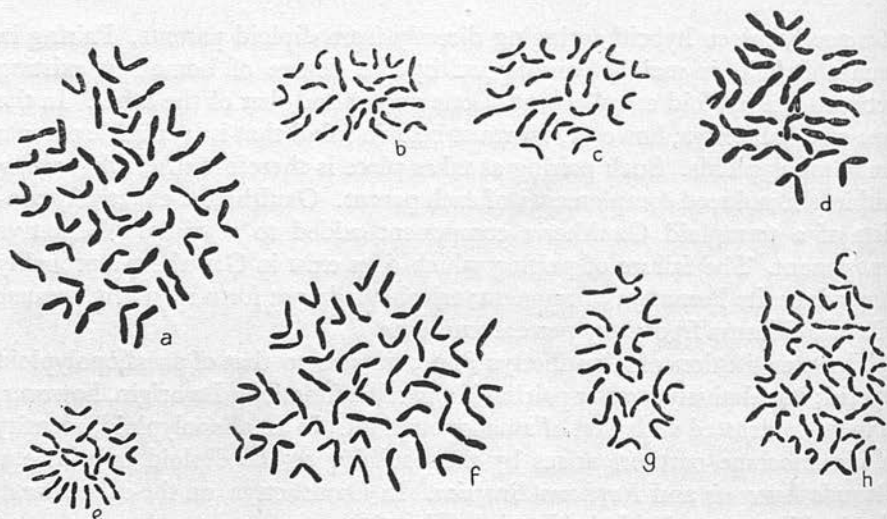


FIG. 4 a-h. Metaphase plates from root-tips of certain Ericaceae. a. *Kalmia polifolia*,  $2n = 44$ ; b. *Leucothoe acuminata*,  $2n = 24$ ; c. *Pieris Mariana*,  $2n = 24$ ; d. *Gaultheria Itoana*,  $2n = 26$ . e. *G. cuneata*,  $2n = 22$ . f. *G. Cumingiana*,  $2n = 44$ . g. *Pernettya tasmanica*,  $2n = 22$ ; h. *P. pentlandii*,  $2n = 44$  ( $\times 3,500$ .)

presumably selfed; three of these, G.P. 2, G.P. 6, and G.P. 7, have been examined and they are all aneuploid, with 71, 79, and 70 chromosomes respectively (Fig. 1). The viability of these aneuploid plants depends on their being high polyploids; a few odd chromosomes disturb their genetic balance less than if they were lower polyploids.

The fertility of G.P. 1 and G.P. 2 is very poor. I raised one seedling from G.P. 2, but this plant died six months after germination, being then only 1 cm. high. Fertile new species could only arise from *Gaulthetia* if by chance a seedling arose with a balanced and paired mixture of *Gaultheria* and *Pernettya* chromosomes. This is not an impossibility, especially since the odd chromosomes could be lost in stages through a number of generations. Physiological sterility would probably act as a greater barrier to this form of speciation than meiotic irregularity due to numerical unbalance of the chromosome complement concerned. Provided there were little physiological sterility, a fertile hybrid could, however, arise direct from two autotetraploid parents, from two auto-octoploid parents, or from one autotetraploid and one auto-octoploid parent.

The cultivated strawberry is a comparable example, it being the fertile octoploid hybrid between two octoploid species, *Fragaria chiloensis* and *F. virginiana* (Darlington, 1932).

Hybridization within and between the two genera *Gaultheria* and *Pernettya* has occurred very commonly in the wild state (Burt and Hill, 1935). It is probable that some speciation in this complex and elsewhere has occurred by the hybridization of autopolyploid species.

#### SUMMARY

Cytological evidence supports the view that *Gaulthetia wisleyensis* is a hybrid between *Gaultheria Shallon* and *Pernettya mucronata*. *Gaulthetia* is heptaploid ( $2n = 77$ ), *Gaultheria Shallon* is octoploid ( $2n = 88$ ), and *Pernettya mucronata* hexaploid ( $2n = 66$ ).

The fertility of *Gaulthetia* depends on the autopolyploid nature of its parents. Hybridization of autopolyploids may be an important mode of speciation.

#### ACKNOWLEDGEMENTS

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#### *Chromosome Numbers (2n) in the Ericaceae*

##### RHODODENDROIDEAE

##### LEDEAE

*Ledum groenlandicum*  
,, *columbianum*

26 Hagerup (1928), Wanscher (1933)  
26 Callan

RHODODENDREAE	
<i>Rhododendron</i> many spp.	26 Sax (1930) and others
" <i>calendulaceum</i> }	52 Sax (1930)
" <i>canadense</i> }	
PHYLLODOCEAE	
<i>Leiophyllum buxifolium</i>	24 Hagerup, 1928
<i>Loiseleuria procumbens</i>	24 " "
<i>Kalmia latifolia</i>	24 " "
" <i>glauca</i>	48 " "
" <i>polifolia</i>	44 Callan
<i>Phyllodoce coerulea</i>	24 Wanscher (1933)
<i>Daboecia cantabrica</i>	24 Maude (1940)
ARBUTOIDEAE	
ANDROMEDEAE	
<i>Cassiope hypnoides</i>	48? Hagerup, 1928
<i>Leucothoe acuminata</i>	24 Callan
<i>Andromeda polifolia</i>	48 Hagerup, 1928
<i>Pieris Mariana</i> }	24 Callan
" <i>Forrestii</i> }	
" <i>lucida</i> }	
" <i>japonica</i> }	
GAULTHERIEAE	
<i>Gaultheria Itoana</i>	26 Callan
" <i>hispida</i> }	22 Callan
" <i>cuneata</i> }	
" <i>antipoda</i> }	
" <i>glomerata</i> }	
" <i>Cumingiana</i> }	44 Callan
" <i>Griffithiana</i> }	
" <i>Shallon</i>	96 Hagerup (1928), 88 Callan
<i>Pernettya tasmanica</i>	22 Callan
" <i>prostrata</i> }	44 Callan
" <i>pentlandii</i> }	
" <i>ciliata</i> }	
" <i>buxifolia</i> }	
" <i>mucronata</i> }	66 Callan
" <i>furiens</i> }	
ARBUTEAE	
<i>Arbutus</i> 2 spp.	26 Hagerup, 1928
" <i>arachnoides</i> }	26 Callan
" <i>xalapensis</i> }	
<i>Arctostaphylos</i> , 2 spp.	26 Hagerup, 1928
"    " <i>bicolor</i> }	26 Callan
"    " <i>pungens</i> }	
VACCINIOIDEAE	
VACCINEAE	
<i>Gaylussacia baccata</i>	24 Longley (1927)
<i>Vaccinium</i> , 5 spp.	24 " " , Hagerup (1928)
" <i>uliginosum</i>	24, 48 Hagerup (1928)
"    3 spp.	48 Longley (1927)
"    3 spp.	72 Longley (1927), Hagerup (1928)

THIBAUDEAE	
<i>Pentapterygium serpens</i>	24 Callan
ERICOIDEAE	
<i>Calluna vulgaris</i>	16 Hagerup (1928), Hahn, 1929 (Tischler, 1931)
<i>Erica</i> , 6 spp.	24 Hagerup (1928), Maude (1940), Wanschler (1933)
<i>E. curvirostris</i>	} 24 Callan
<i>E. sessiflora</i>	
<i>E. Willmoreana</i>	
<i>Bruckenthalia spiculiflora</i>	36 Hagerup (1928), Callan

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HETEROCHROMATIN IN TRITON

By

H. G. Callan

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## Heterochromatin in *Triton*

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(Communicated by C. D. Darlington, F.R.S.—Received 8 August 1941)

[Plate 13]

When subjected to low temperature, certain segments of the chromosomes of *Triton vulgaris*, *T. palmatus* and *T. cristatus* are heterochromatic. At mitosis these segments are undercharged and at meiosis uncharged with nucleic acid.

These segments show the same type of allocyclic behaviour as do similar segments in *Paris*, *Trillium* and *Fritillaria*. They form Feulgen-positive chromocentres in all diffuse nuclei except pachytene, which is diffuse in *Triton*.

The availability of nucleic acid at the stage when the chromosomes normally spiralize is now shown to be a condition of that spiralization. The diffuse pachytene without chromocentres is followed by meiosis with unspiralized heterochromatin: the diffuse resting nucleus with chromocentres is followed by mitosis with spiralized heterochromatin.

With certain exceptions heterochromatin seems to be confined to those parts of chromosomes where chiasmata and crossing-over rarely occur.

## 1. INTRODUCTION

Longitudinal differentiation of the chromosomes is a condition underlying genetic analysis. This differentiation is visible equally at the pachytene stage of meiosis and in the salivary-gland chromosomes of Diptera. The typical chromosome at metaphase, however, is optically homogeneous. We know that this anomaly results from certain adaptations concerned with cell division. The chromosomes spiralize and become charged with nucleic acid preparatory to their anaphase movement on the spindle (Caspersson 1937, Summary; Darlington 1935; Darlington and Upcott 1939; *et alii*). Centromeres and nucleolar organizers are frequently visible because they do not behave like the other parts of chromosomes.

Since the work of Henking (1891) on the Hemipteran *Pyrrhocoris*, it has been known that the sex chromosomes of many insects and mammals 'condense' precociously during the spermatocyte prophase and then stain with basic dyes as intensely as the autosomes do at first meiotic metaphase. This reaction was termed *heteropycnosis* by Gutherz (1907). Heitz in 1928 introduced the morphological term *heterochromatin* to describe a particular kind of 'chromatin' which was distinguishable from another kind of chromatin, *euchromatin*, in its staining properties at telophase and prophase of mitosis. He at first thought that heterochromatin was specifically concerned with sex, but this view has since been discarded.

The problem of this reaction and substance has been extended and clarified by the work of Darlington and La Cour (1938, 1940, 1941). They find that certain regions of the mitotic chromosomes of species of *Paris*, *Trillium* and *Fritillaria* which appear euchromatic at metaphase when the plants are kept at normal temperatures, are heterochromatic when the plants are kept at low temperatures. These regions exhibit the phenomenon of heteropycnosis in that they are over-condensed in resting nuclei. Darlington and La Cour (1940) have accordingly introduced the term *allocyclus* to describe the specific reaction (heteropycnosis) of the specific substance (heterochromatin) in terms of spiralization and nucleic acid charging. Similar conditions have now been found to obtain in the newt, *Triton* spp.

## 2. MATERIAL AND METHODS

Mitoses were studied in the tail tips of larval *Triton vulgaris* collected and reared at Merton. Adult males of *Triton vulgaris*, *T. palmatus* and *T. cristatus*, collected in a pond on Ranmore Common, Surrey, in the spring of 1940, supplied meiotic material. These males were placed on damp moss after the breeding season, and their testes were fixed during the early summer.

Acetic alcohol was found to be the most suitable fixative for *Triton*. Material was subsequently stained in iron aceto-carmine or by means of the Feulgen technique, tapped out on a slide and covered. Slides were made permanent according to the technique of La Cour (1937). The Feulgen technique was used only for mitotic material and as a test for nucleic acid. The hydrolysis involved in this technique causes the huge meiotic chromosomes to soften and contract, whereas with aceto-carmine they are well preserved.

Tail tips were examined untreated, after immersion in colchicine solution, and after exposure to low temperatures. It is difficult to analyse metaphase plates in the untreated larvae because the spindle is narrow and the chromosomes are crowded on its periphery. A few cells in larvae immersed in 1% colchicine solution for 2 days were suitable for examination of the individual chromosomes, since the spindle is suppressed and the slightly super-spiralized chromosomes lie spaced out in the cytoplasm.

Temperature treatment, however, was the basis of manipulation. The minimum cold exposure to show heterochromatin is 3 days. Abnormal spindles arise in the mitotic cells of larvae exposed to 3° C (Barber and Callan, unpublished). The rate of entry of resting nuclei into mitosis falls off at low temperatures. It is thus rare to find analysable diploid complements which have reached metaphase spiralization while under the action of low temperatures. The only exposure which was found suitable therefore was one of 3 days' duration.

Testicular material was examined untreated and after exposure to low temperature. Temperatures of 3–6° C inhibit spindle formation in testis cells without preventing first spermatocytes at pachytene from reaching metaphase spiralization. Some material was fixed after 2–8 days of this treatment, but it was found that the bivalents were more easily examined when lying stretched on a spindle. This condition was realized by removing the newts from the refrigerator after 2 days at 6° C and then keeping them for 2 hr. at 18° C before fixing the testes.

By varying temperatures it is therefore possible to put the bivalents on or off the spindle as required for observation or experiment and at the same time control their spiralization and nucleic acid metabolism.

### 3. MITOSIS

The mitotic chromosomes of *Triton vulgaris* kept at 18° C are shown in the upper column of figure 1. The larva had been given colchicine treatment, consequently the chromosomes are somewhat super-spiralized and

some have widely separated chromatids. There are six pairs of large chromosomes with median (*M*), two pairs of large chromosomes with submedian (*S*), three pairs of small chromosomes with submedian (*s*) and a single pair of small chromosomes with median (*m*) centromeres. Three pairs have secondary constrictions, two *M*'s and one *S*. Similar constrictions have been described by Parmenter (1920) in *Amblystoma tigrinum*, Janssens (1901) in *Triton* spp. and Fankhauser (1934) in *T. palmatus*.

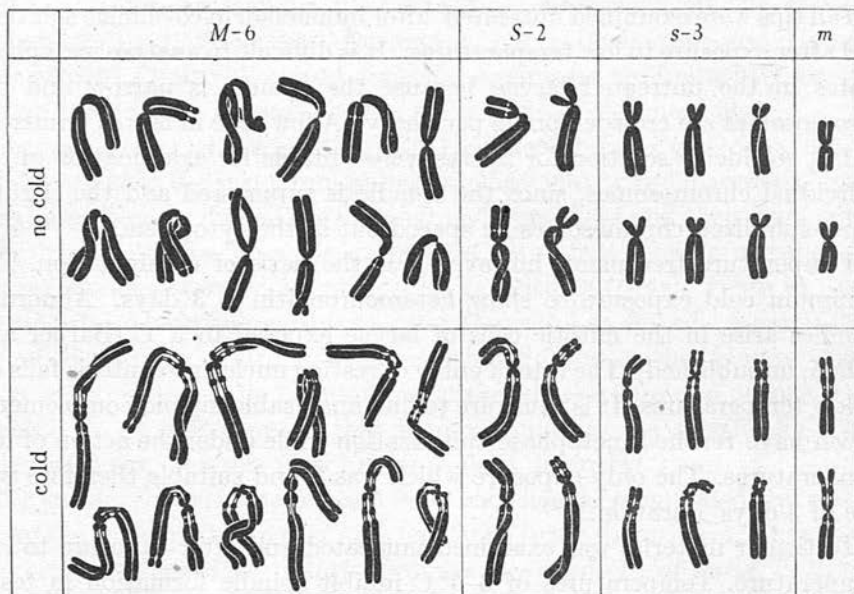


FIGURE 1. Mitotic chromosomes of *Triton vulgaris*. Feulgen.  $\times 1200$ .

The mitotic chromosomes of a typical cold-treated larva are shown in the lower column of figure 1. It will at once be seen that the number of constrictions has increased enormously. So far as one can judge they are constant from cell to cell in one individual larva: this complement has 74. Almost all of the constrictions are crowded into the proximal regions of the chromosomes. Each constriction is very short unless stretched during preparation. Through it the deeply stained euchromatin segments are joined by a thinner under-stained strand just as in the plant material of Darlington and La Cour. The constrictions present in untreated material, and those induced by the action of low temperature are heterochromatic segments. They are deficient in nucleic acid and they produce a spiral of narrower diameter than that of the euchromatin.

They have the same type of allocyly as heterochromatic segments in plants. Thus resting epidermal nuclei have Feulgen-positive bodies lying in the region around the polar depression, i.e. close to the centromeres, which represent the over-condensed phase of the heterochromatic segments (figure 2).

The distribution of heterochromatin in *Triton vulgāris* closely corresponds to that found in *Fritillaria pudica* (Darlington and La Cour 1941). This plant species also has in the main short proximal segments.

The full development of under-stained regions, i.e. of nucleic acid deficiency, is first found in a few cells of larvae kept for 3 days at 3° C. Many cells are held at metaphase owing to anaphase suppression, but only those which have developed right through from the preceding resting stage in the cold give the complete expression. Cells which were in prophase when the treatment started show ill-defined heterochromatic segments. These are visible as slight constrictions, but they stain almost as intensely as euchromatin.

The effect of heterochromatin on anaphase can only be checked when larvae held at low temperatures for 3 or 4 days are released for an hour at room temperatures before fixation. A few cases of the sticking observed by Darlington and La Cour in *Paris* and *Trillium* (1938, 1940) were noticed. Other spindle abnormalities exist, however, and these make interpretation uncertain.

#### 4. MEIOSIS

The normal meiotic chromosomes of *Triton vulgāris* and *T. palmatus* are shown in the upper columns of figures 3 and 4. The two species have generally similar complements. They differ in that *T. palmatus* has only one pair of large chromosomes which are markedly submedian (*S*), and no small median pair. Both species have distally localized chiasmata. *T. palmatus* has more extreme localization than *T. vulgāris* (Callan and Koller, unpublished).

While lying on the spindle at first metaphase, the bivalents stretch somewhat unevenly, giving slight indication of the position of the heterochromatin segments. Klingstedt (1936) has described similar constrictions in the normal meiotic chromosomes of the grasshopper *Chrysochraon*. The

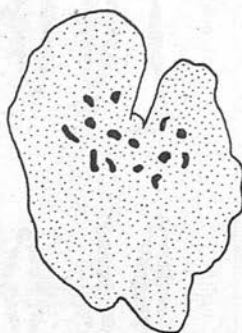
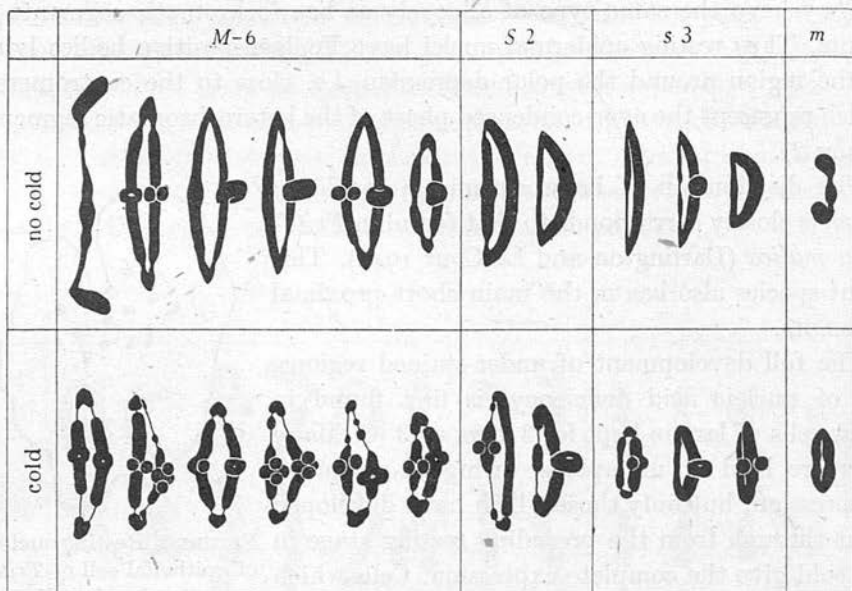
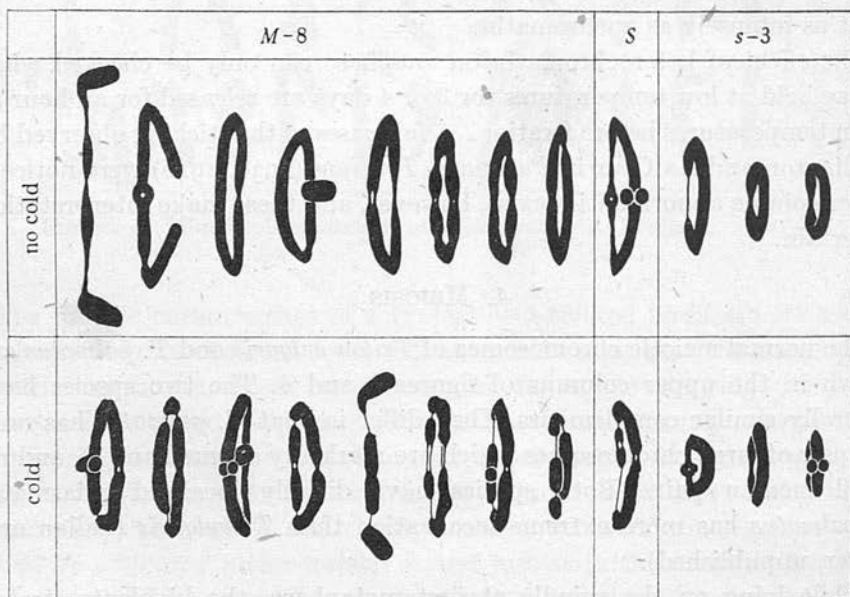


FIGURE 2. Resting nucleus of epithelial cell of *Triton vulgāris*, showing disposition of chromocentres around the polar depression. Feulgen.  $\times 1800$ .

FIGURE 3. Meiotic bivalents of *Triton vulgaris*. Aceto-carmine.  $\times 1200$ .FIGURE 4. Meiotic bivalents of *Triton palmatus*. Aceto-carmine.  $\times 1200$ .

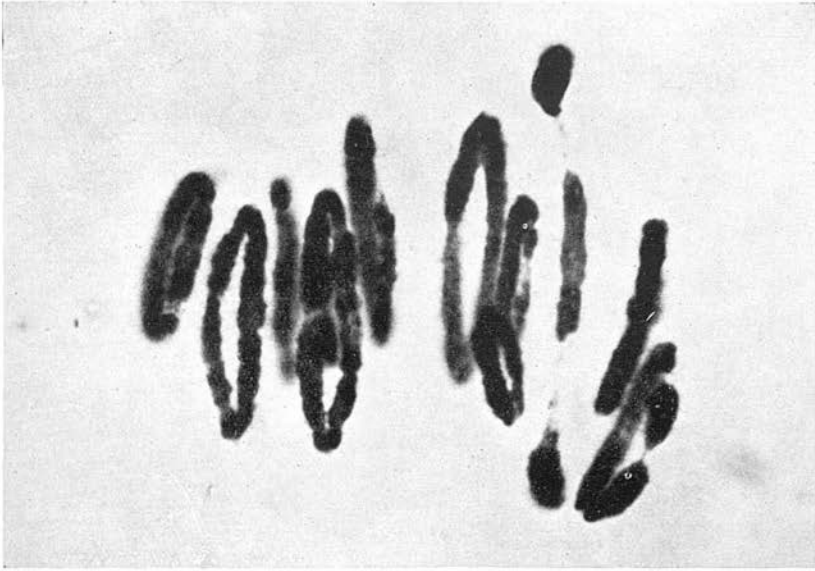


FIGURE 5. First metaphase of meiosis in *Triton palmatus*. 2 days 6° C, 2 hr. 18° C. Aceto-carmin.  $\times 2000$ . Note stretched heterochromatic segments in *M* bivalent with single terminal chiasma.



FIGURE 6. First metaphase of meiosis in *Triton vulgaris*. 2 days 6° C, 2 hr. 18° C. Aceto-carmin.  $\times 2000$ . Note proximal heterochromatic segments in all orientated bivalents. The *S* bivalent in the middle of the plate may be heterozygous for a segment in the short arm.





heterochromatin interpretation might also be given to the constrictions seen at anaphase in the grasshopper *Stenobothrus* by Belar (1928, figure 266). Belar interpreted these constrictions as indicating places where the chromatids had broken at crossing-over.

In great contrast with this normal-temperature behaviour is that of the bivalents which have passed from pachytene to metaphase while under the influence of low temperature. Those shown in the lower columns of figures 3 and 4 have been subjected to 2 days at 6° C followed by 2 hr. at 18° C (see plate 13). As explained earlier, the meiotic spindle is inhibited at 6° C, and the short release before fixation was given in order that spindles should be formed.

The heterochromatin segments are often as clearly defined as they are in mitosis. They are completely Feulgen-negative. Each consists of a very thin non-staining strand between two blocks of euchromatin. Forty such segments are visible in the cell from *T. vulgaris*, and eighteen in that from *T. palmatus*. It is not possible to arrive at any conclusions about the constancy of regions from cell to cell, nor about possible hybridity. Only those segments which are seen from a suitable aspect can be identified with certainty. The segments are so short that in unstretched bivalents they are frequently obscured by nearby coils of the euchromatin spiral, and such segments resemble the constrictions between successive gyres. A greater proportion are rendered visible when the bivalents are stretched on a spindle, but one is never able to count as many segments at meiosis as at mitosis.

The unstained regions undergo great stretching in single-chiasma bivalents and in bivalents which are slow to separate at anaphase owing to their having formed more chiasmata than usual. Despite the fact that they are so much weaker than euchromatin in resisting longitudinal stress, no cells have been observed in which breakage has occurred. Nevertheless, it must be recognized that these segments constitute a source of weakness to the continuity of the chromosome thread, and that infrequent breakages at these points would provide the variation necessary for the play of selection.

Chromosomes at second meiotic metaphase also exhibit heterochromatic regions, if they have arisen from the preceding interphase under the action of low temperature. They are unsuitable for study, since they are small and greatly contracted.

The resting stage nuclei between spermatogonial divisions contain Feulgen-positive bodies similar to those in resting epidermal cell nuclei. The same is true of first and second spermatid resting nuclei. Pachytene is the last critical diffuse stage before first meiotic metaphase. This stage, unlike all other diffuse stages, does not contain nucleic acid aggregates.

It is clear from the examination of a few cold-treated meioses in *Triton cristatus* that this species also possesses proximal heterochromatic regions. It is, however, unsuitable material for a study of heterochromatin, since the first spermatocytes show much less localization of chiasmata than do those of *T. vulgaris* or *T. palmatus*. Chiasmata are formed in regions where heterochromatin is present also.

#### 5. THE DETECTION OF HETEROCHROMATIN

Darlington and Upcott (1941) have suggested that the so-called 'inert' genes situated in heterochromatin segments are concerned with the metabolism of nucleic acid necessary for the efficient working of the cell cycle. If this is the case, we may presume that all organisms carry such inert genes, and we must ask ourselves what are the factors which determine that heterochromatin should be visible at metaphase in one organism and not in another.

Darlington and La Cour (1940) have discussed the phenomenon of allocyclic behaviour in terms of the 'supply and demand' of nucleic acid. Little need be added to their account. The detection of heterochromatin depends primarily on passing below the concentration threshold for the differential supply of nucleic acid during the prophase stages. Some organisms perform the natural experiment. Parts of the sex chromosomes of male Orthoptera and Mammalia, etc., show at certain stages and in certain cells the typical properties of heterochromatin. The same is true of certain individuals of *Mecostethus grossus* (Callan, unpublished) where proximal regions of certain of the autosomes behave as heterochromatin at meiosis, although they are not distinguishable at the metaphase of mitosis.

Low temperature is probably merely one of the agents which could assist in the detection of heterochromatin. When low temperature is the agent used, two conditions must be satisfied:

(a) A temperature must be applied sufficiently low to pass below the nucleic acid threshold, when the competition between genes for their supply of nucleic acid (this will be discussed under *Mecostethus*) results in the less 'active' genes obtaining little or none.

(b) The cell must be able to reach metaphase from a diffuse stage whilst under the influence of low temperature.

When an organism does not exhibit heterochromatin after a certain treatment, the conclusion to be drawn is not necessarily that it possesses no heterochromatin; one or other of these conditions may not have been satisfied. Thus from their work on root-tip mitoses of *Trillium*, Darlington

and La Cour have shown that this plant possesses heterochromatin. Cold-treated pollen-grain divisions show no heterochromatin, however, owing to the enormous length of time between the previous diffuse stage and the pollen-grain metaphase. This period has never been covered by the treatment.

The newt provides very favourable meiotic material for the demonstration of heterochromatin. Its upper temperature limit is 6° C, at which temperature all processes but spindle formation continue. Also important is the fact that pachytene is a diffuse stage and lengthy treatment is thus unnecessary: only the pachytene-metaphase period needs to be covered.

## 6. HETEROCHROMATIN AND SPIRALIZATION

It has been suggested that heterochromatic regions do not have a spiral structure (Muller and Prokofieva 1935). White (1940) has shown that this view is manifestly inapplicable to the sex chromosomes of Orthoptera. Darlington and La Cour come to the same conclusion in their work on *Paris* and *Trillium* (1940) and in fact state that 'nucleic acid seems to have no necessary relation to spiralization'. The proximal heterochromatin segments in the autosomes of *Mecostethus* (Callan, unpublished) have approximately the same diameter of cross-section as euchromatin regions, and they may thus be considered to have the same type of spiral structure as euchromatin. Similarly, heterochromatin visible at mitosis in *Triton*, though of smaller diameter of cross-section than euchromatin, is considerably wider than the earliest prophase threads and thus probably has a spiral structure.

The heterochromatin visible in *Triton* at meiosis, however, is as thin as the leptotene or zygotene threads, despite fixation in acetic alcohol and staining in aceto-carmin, a technique which swells the chromosomes. When stained by means of the Feulgen technique, the tiny strands joining blocks of euchromatin are entirely unstained and in fact invisible. I am therefore convinced that neither minor nor major spirals exist in these regions. In the light of this evidence we must reconsider the relationship between nucleic acid and spiralization.

The sex chromosomes of Orthoptera spiralize while they are heavily charged with nucleic acid. If they become under-charged during meiosis, the loss of nucleic acid occurs during diakinesis and metaphase and subsequent to spiralization. The same is true of the autosomal heterochromatin in *Mecostethus*. At no stage are the heterochromatic regions of *Paris*, *Trillium* and *Fritillaria* completely negative to Feulgen, and we can

thus presume that nucleic acid is always present during spiralization in these species.

During meiosis in *Triton* subjected to low temperature, the heterochromatic regions are completely negative to Feulgen throughout diplotene, diakinesis and metaphase, while in the diffuse pachytene stage there are no Feulgen-positive chromocentres. This fact and the absence of spiralization may be connected. We may in fact suppose that, while heterochromatin may or may not assume a spiral state, if it is utterly deficient in nucleic acid throughout the cell cycle, it cannot spiralize. This conclusion has important chemical and physical implications when we attempt to analyse the molecular mechanics of the chromosomes.

It is of interest to note that *Paris japonica* a species closely related to the *P. polyphylla* which has spiralized heterochromatin, has at mitosis thin thread-like regions very similar to those found in *Triton* at meiosis (Darlington and La Cour 1940). This argues against any major inherent difference between spiralized and unspiralized heterochromatin. It is a character of the cell, not of the segment.

#### 7. HETEROCHROMATIN AND CHIASMA LOCALIZATION

Darlington has suggested that heterochromatin will develop in those parts of the chromosomes where chiasmata are rarely formed. When part of a chromosome fails to form chiasmata, recombination of the genes in this part is thereby prevented and these genes will finally mutate to inertness. The relationship between chiasma localization and heterochromatin regions is, however, complex (see table 1). *Paris polyphylla*, *Trillium* spp., *Adoxa moschatellina* (Geitler 1940), *Triton vulgaris* and *T. palmatus*, *Chrysochraon dispar* and the Y-chromosome of *Cricetus auratus* (Koller 1938) are examples which support Darlington's hypothesis. *Mecostethus grossus* is also no exception, since although both chiasmata and heterochromatin regions are proximally localized, chiasmata are never formed in the heterochromatin of this species. On the other hand, *Fritillaria pudica*, other *Trillium* spp., *Zea Mays* (Longley 1938; Darlington and Upcott 1941), female Orthoptera (McNabb 1928), *Triton cristatus* and *Drosophila melanogaster* are examples in which chiasmata are formed in the heterochromatin.

Darlington has put forward the further hypothesis that in such species as these the blocks of heterochromatin occurring in chiasma-forming regions are adapted to regulate the cross-over frequency in active regions nearby. The proximal heterochromatin in the X-chromosome of *Drosophila*

is a case in point. It appears to be specifically adapted to achieve reciprocal cross-overs with the Y at meiosis in the male (Darlington 1934; Philip 1935).

TABLE 1. HETEROCHROMATIN AND CHIASMA DISTRIBUTION  
(EXCLUDING SEX CHROMOSOMES)

organism	initiation of pairing	chiasma localization	hetero- chromatin distribution	author
<i>Paris polyphylla</i>	procentric	intermediate	distal	Darlington and La Cour (1938)
<i>P. japonica</i>	"	"	intercalary	Darlington and La Cour (1940)
<i>Adoxa moschatellina</i>	"	maximum	distal	Geitler (1940)
<i>Trillium sessile</i>	"	intermediate	mainly distal	Darlington and La Cour (1940)
<i>T. recurvatum</i>	"	"	" "	" "
<i>T. stylosum</i>	"	"	" "	" "
<i>T. grandiflorum</i>	"	"	" "	" "
<i>T. erectum</i>	"	"	distal and proximal	" "
<i>T. kamschaticum</i>	"	"	" "	" "
<i>Fritillaria pudica</i>	"	minimum	proximal	Darlington and La Cour (1941)
<i>Mecostethus grossus</i>	"	maximum	"	Callan (unpub.)
<i>Triton palmatus</i>	proterminal	maximum	proximal	Callan
<i>T. vulgaris</i>	"	"	"	"
<i>T. cristatus</i>	"	intermediate	"	"
<i>Chrysochraon dispar</i>	"	maximum	"	Klingstedt (1936)
<i>Zea Mays</i> (with knobs)	"	intermediate	distal	Longley (1938)
<i>Zea Mays</i> (with B-chromosomes)	" ?	minimum	entire	Darlington and Upcott (1941)

Apart from any genetic inferences to be drawn from the distribution of heterochromatin and chiasmata, we must bear in mind that the mechanical properties of heterochromatin, its allocyclic behaviour, have some relation to the three prime variables of meiosis (Darlington 1940) which determine the distribution of chiasmata. In some organisms and under some conditions, heterochromatin is not allocyclic during meiosis. In these cases we have no evidence or a priori reason for supposing that heterochromatin behaves differently from euchromatin. In tetraploid spermatocytes of the grasshopper *Schistocerca*, the two X-chromosomes are allocyclic and form no chiasmata (White 1933). In the female, on the other hand, the two X-chromosomes are not allocyclic, and they form chiasmata like the

autosomes (McNabb 1928). The heterochromatic *B*-chromosomes of *Zea Mays* which are allocyclic at meiosis form fewer chiasmata per unit length than the euchromosomes (unless the plant is trisomic for *B*'s when the 'triploid tie effect' counterbalances the mechanical properties of the heterochromatin (Darlington and Upcott 1941). In *Mecostethus grossus* the allocyclic proximal regions of the autosomes do not form chiasmata, and in fact the distance between the proximal chiasma and the centromere (the *differential* distance of Mather 1940) is precisely limited by the length of the intervening heterochromatic segment.

The analysis of the special problems connected with inert genes is only in its infancy. It is already clear, however, that by contrasting the properties of inert and active genes we have a further profitable line of approach to the general problems of heredity.

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# The effects of cold and colchicine on mitosis in the newt

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[Plates 9–11.]

The application of cold or colchicine to dividing epidermal cells of the newt larva produces by similar means an arrest of mitosis at metaphase.

The cells respond to both agents by complete suppression or by abnormal development of the spindle. The various types of abnormal spindle which are produced can be described in terms of the complete or incomplete inactivation of centromeres, centrosomes or both. Spermatogonia and spermatocytes, unlike the epidermal cells, invariably show complete spindle suppression during cold treatment.

The more obvious differences between the effects of the two agents are due (a) to the greater severity of spindle suppression produced by colchicine, and (b) to the slowing up of all cell processes produced by cold. Thus while both treatments lead to the production of relaxed tetraploid cells, these occur more commonly after colchicine treatment.

The untreated epidermal cell swells up during anaphase. This swelling is exaggerated in cells held at metaphase under the action of cold or colchicine.

The inactivation effects can be described in terms of a tactoid theory, as due to surface changes in centrosomes or centromeres which prevent them orientating or breaking down spindle molecules.

## 1. INTRODUCTION

The progress of cell division may be modified or suppressed by a great variety of agents. Subnormal temperature is known to interfere with the mitotic spindle in plant cells (e.g. Blakeslee & Cartledge 1927, on *Datura*; Darlington & La Cour 1940, on *Trillium*, etc.). Various drugs, including colchicine, have comparable effects.

The action of colchicine was first noticed by Dixon (1905) in leucocytes. Dixon interpreted the increase he found in the frequency of metaphases as due to the action of the drug in stimulating mitosis. Recently extensive and detailed studies have been carried out by various workers, notably Lits (1934), Dustin (1934), Brues & Cohen (1936) and Ludford (1936). Brues & Cohen were able to show that there was a precise concentration of colchicine in the presence of which regenerating liver cells of the mouse would pass into mitotic activity at the normal rate but would not proceed beyond metaphase; this stage therefore accumulated throughout the tissue. Below this concentration the rate of entry of cells into mitosis was normal, but some of the cells which reached metaphase were able to continue into anaphase and telophase; above this concentration there was a depressant action on the cells about to enter mitosis. Ludford determined the effective concentrations of various drugs necessary to inhibit anaphase in tissue cultures, and demon-

strated the quite remarkable specificity of action of colchicine at great dilutions (up to 1 part in 100,000,000).

The effects of colchicine on plant tissues have recently become widely known owing to their utilization in producing polyploids (Blakeslee & Avery 1937; cf. Darlington & La Cour 1942). This action has been shown to have the same type of cell effect as that described for animals. Thus with some variation in threshold between different species, it leads to complete suppression of the spindle. The chromosomes reach metaphase or super-metaphase spiralization and lie scattered about the cell. After some delay the centromeres divide, but no separation of the sister chromatids into two independently organized nuclei takes place. A tetraploid cell is the result (Levan 1938). The effects of cold on plant mitosis are similar but again with differences of threshold (Darlington & La Cour 1940).

In general Ludford's work indicates that the action of colchicine on chromosome movement takes effect in a similar way in animal cells. It seemed likely, however, that in view of the difference in organization of the animal spindle, the similarity in action of cold and colchicine which held good in plants might break down in animals.

We have therefore carried out comparative tests with an animal chosen for the accuracy with which we have found it possible to record and describe the movements of the chromosomes, namely, the newt (*Triton vulgaris*,  $2n = 24$ ). In the following account we shall deal first with the observed and qualitative effects of treatment together with their quantitative recording and the immediate developmental inferences which have to be drawn. Secondly, we shall deal with the more indirect inferences about cell mechanics which are revealed by the type of breakdown the spindle undergoes.

The experiments were carried out on half-grown larvae. In the cold-treatment experiments, larvae were kept in water at a temperature of  $3^{\circ}\text{C}$ . Lower temperatures completely suppressed mitosis. Colchicine treatment consisted in the immersion of larvae in a 1% solution at room temperature ( $18\text{--}20^{\circ}\text{C}$ ).

Fixations of amputated tail tips were made in acetic alcohol after various periods of treatment. The tails were stained in Belling's aceto-carmine, or by means of the Feulgen technique, and pressed out for examination of the epithelial cells. The same methods were used with testes.

## 2. THE ARREST OF MITOSIS AT METAPHASE

The most striking effect of cold and of colchicine is the arrest of mitosis when the chromosomes have reached metaphase spiralization. This arrest leads to an accumulation of cells at this stage. In figures 1 and 2 counts of the numbers of cells at the various mitotic stages in a standard area of tail (the tail fin epidermis behind the anus in half-grown larvae of a standard size) are plotted against the time of treatment.

Cold, it will be seen (figure 1, table 1) has an immediate and general depressant

effect on the initiation of mitosis. The number of prophases is reduced by 75 % within 12 hr., and remains more or less constant at this low level for over 4 days. But the most significant effect is that on the anaphases. After 12 hr. they disappear completely. The number of metaphases, on the other hand, increases from 33 in the controls to 86 in a period of 8 days. Evidently the cells coming into mitosis are held at metaphase. The metaphases are of various types, which we shall consider later.

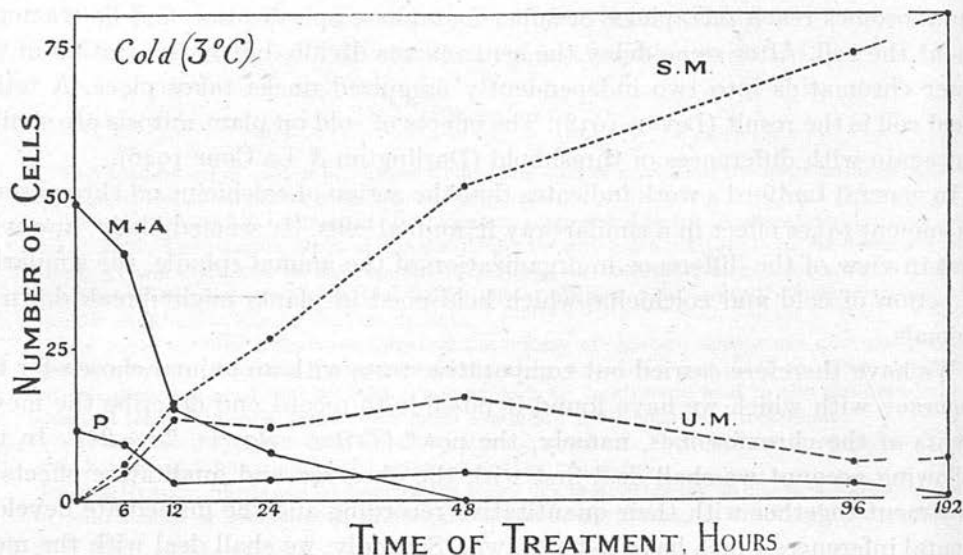


FIGURE 1. Graph showing frequency of cell types in the tail epidermis of the newt plotted against duration of cold treatment ( $3^{\circ}\text{C}$ ). The data are from table 1. The four lines give number of prophases (*P.*), metaphases with bipolar spindles and anaphases (*M.+A.*), and two categories of arrested metaphase, the *star* (*S.M.*) and *unorientated* metaphases (*U.M.*).

TABLE 1. FREQUENCY OF DIFFERENT TYPES OF CELL AFTER COLD TREATMENT ( $3^{\circ}\text{C}$ ). THE FIGURES ARE THE MEANS OF COUNTS OF THE CELLS IN THE POST-ANAL TAIL FINS OF THREE LARVAE

time of treatment hr.	prophase	bipolar metaphase	<i>star</i> metaphase	<i>unorientated</i> metaphase	total metaphase	anaphase
0	11.7	33.3	—	—	33.3	15.3
6	9.3	31.0	6.0	4.3	41.3	9.3
12	3.0	12.3	16.0	13.3	41.7	3.0
24	3.7	7.3	26.3	12.0	49.0	—
48	4.3	—	51.7	17.0	68.7	—
96	2.3	—	73.3	6.7	80.0	—
192	0.7	—	80.0	6.3	86.3	—

The initial effects of colchicine are similar (figure 2, table 2). They differ from those of cold in that colchicine does not at once reduce the number of cells coming into prophase. There is, however, an immediate reduction in the number of anaphases, and the few that continue frequently fail to separate the two daughter

nuclei completely (figure 21, plate 10). There is an even greater accumulation of cells with metaphase chromosomes. This means that the chromosomes are arrested at metaphase as they are during cold treatment, but that prophase is not slowed down immediately by the drug as it is by the low temperature.

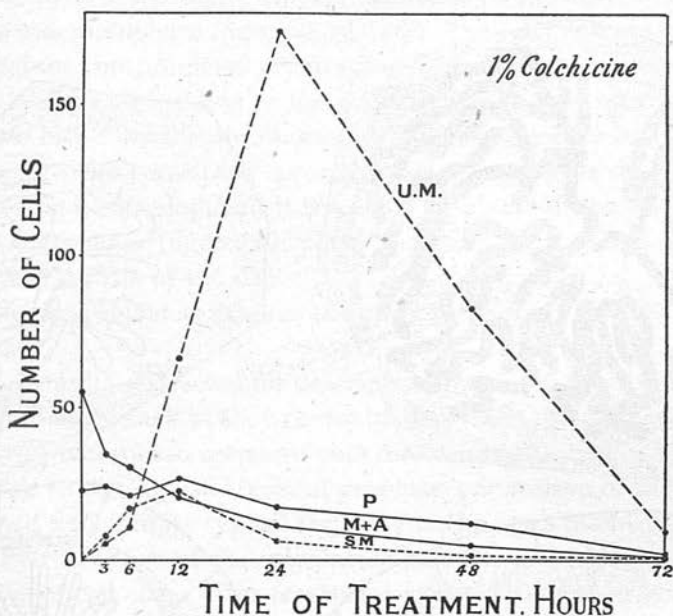


FIGURE 2. Graph showing changes in cell frequencies plotted against duration of colchicine treatment. The data are from table 2.

TABLE 2. FREQUENCY OF DIFFERENT TYPES OF CELL AFTER COLCHICINE TREATMENT (MEANS OF COUNTS FROM 3 LARVAE)

time of treatment hr.	bipolar		star	unorientated	total	anaphase
	prophase	metaphase	metaphase	metaphase	metaphase	
0	22.3	25.0	—	—	25.0	30.7
3	24.0	15.7	7.7	6.3	29.7	20.0
6	20.3	15.0	16.3	10.7	42.0	15.7
12	27.0	12.3	20.7	66.3	99.3	8.3
24	17.7	5.0	6.7	175.3	186.0	6.7
48	12.0	0.3	1.7	83.3	85.3	4.3
72	2.3	—	—	9.7	9.7	1.0

### 3. THE ABNORMAL CELL TYPES

Cold and colchicine have no visible effect on the form or manner of prophase (figure 3). But among the arrested metaphases the following types of abnormal chromosome development can be distinguished:

(a) The *Star* metaphase (figure 6; figure 11, plate 9). The undivided centromeres come together at one focus with the arms of the chromosomes radiating outwards in all directions. Odd chromosomes are frequently left out (figures 13, 14, plate 9).

In a small proportion of such cells two or three centromere-foci have been seen to give a pseudo-anaphase appearance (figure 15, plate 9).

(b) The *Distorted-Star* metaphase (figures 16, 17, plate 10). This type is similar to the star except that it is not radially symmetrical. The centromeres come

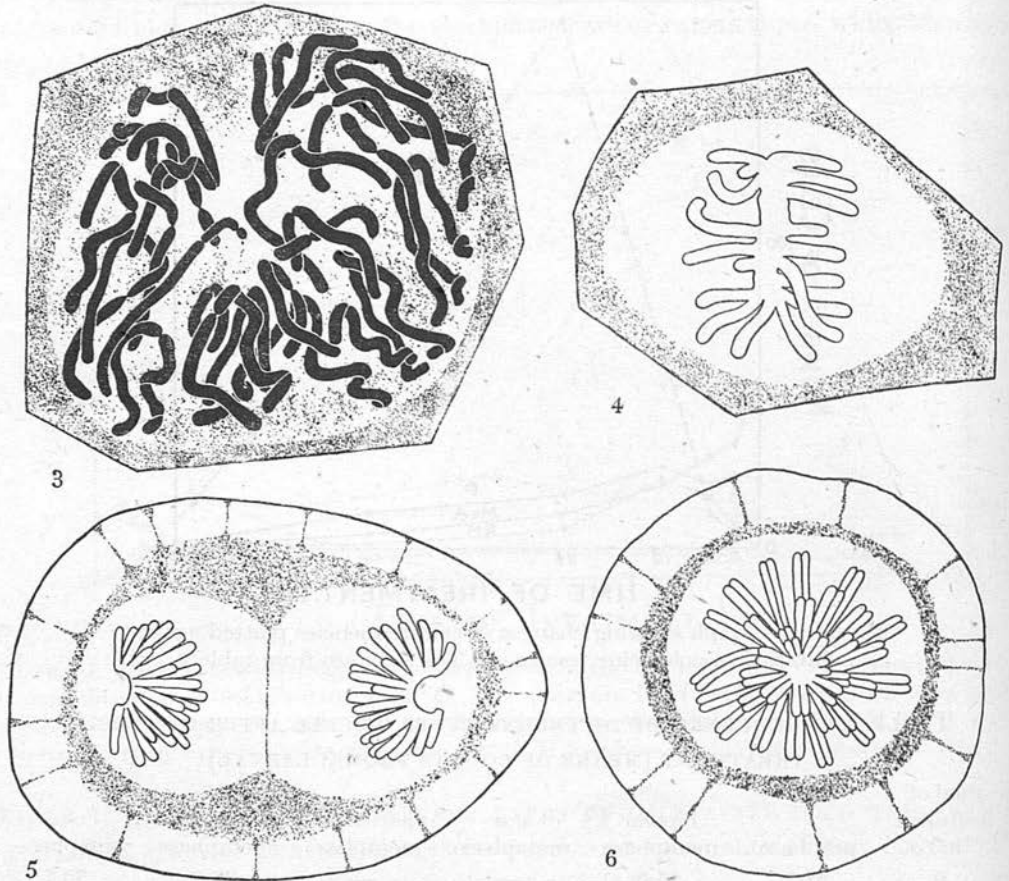


FIGURE 3. Normal prophase. Note disposition of centromeres as derived from the preceding telophase, and absence of cytoplasmic vacuoles. Aceto-carmin.  $\times 1600$ .

FIGURE 4. Normal metaphase. Note absence of cytoplasmic vacuoles. Iodine-alcohol.  $\times 1200$ .

FIGURE 5. Normal anaphase. Note vacuolization of cytoplasm outside spindle area. Iodine-alcohol.  $\times 1200$ .

FIGURE 6. *Star* metaphase after 5 days at  $3^{\circ}\text{C}$ . Note vacuolation of cytoplasm outside the spindle area. Iodine-alcohol.  $\times 1200$ .

together at one focus, but at one side there is a gap around which the chromosome arms are stretched. This leads in the extreme condition to a flattened star pushed against one side of the cell.

(c) The *Exploded* metaphase (figure 18, plate 10). The centromeres remain completely unorientated, but the chromosomes are pushed bodily to the boundary of

the cell, many becoming stretched. The centromeres themselves may or may not be stretched, indicating that they play no specific part in the movement. In the final condition the chromosomes may become segregated in several groups, but no separation of sister chromatids into two different nuclei takes place (figure 19, plate 10). The products of this segregation are unbalanced and ultimately degenerate.

(d) The *Prophase* metaphase (figure 9, plate 9). The fully contracted metaphase chromosomes retain the prophase orientation, with the centromeres directed to one side of the nucleus. According to the compactness of the resting nucleus from which they have come, so the chromosomes lie close together or far apart. This type corresponds to the transitory 'prometaphase' in untreated cells, but owing to the failure of spindle development it is a fairly stable condition under treatment.

(e) The *Ball* metaphase (figure 10, plate 9). The chromosomes form a closely tangled ball in the middle of the cell.

Types *a* and *b* are grouped as Stars in the graphs, types *c*, *d* and *e* as unorientated metaphases.

The types of metaphase selected for description are of course arbitrary and show a regular intergradation, such as they no doubt sometimes undergo in development. Thus the prophase-metaphase seems to pass direct into the ball. In this cell type it is often possible to distinguish traces of prophase orientation of the centromeres. Similarly the star, which in its typical form shows the arms radiating straight out, may also pass by relaxation of the orientation into the ball (figure 8).

All these types of cell occur after treatment either with cold or with colchicine. But their proportions differ. The commonest type in the cold is the star (over 90 %). In colchicine on the other hand, the ball is the commonest (75 %), and stars as a rule appear only in the early fixations (up to 24 hr.). The other types are rarer, the exploded type occurring sporadically throughout the period of treatment.

Spermatogonia and spermatocytes were similarly subjected to cold treatment. They also are arrested at metaphase. Unlike epidermal cells, however, all the arrested metaphases are of the prophase-metaphase type.

Both cold and colchicine treatments frequently seem to disturb the gross timing relations of the processes responsible for cell division. This appears in two ways. First both star and exploded metaphases may be formed by chromosomes whose spiralization has reached only the mid-prophase state (figure 12, plate 9). Possibly the precocious breakdown of the nuclear membrane, whose equilibrium during prophase is inherently unstable, is the conditioning factor.

The second timing irregularity is shown by what we may call *swelling*. Previous to anaphase in the untreated epithelial cells, the cell outline is polygonal (figures 3, 4). During anaphase, however, the dividing cell swells by the intake of liquid and forms an even, rounded outline at the expense of the neighbouring cells. This liquid forms vacuoles in the cytoplasm immediately inside the cell boundary and outside the spindle (figure 5). The vacuolation of the anaphase cytoplasm may be related to 'bubbling' at anaphase described by Strangeways (1922) in chick fibroblasts and by Barber (1939) in *Tradescantia* hair cells.

The various types of cell containing arrested metaphases invariably develop this swelling to excess (figure 6). Their cytoplasm is in fact in a condition of prolonged and thereby exaggerated anaphase although the chromosomes are still in metaphase. The intake of water may be due to an increase of osmotic pressure inside the cell membrane; this increase may be ascribed to a repartitioning of the bound and free water during chromosome spiralization and spindle growth, resulting in a decrease of free water and hence an increase in solute concentration. Guinochet (1940) has demonstrated that under the action of colchicine the osmotic pressure of the cell sap of wheat seedlings increases from 7.1 to 11.1 atmospheres. Normally the ensuing telophase, with the breakdown of the spindle and the reconstitution of resting nuclei, will liberate the cytoplasm from this stress. The arrested metaphase cell, on the other hand, must remain abnormally swollen until its chromosomes lapse into a resting stage or until disintegration takes place. In consequence cold and, even more, colchicine seem to disturb the gross water relations of the larvae. After heavy treatment the whole animal often swells enormously. We have as it were induced intracellular dropsy.

Under continued treatment anaphase is frustrated in all types of arrested cells. In colchicine the arrest is followed after a few hours by a lapse of the chromosomes into a normal resting nucleus or by degeneration into pycnotic masses. The sequence of events is similar to that described by Levan (1938) in *Allium*. The chromatid attraction lapses and division of the centromere takes place, but the two chromatids remain parallel. Either a single resting nucleus, which will be tetraploid, is produced, or several unbalanced nuclei which will degenerate.

In the cold, on the other hand, the arrested metaphases persist for several days and the return to the resting stage so as to produce a tetraploid cell (figure 20, plate 10) is much rarer, or slower, than with colchicine. Evidently the cold holds up not only the spindle but all other cell processes with it. In particular, low temperature has an effect on the processes of despiralization and loss of attached nucleic acid by the chromosomes: many nuclei in metaphase or anaphase commence the telophase processes, only to degenerate before telophase is completed. Degenerating nuclei are characterized by their heavy staining and many-pointed periphery, associated with contraction.

#### 4. RECOVERY

When larvae are taken from the cold and placed in water at room temperature (18–20° C), the arrested cells recover rapidly. After 1–2 hr. anaphases are frequent. Many of them are multipolar (figure 22, plate 11). Some have incompact spindles such as occur naturally in certain plants (figure 23, plate 11) (cf. Darlington & Thomas 1937, and Clarke 1940). In some recovered anaphases, after colchicine as well as after cold, the chromatids stick, either terminally or elsewhere. Occasionally, however, sticking has also been observed in the controls (figure 7; figures 25, 27, plate 11).

The sticking seems to lead to a structural change. For example acentric fragments (figure 26, plate 11) sometimes accompanied by a bridge, have been observed. Darlington & La Cour (1940) have shown a similar increase in the frequency of structural change after cold treatment in *Trillium*. Here the increase was correlated with the nucleic acid starvation of the heterochromatic regions of the chromosomes at low temperatures. Similar heterochromatic regions are revealed in *Triton* under these conditions (Callan 1942), and the structural changes may thus be caused by the same reproductive error as in *Trillium*.

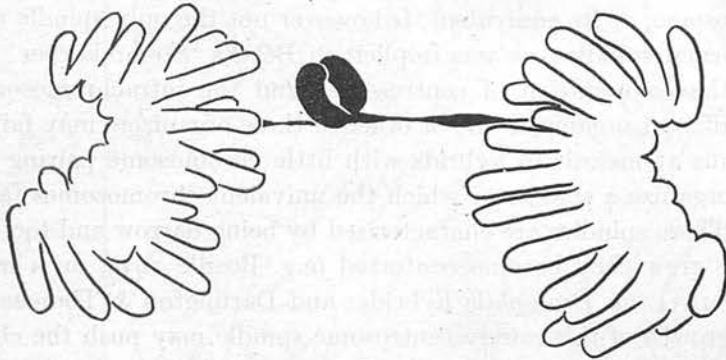


FIGURE 7. Intercalary and terminal (?) sticking at anaphase. No treatment. Aceto-carmin.  $\times 1600$ .

Recovery after colchicine treatment is much slower than after cold treatment. The drug remains in the cells; the temperature can be changed at once. In consequence the larvae rarely recover completely from the colchicine treatment; nearly all of them die. Among the survivors only a few divisions have been seen. Most of these are diploid, but one approximately tetraploid cell with evidence of secondary pairing was seen in a fixation 5 days after the end of the treatment. Later fixations showed only diploid divisions.

##### 5. THE ORIGIN AND STRUCTURE OF THE SPINDLE

Numerous theories have been put forward to account for the origin and properties of the spindle (cf. Gray 1931). Most of these speculations were premature attempts to explain the spindle in terms of physico-chemical concepts.

The earlier investigators (Boveri, Wilson, etc.) showed that in animal cells spindles are formed in relation to extranuclear bodies, the centrosomes. In plants, except in some of the lower groups (Algae, Fungi) centrosomes have not been recognized. However, as Darlington & Thomas (1937) have shown in a grass, corresponding pole determinants must exist in the higher plants, but they are more diffuse than in animal cells.

In many animal cells the centrosomes have continuity from one division to the next. It therefore seemed justifiable to speak of them as cell organs, particularly



in those cases where, as basal granules, they are associated with cilia or flagella. However, the evidence from experiment runs counter to this view. Morgan (1896) and McClendon (1908), by exposing echinoderm eggs to hypertonic sea water, have induced subsidiary cytasters associated with polar granules which seem to have arisen *de novo* in the cell. The evidence indicated that this agent had not acted by stimulating the division of preexisting centrosomes, but rather by altering the surface properties of other granules already distributed in the cell. Similarly the surface of the cell can take over centrosome functions, as in the 'monopolar' spindles of *Sciara* (Metz 1933).

The centrosome, or its equivalent, is however not the only spindle organizer in the cell. Normal spindles, as was implicit in Bělař's 'Stemmkörper' theory, are formed by the co-operation of centrosomes and the intrachromosomal centromeres. In different organisms one or other of these organizers may fail to operate properly. Thus at meiosis in hybrids with little chromosome pairing the centrosomes may organize a spindle to which the univalent chromosomes fail to attach themselves. These spindles are characterized by being narrow and too long for the cell. As they grow they become contorted (e.g. Beadle 1933, on a mutant *Zea*; Dobzhansky 1934, on *Drosophila* hybrids; and Darlington & Thomas 1937, on a grass). The growth of this purely centrosome spindle may push the chromosomes about the cell just as acentric fragments are pushed about by the growth of a normal spindle. The centromeres remain inactive.

In other organisms the centrosomes may fail. Thus in the grass hybrid (Darlington & Thomas 1937), in a mutant *Zea* (Clarke 1940), or in some coccids as a normal process (Schrader 1932), purely centromere spindles are formed at meiosis. These spindles are characterized by being made up of several discrete spindles, one per bivalent, arranged more or less parallel, but often diverging at the ends. Each subsidiary spindle has the structure of a centrosome spindle except that the fibrils diverge from the centromere. The centromere is behaving like a centrosome. When anaphase occurs, several micronuclei may form at the ends of the cell instead of a single nucleus. Evidently the centrosome is necessary in most cells to bring the spindle to a focus at the poles.

We have seen that under the influence of cold or colchicine several abnormal types of chromosome movement and orientation take place in the epidermal cells of the newt. These various types can readily be interpreted in terms of varying degrees of centromere and centrosome activity (figure 8).

In the most extreme types, viz. the ball and prophase metaphase, both centrosome and centromere are inactivated. No directed chromosome movement takes place, and the movements which do occur are internal. They result from the coiling and uncoiling of the chromosomes and the lapse of chromatid attraction.

In the exploded metaphase the chromosomes are pushed bodily about the cell, probably owing to the formation of a purely centrosome spindle: The centromeres are inactive, and the chromosomes thus passive in their reactions to this spindle. The centrosome spindle may be a monaster, when the chromosomes are pushed

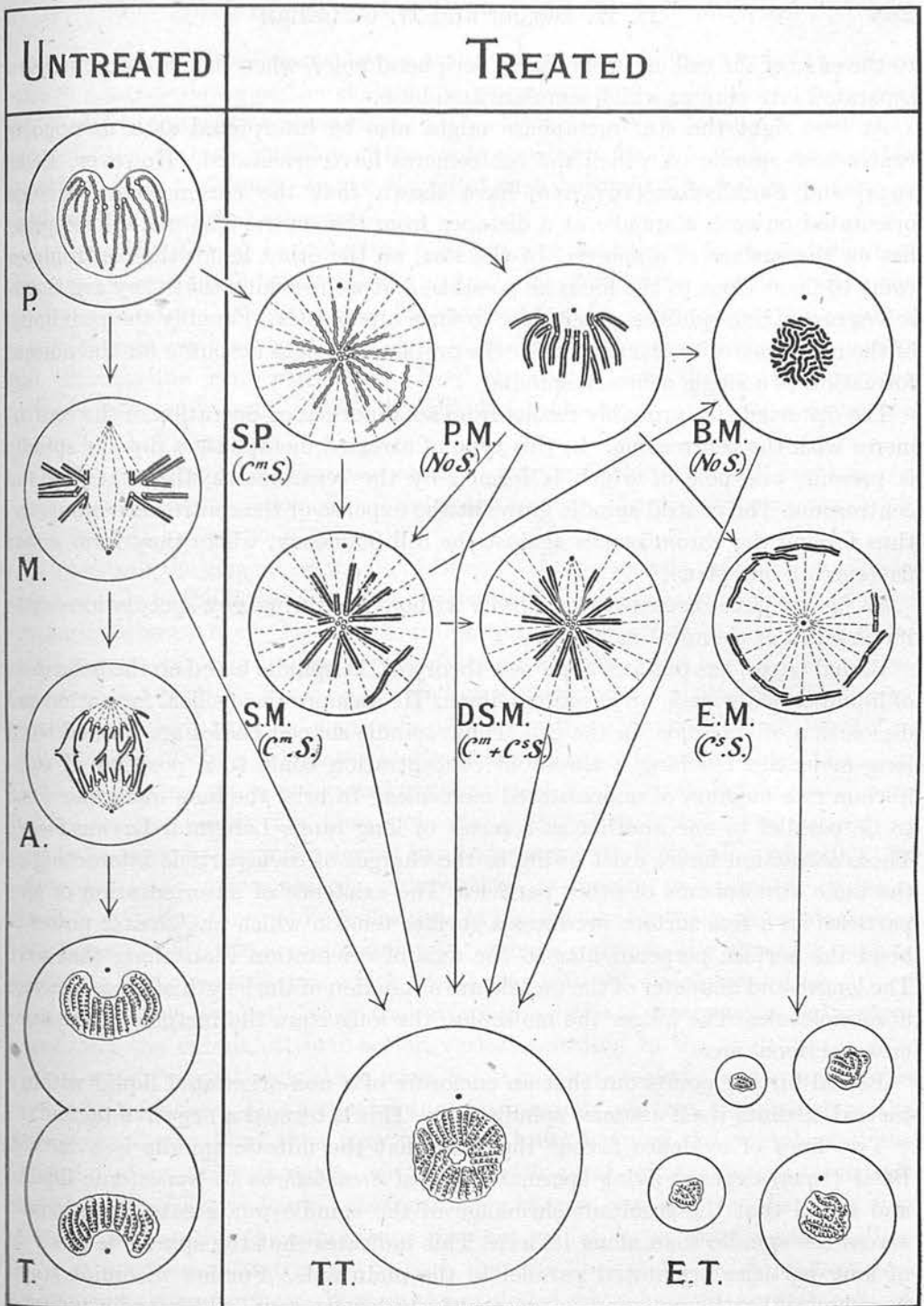


FIGURE 8. Diagram showing the relationships of types of spindle produced after cold and colchicine treatment of the newt larva.

*P.* = prophase, *M.* = metaphase, *A.* = anaphase, *T.* = telophase, *S.P.* = star prophase, *P.M.* = prophase metaphase, *B.M.* = ball metaphase, *D.S.M.* = distorted star metaphase, *E.M.* = exploded metaphase, *T.T.* = tetraploid telophase, *E.T.* = exploded telophase,  $C^m.S.$  = centromere spindle,  $C^s.S.$  = centrosome spindle.

to the edge of the cell, or it may have peripheral poles, when the chromosomes are separated into clumps which simulate anaphase.

At first sight the star metaphase might also be interpreted as a monopolar centrosome spindle on which the centromeres have orientated. However, Bělař (1933) and Fankhauser (1934 *a, b*) have shown that the chromosomes become orientated on such a spindle at a distance from the centre. The metaphase plate lies on the surface of a sphere. In the star, on the other hand, the centromeres come to lie as close to the focus as possible, and we conclude that they are themselves organizing spindles which close to form a monaster. Possibly the proximity of the centromeres to one another in the prophase nucleus accounts for the normal formation of a single common spindle.

The distorted star probably results from an abnormal co-operation of the centromeres with the centrosome. In this type of arrested metaphase a dipolar spindle is present, one pole of which is formed by the centromeres, the other by the centrosome. The central spindle grows at the expense of the centromere monaster, thus forcing the chromosomes against the cell boundary, where they form a star flattened in one plane.

So far we have discussed the spindle without venturing any speculations as to its physical or chemical nature.

Bernal (1940) has put forward a new theory of the spindle based on the behaviour of liquid crystals made up of protein fibres. He assumes the cyclical formation and dissolution of 'tactoids' in the cell. These spindle-shaped bodies are formed when long molecules reaching a threshold concentration come to a position of equilibrium in a medium of unorientated molecules. In brief the long molecules come to lie parallel to one another as a result of long range Langmuir-Levine forces. These attraction forces exist owing to the charges of each particle interacting on the ionic atmospheres of other particles. The existence of an orientation of long particles on a free surface produces a surface tension which has greater power to bend the surface perpendicular to the axis of orientation than along that axis. The length and diameter of the tactoid are a function of the length of its component fibre molecules. The longer the molecules, the longer are the tactoids of the same cross-sectional area.

Bernal further points out that an enclosure of a non-orientated liquid within a tactoid medium itself assumes spindle form. This is termed a negative tactoid.

Two lines of evidence favour the view that the mitotic spindle is a tactoid. Bělař (1929) exposed living spermatocytes of *Stenobothrus* to hypertonic liquids, and found that the resultant shrinkage of the spindle was greater transversely across the spindle than along its axis. This indicates that the spindle is made up of long particles orientated parallel to the main axis. Further, Schmidt (1937) has shown that living spindles are positively birefringent, which again indicates the same type of particle orientation.

Bernal suggests that at mitotic prophase a progressive chemical change occurs within the cell which results in the formation of long protein molecules. As prophase

proceeds the particles become longer and form parallel chains. Owing to certain surface characteristics of the centrosome granules, these chains come to lie perpendicular to the centrosome surface. Such a condition, the monaster, is inherently unstable, but with the division of the centrosome into two parts, the more stable spindle tactoid is formed. As the length of each individual particle increases, so the length of the spindle increases.

At this stage Bernal assumes that owing to a different surface characteristic of the centromeres, local dissolution of the long molecules of the spindle close to the centromeres occurs. Negative tactoids are thus formed, which pass into a position of equilibrium on the equatorial plate of the spindle, carrying the centromeres and chromosome arms with them. Now the centromeres divide: the negative tactoids increase in size, forcing the divided centromeres apart, this being the motive force of anaphase.

Finally, a retrogressive chemical change comes over the cell which involves the breakdown of the long molecules forming the spindle. The divided cell passes from telophase to a resting stage.

If we accept the general principles of Bernal's theory, certain new interpretations may be made in the light of our experiments with cold and colchicine and of other cytological observations. The surface properties of the centromere are not identical at all stages and in all cells. Probably in most cells, prior to metaphase, the surface of the centromere resembles that of the centrosome in that fibre molecules set perpendicularly to it. In plant cells with no centrosomes the wave of orientation of the fibre molecules originates at the centromeres. The centromere, however, unlike the centrosome, is not radially symmetrical, with the result that centromere spindles are not asters. The fibre molecules converge to two sides of the centromere only, forming a spindle shaped like two cones with their apices abutting. In cells possessing centrosomes, the centromeres co-operate to a greater or lesser extent in orienting the spindle molecules. At metaphase, however, the centrosomes have come to play the predominant role, perhaps owing to the changing surface conditions of the centromeres which are passing into the dissolution phase.

Cold and colchicine both act on the surface properties of centrosomes and centromeres, and the extent of their action varies according to the stage of the cell. (i) Cells which were at metaphase when the treatment started are prevented from passing to anaphase. This indicates that both agents prevent the change at the centromere surface and thus stop the local breakdown of the spindle and the formation of negative tactoids. (ii) Cells which enter mitosis during treatment may also show suppression of this centromere change, viz. star and distorted star metaphases. (iii) But the suppression may act earlier, by so affecting the surface of the centromere or centrosome or both that the fibre molecules do not set perpendicular to these surfaces. Thus in the exploded metaphase the centromeres, in the star the centrosomes, and finally in the ball and prophase metaphases both centromeres and centrosomes are inactivated.

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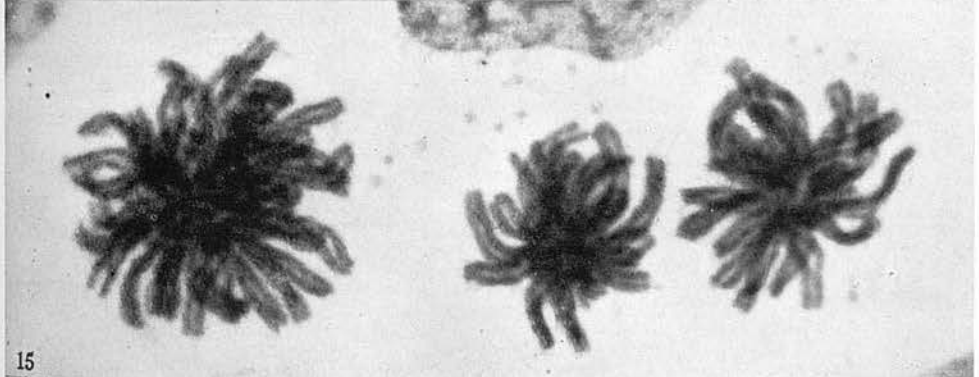
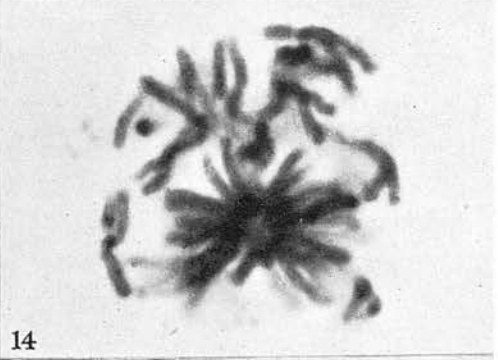
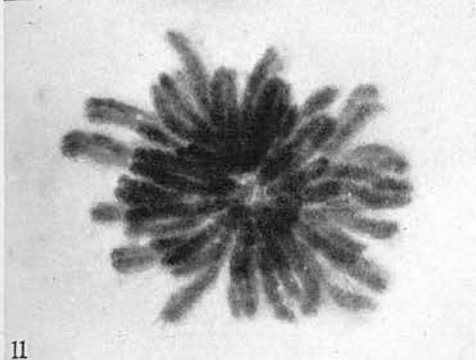
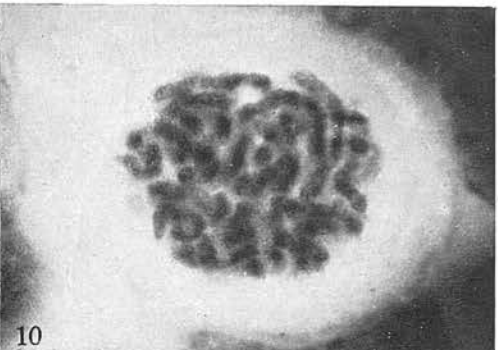
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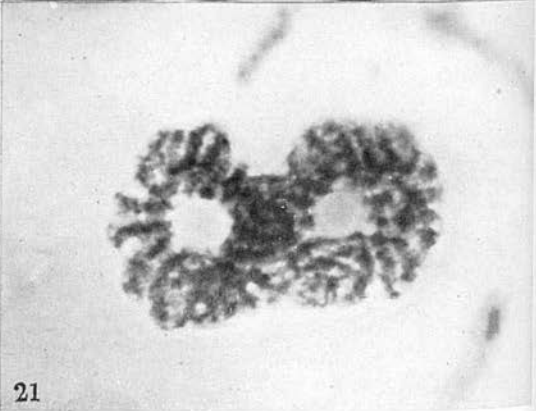
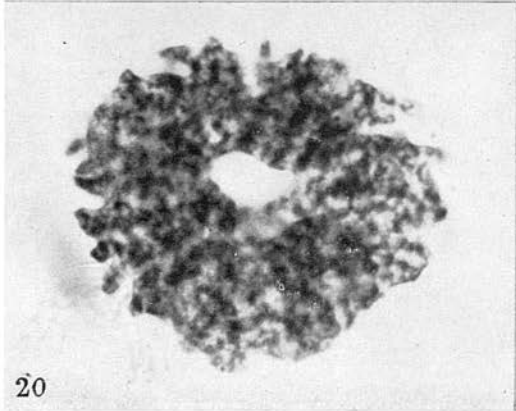
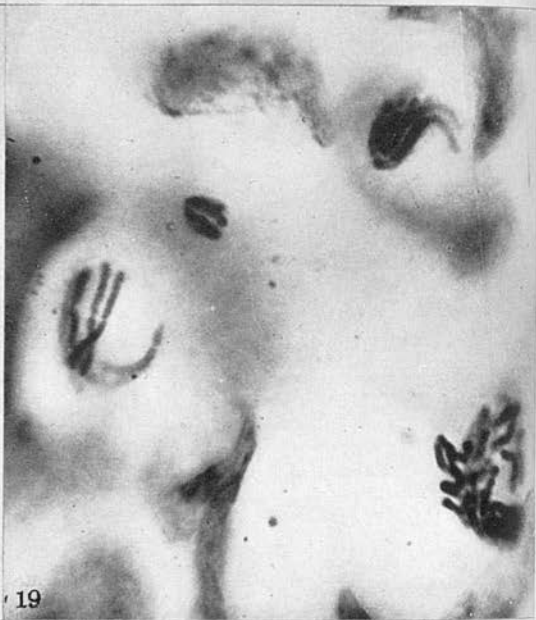
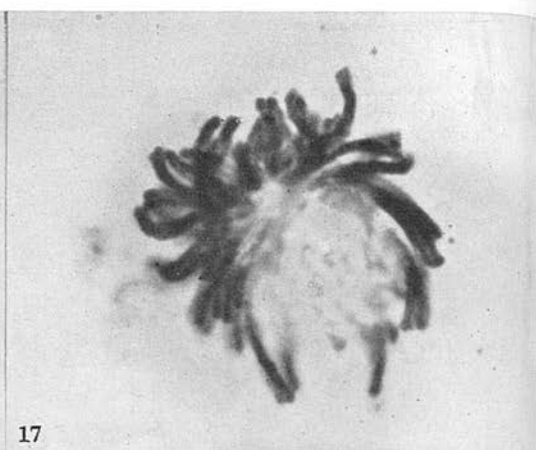
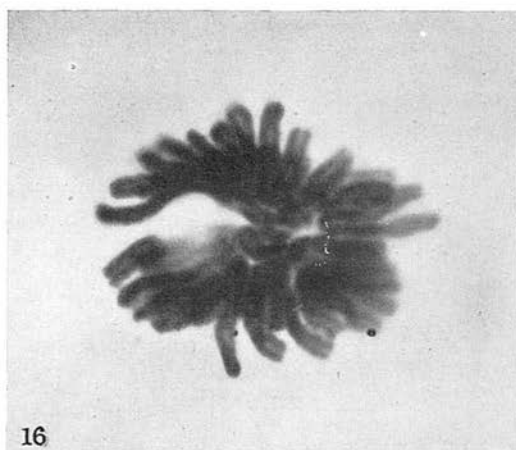
## DESCRIPTION OF PLATES 9-11

Photographs all  $\times 1750$  approx.

## PLATE 9

- FIGURE 9. *Prophase* metaphase. 12 hr., 1 % colchicine. Feulgen.  
 FIGURE 10. *Ball* metaphase. 24 hr., 1 % colchicine. Feulgen.  
 FIGURE 11. *Star* metaphase. 4 days, 3° C. Aceto-carmine.  
 FIGURE 12. *Star* with prophase spiralization. One chromosome has failed to orientate. 6 hr., 3° C. Feulgen.  
 FIGURE 13. Incomplete *star* with two foci. 3 days, 3° C. Feulgen.  
 FIGURE 14. Incomplete *star*. 6 hr., 1 % colchicine. Feulgen.  
 FIGURE 15. Simple and bifocal stars. 12 hr., 1 % colchicine. Feulgen.



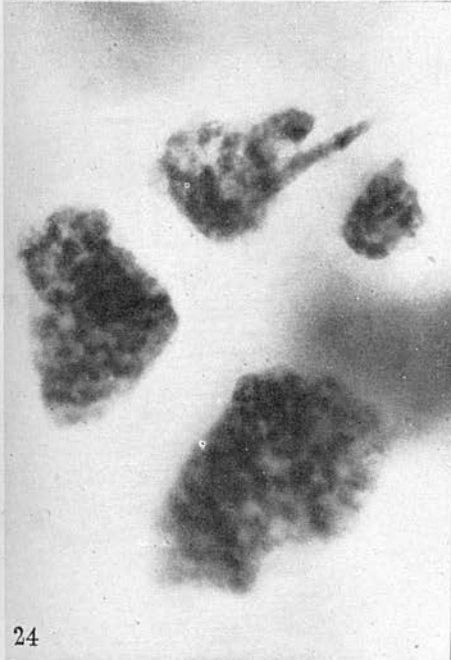




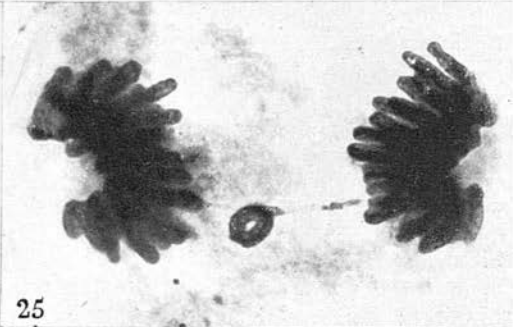
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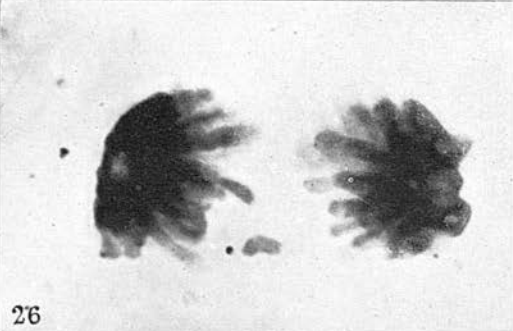
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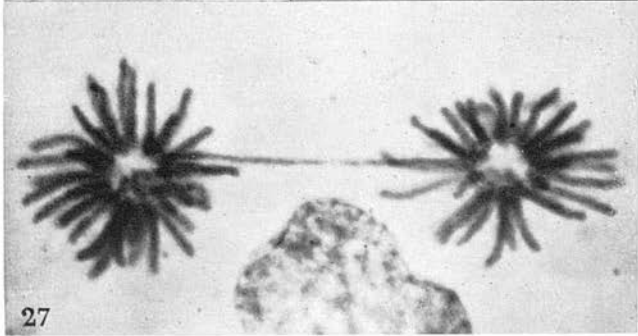
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PLATE 10

FIGURE 16. Early stage *distorted star*. 6 hr., 1 % colchicine. Feulgen.

FIGURE 17. Later stage *distorted star*. 1 day, 3° C. Feulgen.

FIGURE 18. Early stage *exploded* metaphase; note stretching of the chromatids. 1 day, 1 % colchicine. Feulgen.

FIGURE 19. *Exploded* nucleus. Note deformation of surrounding resting nuclei and the pseudo-anaphase appearance of the groups of chromosomes. The centromere of one chromosome in the group on the left has divided. 2 days, 1 % colchicine. Feulgen.

FIGURE 20. Tetraploid resting nucleus resulting from relapse of an arrested metaphase. 2 days, 3° C. Feulgen.

FIGURE 21. Tetraploid telophase resulting from a partially arrested anaphase. 6 hr., 1 % colchicine. Feulgen.

PLATE 11

FIGURE 22. Quadripolar anaphase. 7 days, 3° C; 2 hr. 18° C. Aceto-carmin.

FIGURE 23. Anaphase with an incompact spindle. 4 days, 3° C; 2 hr., 18° C. Feulgen.

FIGURE 24. Unbalanced resting nuclei derived from a quadripolar anaphase. 4 days, 3° C; 3 hr., 18° C. Feulgen.

FIGURE 25. Intercalary sticking at anaphase. No treatment. Aceto-carmin.

FIGURE 26. Fragment at anaphase. 4 days, 3° C; 2 hr., 18° C. Aceto-carmin.

FIGURE 27. Terminal sticking at anaphase. No treatment. Feulgen.

## LETTERS TO THE EDITORS

*The Editors do not hold themselves responsible for opinions expressed by their correspondents. No notice is taken of anonymous communications.*

## Distribution of Nucleic Acid in the Cell

In a recent article, Stedman and Stedman<sup>1</sup> claim to have discovered a new protein constituent of chromosomes, termed "chromosomin". They allege that Feulgen's reaction does not demonstrate the location of thymonucleic acid in the cell and they conclude that "the staining properties of the chromosomes towards both basic dyes and Feulgen's reagent are due to their content of chromosomin and the evidence indicating the presence in them of nucleic acid is shown to be fallacious". The argument runs as follows: Feulgen's reagent is colourized when in contact with the hydrolysis product of thymonucleic acid. The colourized product (see Baker<sup>2</sup>) is freely soluble in water, and it may then act as an ordinary basic stain and become attached to the acidic "chromosomin". Thus it is sufficient for thymonucleic acid to be present anywhere in the nucleus for the chromosomes to stain with Feulgen's reagent.

Although these authors do not deny that there may be small amounts of nucleic acid in the chromosomes, they lead one to infer that "there is little material left to form the nuclear sap unless this contains some nucleic acid", and indeed they suggest that the spindle at metaphase is a gel of nucleic acid.

The experiments of Mazia and Jaeger<sup>3</sup>, however, show this position to be untenable. These workers digested salivary gland cells of *Drosophila* with nuclease, and then tested for products of thymonucleic acid by means of the Feulgen reaction: controls showed the chromosomes stained and the cytoplasm unstained, while the treated material showed the chromosomes unstained and the cytoplasm stained. The protein constituents of the chromosomes were nevertheless still intact in the digested cells. Nuclease-treated glands, tested by the ninhydrin reaction for protein distribution, showed the chromosomes staining more intensely than the cytoplasm. The action of nuclease is to break up thymonucleic acid, and the products of hydrolysis then become distributed through the cell: these products colourize with Feulgen's reagent, but the dye-stuff so formed does not then become differentially attached to the residual protein of the chromosomes.

The Feulgen reaction is specific for aldehydes and the pentose radicals of thymonucleic acid. Now, however, the properties of the two other constituents of nucleic acid molecules have enabled nucleic acid distribution to be examined, and the results obtained corroborate or overlap the evidence from the Feulgen reaction. The purine and pyrimidine radicals determine the characteristic absorption spectrum of nucleic acid with a well-marked maximum at 2600 Å. in the ultra-violet. Caspersson<sup>4</sup> has shown that the chromosomes have a maximum absorption of ultra-violet light at this wave-length, at which indeed their absorption is vastly greater than that of all other cell components. When the same methods were applied to salivary gland chromosomes, Caspersson likewise showed that the bands which are stained with such nuclear dyes as carmine were also the regions of the chromosomes with a high content of nucleic acid. Moreover the contrast between the absorption by the chromosomes and by the rest of the cell (including the spindle) of light of this wave-length remains clear-cut when the protein constituents of the chromosomes have been digested by trypsin; to prove this, it was necessary, of course, that the thymonucleic acid liberated during the digestion should be precipitated by combination with lanthanum ions in the medium.

These experiments did not rule out the presence of nucleic acid derivatives in other parts of the cell. Caspersson and Schultz<sup>5</sup>, again by means of the ultra-violet absorption technique, demonstrated concentrations of ribose nucleotides in the nucleoli and cytoplasm of egg cells and rapidly dividing tissues where the Feulgen reaction gave negative results.

The third line of independent evidence for the distribution of nucleic acid in the cell is available from the micro-incineration experiments of Barigozzi and Norberg. Barigozzi<sup>6</sup> showed that the dark-staining bands of the salivary gland chromosomes give concentrations of phosphorus when incinerated. This we should expect since nucleic acid contains phosphorus in every nucleotide whereas most proteins contain little or none. Norberg's<sup>7</sup> results, based on new photometric methods, confirm and extend those of Barigozzi.

Thus while the results of Stedman and Stedman may require that our views on the protein constituents of the chromosomes be modified, there would seem no justification for radical change in the theory of the distribution of nucleic acid in the cell and of its important relationship to various cell functions<sup>8</sup>.

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Sept. 14.

<sup>1</sup> Stedman, E., and Stedman, E., *NATURE*, 152, 267 (1943).

<sup>2</sup> Baker, J. R., in "Cytology and Cell Physiology", edited by Bourne, G. (Oxford: Univ. Press, 1942).

<sup>3</sup> Mazia, D., and Jaeger, L., *Proc. U.S. Nat. Acad. Sci.*, 25, 456 (1939).

<sup>4</sup> Caspersson, T., *Skand. Archiv. Physiol.*, Suppl. 8 (1936).

<sup>5</sup> Caspersson, T., and Schultz, J., *Proc. U.S. Nat. Acad. Sci.*, 26, 507 (1940).

<sup>6</sup> Barigozzi, C., *Bull. Histol. Tech. Micro.*, 15, 213 (1935).

<sup>7</sup> Norberg, B., *Acta Physiol. Scand.*, 5, Suppl. 14 (1942).

<sup>8</sup> Darlington, C. D., *NATURE*, 149, 66 (1942).

## Distribution of Nucleic Acid in the Cell

THE statements which Stedman and Stedman<sup>1,2,3</sup> have published in NATURE must, it seems, be considered from two points of view. First, there is their chemical analysis leading to the discovery of "chromosomin". Secondly, there are the biological consequences which they have inferred from this discovery. Clearly, the chemical analysis is the foundation of everything else; but since no particulars of this analysis have been published, we can deal only with the superstructure.

The chief of Stedman and Stedman's inferences concerns the distribution of thymo-nucleic acid in the nucleus. They infer that the bulk of the thymonucleic acid lies in the nuclear sap, not in the chromosomes. Their own tables, however, demonstrate the contrary. They have determined the proportion of thymonucleic acid in the dry weight of nuclei varying between the extremes of no nuclear sap in cod sperm to, say, 90 per cent in carcinoma cells. The small variation in proportion of thymonucleic acid is not correlated with the proportion of nuclear sap. Yet it is on the basis of these results that Stedman and Stedman proceed to dismiss the work of Mazia and Jaeger, Caspersson, Norberg and Barigozzi<sup>4</sup>, who by four different methods have converged on the conclusion that thymonucleic acid exists in the cell only in structural relationship with the chromosomes.

This conclusion continues to be reinforced by new evidence. For example, Claude and Potter<sup>5</sup> have recently, by a simple technique, isolated chromosome threads from resting nuclei. By chemical analysis of the threads they conclude that 40 per cent is thymonucleic acid, which agrees closely enough with the proportion determined by Norberg, and the threads were, of course, Feulgen positive. Thus thymonucleic acid was evidently attached to the chromosome threads before their isolation. Stedman

and Stedman's theory of indirect staining by Feulgen's reagent becomes meaningless in the light of this experiment. On the other hand, Choudhuri's observation<sup>6</sup> of the staining of chromosomes by "developed Feulgen reagent", which has been used by Stedman and Stedman in support of their theory, is, of course, merely another example of the use of a basic dye.

Stedman and Stedman's view that thymonucleic acid is the main solid constituent of the nuclear sap leads them further: "it is an attractive hypothesis", they say<sup>1</sup>, "but one for which there is no direct experimental proof, that the spindle which is formed at metaphase is a gel of nucleic acid". Later<sup>2</sup> they state, without additional evidence, that "there seems little doubt that nucleic acid is concerned mainly with spindle formation".

Darlington<sup>7</sup> has given reasons for believing that the development of the spindle depends on a reaction between the centromeres of the chromosomes and fibre-forming molecules of a type not usually found within the nuclear membrane. The spindle can therefore develop only after the breakdown of the nuclear membrane. With regard to the nature of these fibre-forming molecules, most workers have contented themselves with the evidence that the spindle, like other cytoplasmic constituents, does not contain thymonucleic acid. On this point the work of Schmidt and others on birefringence is directly significant. Schmidt<sup>8</sup>, Runström<sup>9</sup> and Nakamura<sup>10</sup> have shown that the spindle fibres are positively birefringent with respect to their length, whereas pure sodium thymonucleate fibres are negatively birefringent, thus giving the peculiar optical properties actually found in the chromosomes.

In the light of this evidence, we feel that the position and function of "chromosomin" in the nucleus will have to be determined in relation to the already well-established position and function of thymonucleic acid.

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<sup>1</sup> Stedman, E., and Stedman E., *NATURE*, **152**, 267 (1943).

<sup>2</sup> Stedman, E., and Stedman, E., *NATURE*, **152**, 503 (1943).

<sup>3</sup> Stedman, E., and Stedman, E., *NATURE*, **152**, 557 (1943).

<sup>4</sup> Callan, H. G., *NATURE*, **152**, 503 (1943).

<sup>5</sup> Claude, A., and Potter, J. S., *J. Expt. Med.*, **77**, 345 (1943).

<sup>6</sup> Choudhuri, H. C., *NATURE*, **152**, 475 (1943).

<sup>7</sup> Darlington, C. D., "Recent Advances in Cytology" (London, 1937).

<sup>8</sup> Schmidt, W. J., "Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma" (Berlin, 1937).

<sup>9</sup> Runström, J., *Protoplasma*, **5**, 201 (1929).

<sup>10</sup> Nakamura, T., *Cytologia Fujii Jub. Vol.*, 482 (1937).

Review copy

# CHIASMA INTERFERENCE IN MOSQUITOES

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(With Five Text-figures)

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## 1. INTRODUCTION

Muller (1916) first demonstrated interference between cross-overs in *Drosophila*. In 1931 Haldane, by consideration of the variance of chiasma frequency data from a number of plants, showed that interference is also a property of chiasmata. The correspondence between these two discoveries is one among the many arguments for the identity of cross-overs and chiasmata.

Abundant evidence is now to hand which proves the existence of cross-over or chiasma interference within the chromosome arm on one side of the centromere. However, when both chromosome arms separated by the centromere are considered together, most of the evidence, both genetical and cytological, demonstrates independence of the two (Mather, 1936*b*). Two geneticists (Graubard, 1932; Schweitzer, 1935) claimed to have shown positive interference across the centromere in chromosome II of *D. melanogaster*, but Stevens (1936) has shown that their conclusions were fallacious, having been based on inconsistent measures of coincidence.

Positive correlation, i.e. negative interference, between cross-overs in regions near to the centromere has been observed in a number of genetical experiments concerned with the IIIrd chromosome of *D. melanogaster* (summarized by Kikkawa, 1935). Newcombe (1941) analysed the data of Lindegren & Lindegren (1937) from *Neurospora* and found similar positive correlation of cross-overs on either side of the centromere in this fungus.

Pätau (1941) has drawn attention, however, to a result obtained by Gowen (1919) in a study of crossing-over in chromosome III of *Drosophila melanogaster*. Pätau's calculations of coincidence by Stevens's method and based on Gowen's data show that whereas between the sections *se-D* and *ss-e*, which are separated by a region containing the centromere, interference is negative, between *se-D* and *e-ro*, which are separated by a larger region containing the centromere, interference is positive. This appears to be the

only example from genetical experiment of positive cross-over interference developed across the centromere.

The evidence from cytology has been in line with that from genetics. Thus Bennett (1938) was able to show independence of the chromosome arms in *Fritillaria chitralensis*, and Barber (1941) found the same condition to hold for *Uvularia perfoliata*. If, on the other hand, any correlation in the formation of chiasmata on either side of the centromere were demonstrable, the correlation was positive (Callan, 1940, in *Anilocra*; Newcombe, 1941, in *Trillium*).

Pätau (1941) has now clearly demonstrated that the opposite condition, i.e. negative correlation between chiasmata formed on either side of the centromere, occurs in two species of the Diptera Nematocera. He states in the summary of his paper that 'bei *Culex pipiens* wahrscheinlich und bei *Dicranomyia trinotata* mit sehr hoher statistischer Sicherung positive Interferenz über das Centromer existiert'. We have been able to confirm and extend Pätau's conclusion as regards chiasma interference across the centromere in *Culex*.

## 2. MATERIAL AND TECHNIQUE

Larvae and pupae of two species of mosquito were collected from stagnant fresh water at the Stazione Zoologica, Naples, during the summer months of 1945. The two species were identified as *Culex pipiens* L. and *Theobaldia (Allotheobaldia) longiareolata* Macq.

The testes from late larval or pupal stages were dissected out under a binocular microscope, transferred for a few moments to a fixative consisting of three parts absolute alcohol:one part glacial acetic acid, and then stained in bulk on a slide in iron acetocarmine. After staining for about 5 min., the testes were squashed out lightly under a coverslip and examined direct. In the case of *Theobaldia* the preparations were made permanent according to the method of La Cour (1937). With *Culex* this was not successful, however, since much of the material failed to stick either to coverslip or slide at separation, and contraction and distortion occurred in that portion remaining attached. *Culex* material was thus examined fresh and thrown away after use. This unfortunate characteristic of the neapolitan *Culex* was not present in *C. pipiens* obtained from a London source and examined by one of us in 1940.

## 3. *CULEX PIPIENS* L. CHIASMA INTERFERENCE WITHIN THE BIVALENT

The diploid complement of *Culex* consists of six chromosomes, all of which possess median centromeres (Stevens, 1910; Moffett, 1936). Two of the chromosome pairs (*M*) are of equal length, whilst the third pair (*m*) is somewhat shorter, the length relationship between them at mitotic metaphase being approximately as three to two. Two spermatogonial metaphases and three second meiotic metaphases are shown in Figs. 1 and 2. The somatic pairing characteristic of dipteran cells was observed in all mitoses. No sex chromosomes are distinguishable.

Six typical first meiotic metaphases are shown in Fig. 3. Evidently the rule is for no chiasma or one chiasma to be formed within a single arm pair. Very occasionally two chiasmata are formed within one arm pair: the frequency of this occurrence varies from individual to individual, the average being 1.74% of the total bivalents observed. There is perhaps a tendency in scoring to exaggerate the numbers of such exceptional bivalents: in normal bivalents the chiasma counts can be relied on as entirely accurate, the con-

The total  $\chi^2$  of 57.889, with 18 degrees of freedom, indicates that the chance of this divergence from expectation being due to sampling errors is very small (at  $P=0.001$ ,  $n=18$ ,  $\chi^2=42.31$ ).

Table 1. *Culex pipiens*

Specimen no.	No. of cells observed	Mean chiasma frequency per cell	Standard error $\pm$	Metaphase terminalization coefficient	Exceptional bivalents %	Index of interference based on 'M' + 'm' data	Index of interference based on 'M' data alone
1	36	3.139	0.107	0.556	0.90	0.808	0.827
9	92	3.500	0.070	0.395	1.05	0.675	0.648
10	111	3.523	0.064	0.557	1.16	0.671	0.712
6	46	3.587	0.101	0.500	3.27	0.529	0.414
8	37	3.595	0.106	0.511	0.00	0.669	0.626
7	38	3.763	0.116	0.548	2.44	0.600	0.534
4	151	3.821	0.064	0.547	1.48	0.577	0.594
2	71	3.873	0.104	0.601	1.78	0.447	0.426
3	53	4.038	0.132	0.533	3.39	0.457	0.476
5	19	4.105	0.202	0.579	4.54	0.465	0.414

Table 2. *Culex pipiens*

(Figures of expectation are given in brackets.)  
No. of chiasmata per cell

Specimen no.	No. of chiasmata per cell					Total cells
	2	3	4	5	6	
1	5 (0.7156)	21 (15.5780)	10 (14.7523)	0 (4.1835)	0 (0.7706)	36
9	1 (1.8287)	51 (39.8105)	34 (37.7004)	5 (10.6911)	1 (1.9694)	92
10	2 (2.2064)	58 (48.0321)	42 (45.4863)	9 (12.8991)	0 (2.3762)	111
6	2 (0.9144)	18 (19.9051)	23 (18.8500)	3 (5.3455)	0 (0.9847)	46
8	0 (0.7355)	18 (16.0107)	16 (15.1621)	3 (4.2997)	0 (0.7921)	37
7	0 (0.7554)	14 (16.4434)	20 (15.5719)	3 (4.4159)	1 (0.8135)	38
4	0 (3.0016)	59 (65.3419)	64 (61.8785)	24 (17.5476)	4 (3.2325)	151
2	1 (1.4113)	25 (30.7225)	31 (29.7187)	10 (8.2506)	4 (1.5198)	71
3	2 (1.0535)	14 (22.9343)	20 (21.7187)	14 (6.1590)	3 (1.1346)	53
5	0 (0.3777)	5 (8.2217)	8 (7.7859)	5 (2.2079)	1 (0.4067)	19
Total cells	13	283	268	76	14	654

Table 3. *Culex pipiens*

Specimen no.		Mean chiasma frequency per cell	Standard error $\pm$	Terminalization coefficient
4	Diplotene	4.064	0.134	0.533
	Metaphase	3.712	0.069	0.547
9	Diplotene	3.556	0.105	0.317
	Metaphase	3.421	0.081	0.395
10	Diplotene	3.476	0.092	0.395
	Metaphase	3.536	0.087	0.557

In specimens 4, 9 and 10 two separate sets of chiasma counts were made for cells in diplotene or diakinesis and for cells at metaphase. Table 3 sets out the mean chiasma frequencies and their standard errors, as observed both before and at metaphase in these three individuals, together with the terminalization coefficients (Gairdner & Darlington, 1931) at the two stages. In specimens 9 and 10 the chiasma frequency differences are not



significant. The difference figure for specimen 4 is on the borderline of significance: however, this is certainly due to our tendency at this stage of the investigation to overcount the number of 'rings' at diplotene. In many bivalents where only one chiasma has occurred there is a stage when the free arms temporarily remain touching at their extremities before pulling entirely apart due to the activation of the centromeres. We were later able to distinguish between this condition and that of a true fully terminalized chiasma: in the former the arm pairs near to the point where they touch lie close together and parallel to one another, whereas in the latter the arm pairs lie extended in line through the terminalized chiasma. It is significant that the higher diplotene chiasma frequency of specimen 4 is not due to an excess in the proportion of bivalents with more than one chiasma in an arm pair: moreover, it would be the first observation of its kind were it found that fully terminalized chiasmata sometimes break their connexion during the passage from diplotene to metaphase. In short we feel confident that in *Culex* the chiasmata visible at metaphase are not less in number than those which originally formed at pachytene.

In all three specimens the terminalization coefficient at metaphase is somewhat higher than that at diplotene. Moffett made a similar observation, and he thus assumed that a slight movement of interstitial chiasmata towards the distal ends of the chromosomes took place between diplotene and metaphase. However, the differences which we have observed are so small that they might equally well be ascribed to the greater ease of resolution between subterminal and terminal chiasmata at diplotene as compared with metaphase.

(a) *The evidence from chiasma number*

For the purpose of argument, let us assume that each chromosome arm of *Culex* has an equal chance of forming a chiasma and also that the two arms of a bivalent separated by the centromere act independently of one another. The chiasma statistics then provide the information necessary for the calculation of a theoretical figure,  $p$ , the chance that one arm pair has of forming one chiasma.  $1-p$ , or  $q$ , is thus the chance of a chiasma failing to form in one arm pair. Then  $p^2$  is the expectation of the formation of two chiasmata, one in each arm,  $2pq$  is the expectation of the formation of only one chiasma, and  $q^2$  is the expectation of chiasma failure in both arms together. In specimen 1, for example, ten bivalents were observed to have formed two chiasmata each (20 chiasmata), ninety-five bivalents formed one chiasma each (95 chiasmata) and five pairs of univalents failed to form chiasmata. A total of 115 chiasmata were thus formed by 110 bivalents, i.e. between 220 arm pairs.  $p$  is thus  $115/220 = 0.5227$  and  $q = 0.4773$ . Table 4 shows the expected and observed proportions (in percentages) of two-chiasmata bivalents, one-chiasma bivalents and univalent pairs in the ten specimens examined. In all of these there is a consistent divergence of the observed from the expected proportions, so clear that a test of significance is unnecessary. The observed proportions of single-chiasma bivalents are always in excess of expectation, whilst univalent pairs and two-chiasmata bivalents show a deficit.

If, therefore, we make the initial assumption that in any one individual of *Culex* each chromosome arm has the same intrinsic chance of forming a chiasma, then it is clear that the arms are not independent of one another and, moreover, the chiasma correlation in the two arms is negative, i.e. there is positive chiasma interference developed across the

centromere. The specimens differ in their degree of divergence from expectation, and it is necessary to arrive at a measure of interference in order to compare one with another.

Suppose that  $a$  = number of bivalents with two chiasmata,  $2b$  = number of bivalents with one chiasma and  $c$  = number of univalent pairs, then the chance of formation of a chiasma in the left (or right) arm in the absence of one in the other is  $p_1 = \frac{b}{b+c}$ , and the chance of formation of a chiasma in the left (or right) arm in the presence of one in the other is  $p_2 = \frac{a}{a+b}$ . We have chosen as *index of interference* the complement of the ratio  $\frac{p_2}{p_1}$  i.e.  $1 - \frac{a(b+c)}{b(a+b)}$ . \* When there is no interference,  $p_1 = p_2$  and the index of interference = 0.

Table 4. *Culex pipiens*

(Figures of expectation are given in brackets.)  
Percentages of bivalent types

Specimen no.	Percentages of bivalent types		
	X/X	X/O	O/O
1	9.09	86.36	4.55
	(27.32)	(49.90)	(22.78)
2	32.58	64.25	3.17
	(41.86)	(45.68)	(12.46)
3	35.08	63.74	1.17
	(44.84)	(44.25)	(10.92)
4	26.55	73.23	0.21
	(39.90)	(46.53)	(13.56)
5	36.51	63.49	0.00
	(46.58)	(43.34)	(10.08)
6	22.97	70.27	6.76
	(33.76)	(48.69)	(17.56)
7	25.00	75.00	0.00
	(39.06)	(46.88)	(14.06)
8	19.81	80.18	0.00
	(35.88)	(48.02)	(16.08)
9	18.08	80.14	1.77
	(33.82)	(48.67)	(17.50)
10	18.77	80.06	1.17
	(34.57)	(48.45)	(16.97)

When interference is complete,  $p_2 = 0$  and the index of interference = 1. The indices of interference for the ten individuals are listed in Table 1.

In general the 'm' chromosomes form fewer two-chiasmata bivalents than do the 'M' chromosomes. We attempted in all cells to distinguish between 'M' and 'm' bivalents. While this is possible in most cases there is a certain initial tendency to exaggerate the number of two-chiasmata 'm' bivalents since such associations often give the general impression of being smaller than those which have formed only one chiasma.

Our deductions as to interference can thus be criticized on the grounds that we make the unjustifiable assumption that the arm pairs of the 'M' and 'm' bivalents have equal chances of chiasma formation. The criticism is valid: however, further reference to Table 1 will show that the indices of interference, whether calculated on pooled 'M' and 'm' data, or based on 'M' data alone, are in all individual cases considerably less than unity: moreover, except in the case of specimen 6, the difference between the two indices calculated for each individual is always less than 0.1 unit. In view of the fact that the 'm'

\* Patau (1941) has used two measures of interference, neither of which is identical with the one arrived at above. Provided the measure is logically derived, however, the precise form of its employment is a matter of convenience only.

bivalents are often difficult to distinguish from the 'M' bivalents, we would place more reliance on the indices calculated on the mixed statistics.

Table 1 shows that mean chiasma frequency per cell and index of interference are inversely correlated, as, indeed, they must be provided univalents are rare. Specimen 6 was abnormal in showing 6.8% univalents (and a higher proportion of these in the 'M' than in the 'm' bivalents, which is also exceptional). There was, furthermore, extensive breakdown of the spindle at first meiotic metaphase in this specimen.

We must, however, now return to consider the initial assumption on which our deduction and analysis of interference is based. Altogether apart from the question of differences between 'M' and 'm' bivalents, we have assumed that cytologically indistinguishable arms separated by the centromere have equal intrinsic chances of taking part in a chiasma. If, on the other hand, one assumes that the arms are independent of one another in the formation of chiasmata, i.e. there is no interference across the centromere, then it is possible to calculate different chances of chiasma formation in the two arms which could give rise to the observed proportions of the various bivalent types. The calculation also involves the assumption that each chromosome of the complement contributes the same inequality.

If  $a$ ,  $2b$  and  $c$  are the numbers of two-chiasmata bivalents, one-chiasma bivalents and univalent pairs respectively, then it can be shown that the 'high' and 'low' chances of chiasma formation ( $p$ ) are given by the two roots of the quadratic equation

$$(a + 2b + c)p^2 - (2a + 2b)p + a = 0,$$

$$p = \frac{a + b \pm \sqrt{(b^2 - ac)}}{a + 2b + c}.$$

Thus in the case of specimen 1, where  $a = 10$ ,  $2b = 95$  and  $c = 5$ , the 'high' chance = 0.9497 and the 'low' chance = 0.0957. The ratio of 'high' to 'low' is 9.92, i.e. one arm has roughly ten times greater prospect of forming a chiasma than has the other. The chance ratios range from 3/1 to 10/1 in the ten specimens examined.

Intrinsic inequalities of chance of these orders of magnitude seem most improbable unless in *Culex* we are dealing with structural heterozygosity of a type and on a scale previously undescribed. Since we have not seen a single bridge and fragment at meiotic anaphases, such a postulate appears unjustified. On the basis of this argument we are thus led to the same conclusion as that arrived at by Patau: minor intrinsic inequalities of chance of chiasma formation may well exist between the chromosome arms of *Culex*; however, chiasma interference across the centromere is probably the main factor which determines the disproportionately large number of chromosomes which have formed chiasmata on one side of the centromere alone.

#### (b) *The evidence from chiasma position*

We have now been able to carry the analysis a step further, thereby clinching the argument. In scoring the chiasmata of *C. pipiens* we scored as 'P' those which occurred within the proximal half of the arm pair and as 'D' those in the distal half. The letter 'O' indicates the absence of a chiasma. Naturally great observational errors are involved, and in any case the absolute figures may have little meaning if there is chiasma movement in this species. The relative proportions of O/P, O/D, P/P, P/D and D/D bivalents, however, throw light on the interference question.

Specimens 2, 4, 9 and 10 were selected as being those where most cells had been analysed. The proportions in which the various kinds of 'M' bivalents were observed are listed in Table 5. Also listed are the proportions to be expected on the assumptions that the two arm pairs of each bivalent have equal pairing chances and behave independently of one another. Both observed and expected percentages were adjusted to omit the figures for *O/O* bivalents (i.e. univalents). In Table 6 the ratios of observed to expected percentages of all the various types of bivalent in the four individuals are listed side by side. Single-chiasma bivalents are, as we already know, in excess of expectation, the excess being greater in the case of *O/P* than in that of *O/D* bivalents. This indicates that a proximal chiasma in one arm is more effective than a distal in suppressing a chiasma in the other arm. Two-chiasmata bivalents show a deficit on expectation: the divergence is greatest in the case of *P/P* bivalents, least in that of *D/D* bivalents, with *P/D* bivalents falling between.

The figures for both one-chiasma and two-chiasmata bivalents thus agree in showing that the power of one chiasma to suppress the formation of another falls off with distance

Table 5. *Culex pipiens*

(Figures of expectation are given in brackets.)  
Percentages of bivalent types

Specimen no.	<i>O/P</i>	<i>O/D</i>	<i>P/P</i>	<i>P/D</i>	<i>D/D</i>
2	13.02 (8.62)	47.95 (38.64)	0.68 (1.75)	10.95 (15.73)	27.39 (35.26)
4	26.45 (15.35)	48.06 (38.93)	0.32 (3.66)	8.39 (18.54)	16.77 (23.51)
9	21.92 (13.18)	56.68 (43.25)	0.00 (2.38)	6.42 (15.59)	14.97 (25.60)
10	20.35 (11.20)	56.64 (44.38)	0.44 (1.80)	3.54 (14.31)	19.03 (28.32)

Table 6. *Culex pipiens*

Ratio of observed/expected frequencies of bivalent types

Specimen no.	<i>O/P</i>	<i>O/D</i>	<i>P/P</i>	<i>P/D</i>	<i>D/D</i>
2	1.510	1.241	0.388	0.696	0.777
4	1.723	1.234	0.087	0.453	0.713
9	1.663	1.311	0.000	0.412	0.585
10	1.817	1.276	0.244	0.247	0.672

across the centromere, just as it does in the more generally known phenomenon where interference is said to be confined within one arm on one side of the centromere.

The data regarding chiasma position can be analysed in another way to demonstrate more precisely the relationship between interference and distance along the chromosome. Thus the proportions of *O/O*, *O/P* and *O/D* bivalents provide the information necessary for the calculation of the chances of formation of *O*, *P* or *D* chiasmata in one arm given no chiasma in the other. If *OO*, *OP* and *OD* stand for the numbers of *O/O*, *O/P* and *O/D* bivalents respectively, then, given no chiasma in one arm,

$$\text{the chance of formation of } O \text{ in the other} = \frac{OO}{OO + \frac{1}{2}OP + \frac{1}{2}OD},$$

$$\text{the chance of formation of } P \text{ in the other} = \frac{\frac{1}{2}OP}{OO + \frac{1}{2}OP + \frac{1}{2}OD},$$

$$\text{the chance of formation of } D \text{ in the other} = \frac{\frac{1}{2}OD}{OO + \frac{1}{2}OP + \frac{1}{2}OD}.$$

Similarly, if  $O/P$ ,  $P/D$  and  $P/P$  are considered together, then, given a proximal chiasma in one arm,

$$\text{the chance of formation of } O \text{ in the other} = \frac{\frac{1}{2}OP}{PP + \frac{1}{2}OP + \frac{1}{2}PD},$$

$$\text{the chance of formation of } P \text{ in the other} = \frac{PP}{PP + \frac{1}{2}OP + \frac{1}{2}PD},$$

$$\text{the chance of formation of } D \text{ in the other} = \frac{\frac{1}{2}PD}{PP + \frac{1}{2}OP + \frac{1}{2}PD}.$$

Finally, if  $O/D$ ,  $P/D$  and  $D/D$  are considered together, then, given a distal chiasma in one arm,

$$\text{the chance of formation of } O \text{ in the other} = \frac{\frac{1}{2}OD}{DD + \frac{1}{2}OD + \frac{1}{2}PD},$$

$$\text{the chance of formation of } P \text{ in the other} = \frac{\frac{1}{2}PD}{DD + \frac{1}{2}OD + \frac{1}{2}PD},$$

$$\text{the chance of formation of } D \text{ in the other} = \frac{DD}{DD + \frac{1}{2}OD + \frac{1}{2}PD}.$$

Table 7 shows the various figures of chance, expressed as percentages, for specimens 2, 4, 9 and 10. Taking specimen 2 as an example and reading the columns vertically, we see

Table 7. *Culex pipiens*

		Specimen no.	Chance of chiasma in other arm		
			O	P	D
Given initial chiasma in one arm	O	2	2.198	20.879	76.923
		4	0.000	35.498	64.502
		9	0.000	27.891	72.109
		10	0.000	26.437	73.563
	P	2	51.351	5.405	43.243
		4	74.546	1.818	23.636
		9	77.359	0.000	22.642
		10	82.143	3.571	14.286
	D	2	42.168	9.639	48.193
		4	53.405	9.319	37.276
		9	60.920	6.897	32.184
		10	57.658	3.604	38.739

Table 8. *Culex pipiens*

		Specimen no.	Chance of chiasma formation in one arm in the presence of a chiasma in the other arm divided by Chance of chiasma formation in one arm in the absence of a chiasma in the other arm	
			P	D
Given initial chiasma in one arm	P	2	0.259	0.562
		4	0.051	0.366
		10	0.135	0.194
	D	9	0.000	0.314
		2	0.462	0.627
		4	0.262	0.578
		10	0.136	0.526
		9	0.247	0.446
		2	0.247	0.446

that the chance of formation of a proximal chiasma in one arm falls from 20.9:9.6:5.4 according as to whether the other arm has formed no chiasma or a distal or a proximal chiasma. The figures in the various cells of this table are reasonably consistent from

specimen to specimen, thus all show the same trends. The reduction in the chance of formation of 'P' or 'D' chiasmata in one arm imposed by 'P' or 'D' chiasmata in the other arm, expressed as ratios of the chances in the absence of a chiasma in the other arm, are measures of interference. They are listed in Table 8. The specimens have been arranged in order of index of interference as computed from the data of chiasma number alone. The seriation of the chance ratios is in tolerably good agreement with this order.

All the evidence thus converges on the view that in *C. pipiens* chiasma number and position are largely determined by chiasma interference, against which the centromere is no barrier.

#### 4. *THEOBALDIA (ALLOTHEOBALDIA) LONGIAREOLATA* MACQ.

The diploid complement of *Theobaldia*, like that of *Culex*, consists of six chromosomes. Two pairs of larger chromosomes are of equal overall length: one of these has median centromeres while in the other the centromeres are very slightly off-median. The third



Fig. 4. *Theobaldia longiareolata*. Three oogonal mitoses.  $\times 3500$ .



Fig. 5. *Theobaldia longiareolata*. Two primary spermatocyte divisions.  $\times 3500$ .

chromosome pair has median centromeres and is shorter than the other pairs, the length relationship between them being approximately as three to two. As in the description of *Culex*, the larger chromosomes will be referred to as 'M' and the smaller as 'm'. Three oogonal mitoses are reproduced in Fig. 4. In Fig. 5 two first meiotic metaphases are shown. Their appearance contrasts markedly with that of the similar stage in *Culex*, since the 'M' bivalents of *Theobaldia* often form two chiasmata and sometimes three within the arm pair.

*Theobaldia* is not so suitable for cytological study as is *Culex*. The configurations of the bivalents are often complex, and unfortunately only a dozen or so cells are to be found at

first meiotic metaphase in any one individual, in contrast to the hundreds of such stages often available in *Culex*.

Table 9 lists the mean chiasma frequency per cell, its standard error and the terminalization coefficient for six individuals. The means at the two extremes of the scale are not significantly different from one another. Thus in order to compare the chiasma statistics of *Theobaldia* with those of *Culex*, the figures for all six individuals have been pooled.

The 'M' bivalents have a mean chiasma frequency of 2.911, standard error  $\pm 0.069$ , whereas the 'm' bivalents have a mean chiasma frequency of 1.953, standard error  $\pm 0.041$ . In contrast to the 'M' bivalents, the 'm' bivalents rarely form more than one chiasma within the arm pair.

Table 9. *Theobaldia longiareolata*

Specimen no.	No. of cells observed	Mean chiasma frequency per cell	Standard error	Terminalization coefficient
1	16	7.438	0.418	0.235
4	9	7.444	0.412	0.254
6	16	7.500	0.408	—
2	9	8.000	0.372	0.208
3	14	8.143	0.433	0.166
5	14	8.143	0.455	0.158

Table 10. *Theobaldia longiareolata*

Bivalent type	Observed	Expected
O/O	0	0.07
O/X	5	3.69
O/XX	2	2.96
O/XXX	0	0.20
X/X	51	47.12
X/XX	69	75.80
X/XXX	3	5.25
XX/XX	33	30.48
XX/XXX	7	4.23
XXX/XXX	0	0.15
Total	170	

Taken separately, neither the 'M' nor the 'm' bivalent statistics provide any evidence of chiasma interference across the centromere. The 'm' bivalents form simple 'rings' almost without exception. The observed and expected figures of the various types of 'M' bivalent association, considered without regard for chiasma position, are listed in Table 10. The figures of expectation are based on the assumptions that the arm pairs have equal intrinsic chances of forming chiasmata and that they behave independently of one another. There is good agreement between observed and expected figures ( $\chi^2=1.148$ ,  $n=3$ ,  $P>0.7$ ).

Unfortunately, the number of cells of *Theobaldia* which were studied is insufficient to provide a comprehensive test of the distribution of chiasma position, but the figures available give no indication of negative correlation between chiasmata on either side of the centromere; in fact, though the deviation is not statistically significant, P/P bivalents are in excess of expectation.

## 5. DISCUSSION

In *Culex* we have clear evidence of chiasma interference across the centromere, whereas in *Theobaldia* there appears to be none. The *Theobaldia* data are admittedly few and incompletely analysed, but we feel sure that even were they more extensive, treatment by

the method applied to *Culex* would fail to give evidence of chiasma interference across the centromere. None of the bivalents of *Theobaldia* have chromosome arms which are distinguishable from one another at meiosis: were it otherwise, a more extensive investigation would have been justified.

*Culex* and *Theobaldia* are closely related organisms, in fact prior to 1902 *Theobaldia* was included in the genus *Culex*. The cytological differences between these two mosquitoes are thus more probably ascribable to quantitative rather than qualitative differences in the laws governing chiasma formation. The logical inference to be drawn from the comparison between them is that chiasma interference is fundamentally the same phenomenon whether it is working within one chromosome arm or across the centromere. *Theobaldia* has evidently a much lower chiasma interference than *Culex*, since its chiasma frequency is roughly twice as great. Thus even were chiasma formation of equal intrinsic likelihood at all points down the length of the chromosome, a more sensitive method of scoring would be required to reveal its action across the centromere. We have, however, good evidence that chiasma formation is not of equal intrinsic likelihood in the various parts of the chromosome (Mather, 1938). This author has designated as the 'differential distance' the mean distance from the centromere at which the most proximal chiasma is formed; and as 'interference distance' the mean distance between adjacent chiasmata in the remainder of the chromosome. Differential and interference distances are often visibly different in length. They seem to be determined by different factors; the older view that the centromere behaves as though it were itself a chiasma in determining the site of formation of other chiasmata appears to be incorrect. In the grasshopper *Mecostethus grossus*, for example, the distance of the proximal chiasma from the centromere seems to be limited quite precisely by the length of heterochromatin adjacent to the centromere. The chiasma falls just outside the heterochromatic region; since the amount of heterochromatin varies between the bivalents of the complement, the differential distance also varies, yet is more or less constant for each individual bivalent (White, 1945; Callan, unpublished).

Pätau has drawn attention to the way in which differential distance and chiasma interference can be expected to interact in determining chiasma distribution and correlation. The region comprised by the differential distance may be one in which chiasma formation is inhibited by some factor such as tardy division of the chromosomes. Symmetrical variation of this distance about the centromere could explain those cases where positive correlation of chiasmata or cross-overs on either side of the centromere has been observed. Proximal localization of pairing could produce the same effect. Moreover, unless chiasma interference is so potent that it can exert an effect not only across the centromere but also across the two differential distances contiguous with it, then clearly interference will be masked by the positive correlation determined by other factors. It is equally clear that we should not expect to find chiasma interference across the centromere in chromosomes where pachytene pairing is incomplete and does not include the centromere regions.

Pätau has also considered the problem of chiasma interference across the centromere in connexion with relational coiling of the chromosomes at pachytene. On Darlington's theory of chiasma formation (Darlington, 1935*b*) the mechanical instability of the chromosomes at the moment of their division or replication is conditioned by the relational coiling between partners. The formation of a chiasma releases the strain imposed by relational coiling. Chiasma interference is then understood as the distance between the



point where the strain was originally released and that where it again rises to a breakage threshold. On Darlington's theory, positive chiasma interference across the centromere can only arise if the direction of relational coiling is the same on either side of the centromere. If, on the other hand, the direction of coiling is opposite in the two arms, then the formation of a chiasma in one arm should increase the chance of formation of another in the other arm. The first-formed chiasma would act as a 'tie', thereby preventing that compensatory unravelling of the relational coiling about the centromere region which would otherwise occur.

Unfortunately, we know little about the direction of relational coiling at pachytene. In most organisms the chromosomes at this stage fix very poorly: moreover, the threads are near to the size limit of resolution and they are generally intertwined in a complex manner. Darlington (1935*a*, 1936*b*) has studied relational coiling at diplotene in *Fritillaria* and *Chorthippus*. He is led to infer that at pachytene there is a marked preponderance of bivalents with opposite directions of coiling in the two arms. These organisms have very large chromosomes in comparison with those of mosquitoes, and we would not be justified in the assumption that what holds for the one is true of the other also. Examination of pachytene in mosquitoes themselves cannot bring any evidence to bear on the question, since the nuclei are small and the chromosomes much intertwined. It is therefore not yet possible to reach any conclusions about the relationship between relational coiling at pachytene and chiasma interference across the centromere.

We have unpublished evidence of chiasma interference across the centromere in a number of other organisms. Thus the chromosomes of the dipteran *Psychoda* sp. almost invariably form a single chiasma in one arm only, though the centromeres are approximately median in position. In the earthworm *Eisenia foetida*, where five of the chromosomes have arms visibly unequal in length, chiasmata can be formed in long or short arms, yet it is most exceptional for them to be formed in both arms of the one bivalent.

The case of *Petunia violacea*, *P. axillaris*, and the hybrid between these two plant species is particularly instructive. Mather (1943*a*) has studied these plants from a genetical standpoint, and he kindly allowed one of us to examine meiosis in his material. Both *P. violacea* and *P. axillaris* have 14 chromosomes, superficially alike and with median centromeres. *P. violacea* has a mean chiasma frequency per bivalent of  $1.09 \pm 0.02$ . It is rare for more than one chiasma to form within an arm pair. The observed proportions of univalent pairs, one-chiasma and two-chiasmata bivalents deviate from the expected proportions (assuming arm equality and independence) in the same way as was found in *Culex*, i.e. bivalents with a chiasma in one arm only are disproportionately common. *Petunia axillaris* has a mean chiasma frequency per bivalent of  $2.06 \pm 0.03$ . The chromosomes of this species frequently form two chiasmata within the arm pair, and the proportions of the various types of bivalent agree closely with expectation in the same way as was found in *Theobaldia*. The hybrid between the two species has a mean chiasma frequency per bivalent of  $1.57 \pm 0.02$ , which is intermediate between the means of the parent forms. The rise in chiasma frequency relative to *Petunia violacea* is consequent on an increase in the number of bivalents which have formed one chiasma in both arm pairs, two chiasmata within a single arm pair being just as infrequent as in *P. violacea*. Since we cannot ascribe this greater regularity of chiasma formation to an increase in structural homozygosity—the opposite must be the case—a fall in the chiasma interference operating across the centromere appears to be the reasonable inference.

It is also possible to explain some observations of Frankel (1940) on various species of *Fritillaria* by assuming chiasma interference across the centromere. In the chromosomes with subterminal centromeres of seven out of eight species, Frankel found an inverse correlation between the number of chiasmata formed in the long arms and the presence or absence of a chiasma in the short arms. Since, however, the chromosomes of these species are very large and show, to a varying extent, incomplete pairing at pachytene, we should not overlook the possibility of this correlation being the result of a pairing phenomenon and not one of interference.

Finally, we must consider the genetic consequences of the chiasma interference which we find in *Culex*. In this organism, interference distance and chromosome length are critically balanced. Each chromosome forms one chiasma, or two when interference permits; the frequency with which two chiasmata are formed varies over a wide range in the specimens studied. Thus our ten specimens show a range of chiasma frequency per bivalent from 1.05 to 1.37, while Moffett found an even greater spread in his six specimens of *Culex*. The same relative variability in the occurrence of one more chiasma than normal will clearly lead to a wider overall range of variation when 'normal' is one chiasma than when it is two or more. We cannot define in precise terms what this exceptional variability in chiasma frequency will give rise to in terms of cross-over variability in specific regions of the chromosome. However, Gilchrist & Haldane (1947) have found an unusually high variability in cross-over frequency between eye colour and sex-determining genes in *C. molestus*, which is what we should also infer from our cytological observations.

#### 6. THE INDEPENDENCE OF THE BIVALENTS OF *CULEX PIFIENS* IN CHIASMA FORMATION

*Culex pipiens* is also a favourable object for the study of chiasma correlation between different bivalents within the same nucleus. Negative correlation between the chiasma or cross-over frequencies of different bivalents has been observed in a number of organisms, though it is by no means universal (Mather, 1936*a et al.*).

If the probabilities of formation of two chiasmata, one chiasma or no chiasma be assumed the same for '*M*' as for '*m*' chromosome pairs in a given individual, and if *a*, *b* and *c* are the numbers of two-chiasmata bivalents, one-chiasma bivalents and univalent pairs respectively, then in the absence of correlation the proportions of cells containing the various combinations of these types of bivalent are given by the terms of the trinomial expansion  $(a+b+c)^3$ . In these calculations, cells containing bivalents with two chiasmata in a single arm pair have been neglected. Table 11 shows the observed and expected numbers of the various cell types in specimens 2, 4, 9 and 10. These have been selected as examples, since in them the greatest numbers of cells were studied. Analyses of  $\chi^2$  show that in all ten specimens there is good agreement between observed and expected figures. (In the four specimens chosen as examples,  $n=3$  in all cases and  $P>0.4$ , 0.9, 0.8 and 0.9 respectively.) We may therefore conclude that there is no correlation between the bivalents of *C. pipiens* in the formation of chiasmata.

The  $\chi^2$  test does not take into account the sign of the deviation of observation from expectation, and thus any slight trend away from randomness cannot be made evident from this test alone. Table 12 shows the sign of the deviation from expectation for certain cell types in the ten individuals. Combinations involving univalents give unreliable figures, since the proportions of these cell types are always low in value. The sign of the

deviations of the remaining four combinations, which appears to be at random, shows clearly that: (1) there is no competition between bivalents within the same nucleus, and (2) high and low chiasma frequencies are not functions of the nuclei as units, i.e. the 'time-limit' to pairing (Darlington, 1936*a*, 1940), plays no part in the determination of the number of chiasmata in the nucleus of *C. pipiens*.

Table 11. *Culex pipiens*  
(Figures of expectation are given in brackets.)

Cell type	Specimen no.			
	2	4	9	10
X/X X/X X/X	4 (2.41)	4 (3.10)	1 (0.54)	0 (0.68)
X/X X/X X/O	10 (14.48)	24 (24.64)	5 (7.28)	9 (8.99)
X/X X/X O/O	3 (0.63)	0 (0.00)	0 (0.13)	0 (0.13)
X/X X/O X/O	28 (28.98)	64 (65.51)	34 (32.34)	41 (39.49)
X/X X/O O/O	3 (2.52)	0 (0.00)	3 (1.17)	2 (1.18)
X/O X/O X/O	22 (19.32)	59 (57.85)	48 (47.87)	57 (57.86)
X/X O/O O/O	0 (0.06)	0 (0.00)	0 (0.01)	0 (0.01)
X/O X/O O/O	1 (2.52)	0 (0.00)	1 (2.59)	2 (2.58)
X/O O/O O/O	0 (0.10)	0 (0.00)	0 (0.05)	0 (0.03)
O/O O/O O/O	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Totals	71	151	92	111

Table 12. *Culex pipiens*  
Sign of deviation of observed from expected frequencies

Cell type	Specimen no.										Total +	Total -
	1	2	3	4	5	6	7	8	9	10		
X/X X/X X/X	-	+	+	+	+	-	+	-	+	-	6	4
X/X X/X X/O	-	-	+	-	+	-	-	-	-	-	2	8
X/X X/O X/O	+	-	-	-	-	+	+	+	+	+	6	4
X/O X/O X/O	-	+	+	+	+	+	-	-	+	-	6	4

7. SUMMARY

1. Analysis of chiasma distribution in the bivalents of the mosquito *Culex pipiens* shows that chiasma interference acts across the centromere in this organism. The strength of the interference varies from specimen to specimen.
2. This interference diminishes as the distance from the chiasma increases.
3. In the mosquito *Theobaldia (Allotheobaldia) longiareolata* chiasma interference is much weaker than it is in *Culex*. Its action cannot be detected across the centromere.
4. Comparison of the two mosquitoes leads to the inference that chiasma interference which crosses the centromere is the same phenomenon as chiasma or cross-over interference which is generally held to be confined within one arm on one side of the centromere.
5. There is no correlation between the chiasmata formed by different bivalents within the same cell of *C. pipiens*.

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# Alcune proprietà fisiche della membrana nucleare

ESTRATTO DA «LA RICERCA SCIENTIFICA»

Anno 18° - 1948

*Supplemento a cura del Centro di studio per la Biologia*

CONSIGLIO NAZIONALE DELLE RICERCHE  
ROMA

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### Ribose Nucleic Acid in the *Drosophila* Egg

Caspersson and Schultz<sup>1,2</sup> have claimed that the presence of an extra Y-chromosome in the female *Drosophila* is correlated with an abnormally high concentration of ribose nucleic acid or allied substances containing the pyrimidine ring in the cytoplasm of oocytes. Their graph, based on quantitative determinations of ultra-violet light absorption, indicates that XXY oocytes contain about 40 per cent more ribose nucleic acid than do XX oocytes. If confirmed, this claim would be evidence for the control exercised by heterochromatin over the synthesis of ribose nucleic acid: and since this substance seems to be closely associated with protein synthesis, the argument might be extended to include a specific connexion between heterochromatin and growth-rate determination.

Brachet<sup>3</sup> has recently described a simple micro-chemical method whereby ribose nucleic acid may be estimated quantitatively. The tissue under examination is hydrolysed and distilled in a current of steam. Furfural, the hydrolysis product of ribose sugar which appears in the distillate, is assayed colorimetrically, making use of the coloured compound which it forms with aniline and acetic acid.

Caspersson and Schultz's contention is not directly open to chemical proof since the collection of a homogeneous batch of *Drosophila* oocytes is impracticable. However, freshly laid eggs can be collected in quantity and these assayed for ribose content.

Such eggs were collected over hourly intervals from the matings XX × XYY and XXY × XY. They were dechorionated *en masse* and fixed in a mixture consisting of equal parts acetone and 10 per cent trichloro-acetic acid. After prolonged extraction in changes of fixative, the eggs were desiccated at 100° C., extracted with hot ether to remove neutral fat, hydrolysed and distilled. Furfural yields gave the following ribose nucleic acid assays:

	Weight of ether-extracted eggs (mgm.)	Yield of furfural (γ)	Estimated ribose nucleic acid (mgm. per gm.)
Eggs from XX females	5.9	11.3	6.48
	6.5	13.1	6.82
	6.6	13.5	6.94
	6.6	11.6	6.00
	7.0	13.3	6.45
	Average		6.54
Eggs from XXY females	4.9	9.6	6.61
	6.5	11.7	6.11
	6.7	12.7	6.41
	7.1	13.1	6.25
	8.7	19.0	7.39
	Average		6.55

From these figures it is evident that there is no significant difference in the ribose nucleic acid content of newly laid eggs deriving from XX and XXY parent flies. In keeping with this result it should be further mentioned that in other experiments, to be described in detail elsewhere, no difference either in developmental-rate or in oxygen consumption was found to exist between these two types of eggs.

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<sup>1</sup> Caspersson, T., and Schultz, J., *Nature*, 142, 294 (1938).

<sup>2</sup> Schultz, J., and Caspersson, T., *Archiv. Exp. Zellf.*, 22, 650 (1939).

<sup>3</sup> Brachet, J., *Enzymologia*, 10, 87 (1941).



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Cleavage rate, oxygen consumption and ribose nucleic  
acid content of sea urchin eggs

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## CLEAVAGE RATE, OXYGEN CONSUMPTION AND RIBOSE NUCLEIC ACID CONTENT OF SEA URCHIN EGGS

by

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### I. INTRODUCTION

At a given temperature the cleavage rates of the eggs of various sea urchin species may differ widely from one another. The nature of the determination of these differences in cleavage rate is not only of general interest but has also practical importance when it is recalled that genetically determined growth rate differences within a single species are reflected in the cleavage rates of the embryonic cells (c.f. PAINTER<sup>1</sup>).

A number of investigators have studied the cleavage rate of sea urchins' eggs, this material offering many special facilities for experimental attack. The early work of DELAGE, GODLEWSKI, NEWMAN, TENNENT and others, which gave results conflicting in many particulars, has been reviewed by MOORE<sup>2</sup>, and the reader is referred to his account.

One line of experiment was the study, within a species, of the relative contributions made by egg cytoplasm, egg nucleus and sperm nucleus to the determination of cleavage rate.

DELAGE<sup>3</sup> cut virgin eggs of *Strongylocentrotus lividus* into nucleated and non-nucleated fragments: when such fragments were subsequently fertilized, the diploid fragment was found to cleave somewhat more slowly than whole egg controls, and the haploid fragment still more slowly.

WHITAKER<sup>4</sup>, working with eggs of the starfish *Patiria miniata*, repeated DELAGE's experiment and confirmed his results. He suggested, however, that the effect might be due to inequalities of yolk distribution in nucleated and non-nucleated fragments rather than directly associated with the nuclear differences. TENNENT, TAYLOR, AND WHITAKER<sup>5</sup> also repeated DELAGE's experiments. In this case the material consisted of eggs of *Lytechinus variegatus*, and once more DELAGE's results were confirmed.

WHITAKER<sup>7</sup> went on to study the rate of cleavage of egg fragments of *Arbacia* which, prior to fragmentation, had been stratified by centrifugation. The cuts were made so as to sever the "light" end of the egg, with nucleus, from the "heavy" anucleate end. The differential distribution of various cytoplasmic materials was found to make no difference to cleavage rate, the centrifuged fragments cleaving at exactly the same rates as comparable non-centrifuged fragments. However, the delay of haploid over diploid fragments was once more confirmed (in this case diploid fragments were found to cleave slightly faster than whole egg controls), and WHITAKER concluded that the nuclear/cytoplasmic ratio is a determining factor in cleavage rate. However, the differ-

ences of rate observed by WHITAKER were in fact very small indeed (of the order of 6 min in 50), and later evidence, which we shall now consider, indicates that his conclusions were not justified.

MOORE<sup>2</sup> has made a very elegant experimental analysis which has exposed the roots of the problem. Advantage was taken of the fact, already mentioned, that the eggs of different species of sea urchins cleave at different rates. The eggs of *Strongylocentrotus franciscanus* at 20° C take approximately 95 min to pass from fertilization to the first cleavage, and succeeding divisions take about 47 min each. The eggs of *Dendraster excentricus* divide nearly twice as rapidly, the first cleavage occurring after 57 min, and the interval between subsequent divisions being approximately 28 min. When MOORE crossfertilized these two species he found that the cleavage rate of the hybrids was precisely that characteristic of the egg and independent of the sperm. This experiment fails to differentiate between the contribution of the egg cytoplasm and egg nucleus. To answer this question, MOORE cut unfertilized eggs into pieces and then made cross-fertilizations. All such fragments, with or without the egg nucleus, were found to cleave at the rate characteristic of the original eggs. These experiments show not only that it is the egg cytoplasm which determines cleavage rate, but also that this rate is, at least within certain limits, independent of egg size. MOORE interprets WHITAKER's results as being ascribable to slight inequalities in the distribution of some critical cytoplasmic material between haploid and diploid egg fragments.

The question which next presents itself is whether, by means of experiment, a factor determining cleavage rate can actually be located within the egg cytoplasm. Such a substance had already been envisaged by LOEB AND CHAMBERLAIN<sup>6</sup> in their attempt to provide a physico-chemical explanation for inter-egg variability of cleavage rate within a single species. The centrifuge experiments of E. B. HARVEY<sup>8, 9</sup> provide relevant information. Under the action of centrifugal force *Arbacia punctulata* eggs stratify into five layers: oil, clear layer, granular layer (the granules being identified as mitochondria), yolk layer and pigment. The oil cap is at the centripetal, the pigment at the centrifugal end of the egg, while the nucleus lies in the clear layer. With stronger centrifuging the stratified eggs may be pulled apart into "lighter" and "heavier" half-eggs, the lighter half containing oil, nucleus, clear layer and part of the granular layer, the heavier half containing the rest of the granular layer together with yolk and pigment.

The light half-eggs, when fertilized, cleave at approximately the same rate as whole eggs, though occasionally the rate is perceptibly higher. The heavy half-eggs, when fertilized, undergo cycles of nuclear division which are somewhat delayed as compared with whole eggs. These nuclear divisions are not at first accompanied by cytoplasmic cleavage; however later the cytoplasm splits up between the nuclei. HARVEY recentrifuged the light half-eggs, which then separate into quarter-eggs, the centripetal quarter-egg containing oil, nucleus and part of the clear layer, while the centrifugal quarter-egg contains the rest of the clear plus the granular layer. When these quarter-eggs are fertilized the nucleated quarter cleaves exceedingly slowly: the granular quarter, on the other hand, cleaves at nearly the same rate as whole eggs. HARVEY<sup>9</sup> found the same rules to hold for centrifuged eggs of *Arbacia lixula* and *Sphaerechinus granularis*. The situation is, however, somewhat different in two other species *Paracentrotus lividus* and *Psammechinus microtuberculatus*, which do not stratify in the same order as do *Arbacia* and *Sphaerechinus*.

MOORE<sup>10</sup> applied HARVEY's centrifuge technique to the eggs of *Dendraster excen-*

*tricus*. The eggs were stratified and pulled into dumb-bell shape but not completely split into fragments. They were then activated parthenogenetically. In these stratified eggs the anucleate centrifugal end always cleaved in advance of the nucleate centripetal end, while in some egg batches so treated, no cleavage of the centripetal end took place at all. Topographically speaking, MOORE's results are the antithesis of HARVEY's results with *Arbacia* and *Sphaerechinus*, but this may well be due to differences in the order of stratification of the various cytoplasmic components. On the basis of HARVEY's results and his own, MOORE concludes that under the action of centrifugal force a "cleavage-substance" can be differentially distributed through the egg cytoplasm and that the rates of cleavage of the various egg regions or fragments are then determined by the relative concentrations of this substance.

However, one point which emerges from the work of HARVEY AND MOORE has been, in my opinion, somewhat overlooked by the latter author. If the word "cleavage-substance" is used to define some stratifiable material of the egg cytoplasm whose concentration is the limiting factor determining normal cleavage rate, then it should be possible not only to decrease its concentration, thereby decreasing the rate, but also to increase its concentration, in which case a faster rate should result. Cleavage rate can undoubtedly be reduced by stratification: but at the same time there is no experimental evidence for a clear cut and striking increase in cleavage rate in any way comparable to the interspecific differences which exist in nature. In the absence of this evidence the reduction of cleavage rate observed in egg fragments and regions produced by centrifugation is better envisaged as the imposition of a new limiting factor rather than the differential distribution of a factor limiting the normal cleavage rate in the entire egg. I therefore suggest that the use of the word "cleavage substance" be discontinued or at least reserved until such time as a normal limiting factor is discovered.

There remains another line of work which bears directly on cleavage rate determination and which we must consider before turning to the experimental results reported in this paper. HÖRSTADIUS<sup>11</sup> has shown that the winter and summer eggs of *Paracentrotus lividus* cleave at different rates when exposed to the same temperature. At 13° C the winter eggs cleave faster than the summer eggs. At 26° C the summer eggs cleave faster than the winter eggs. This phenomenon, which may be best described as acclimatization to the normal environmental temperature, has also been studied by FOX<sup>12, 13</sup> from a somewhat different point of view. FOX determined the cleavages rates of closely related species of *Psammechinus* and the same species of *Paracentrotus* from different localities. He found that at a given temperature (ca. 20° C) the material deriving from colder waters cleaved more rapidly than that deriving from warmer waters.

It will thus be clear that although there are specific differences in cleavage rate, these rates are not rigidly controlled and may undergo adaptation. HÖRSTADIUS has suggested that this adaptation involves alterations in the dispersion of the protoplasmic colloids.

## II. CLEAVAGE RATES OF THE NEAPOLITAN SEA URCHIN SPECIES

*Psammechinus microtuberculatus* (Blainv.), *Sphaerechinus granularis* (Lam.), *Arbacia lixula* (L.) and *Paracentrotus lividus* (Lam.) are four species of sea urchins which occur commonly in the Gulf of Naples. For the purposes of the experiments to be described in this paper, these species were used during the months of March and April 1947.

TABLE I

Species	Individual	Time in minutes between:		
		fertilization and first cleavage	first and second cleavage	second and third cleavage
<i>Psammechinus microtuberculatus</i> (Blainv.)	A	58	38	34
		58	37	35
	B	63	37	33
		62	37	34
	C	63	37	36
62		39	37	
Averages	61.0	37.5	34.8	
<i>Paracentrotus lividus</i> . . . . . (Lam.)	A	74	43	43
		74	43	44
	B	79	43	45
		79	43	45
	C	76	42	45
		77	43	—
D	74	43	44	
	75	42	44	
Averages	76.0	42.7	44.3	
<i>Arbacia lixula</i> . . . . . (L.)	A	97	54	52
		100	53	55
	B	95	52	57
		97	52	58
	C	102	53	55
		105	57	54
D	99	56	55	
	100	56	55	
Averages	99.4	54.1	55.1	
<i>Sphaerechinus granularis</i> . . . . . (Lam.)	A	103	57	56
		101	57	58
	B	99	59	54
		100	58	52
	C	99	58	57
		101	57	55
Averages	100.5	57.7	55.3	

Eggs and sperm were collected by clipping away the oral half of adult specimens and inverting the aboral half over bowls containing filtered sea water. This method of egg collection is preferable to the excision of whole ovaries, as the egg batches so obtained contain hardly any immature oocytes.

The egg batches were repeatedly rinsed with large quantities of clean sea water, and trial inseminations were made about one hour after shedding. Occasionally, for reasons which are not apparent, seemingly mature eggs cannot be fertilized. Such egg batches were rejected. When trials showed that effectively 100% fertilization followed insemination, small quantities of eggs from the same parent batch were transferred to 100 ml Erlenmeyer flasks containing about 40–50 ml of clean sea water: the flasks were then placed in a water bath maintaining a temperature of  $18^{\circ} \pm 0.2^{\circ}$  C. The temperature of the circulating sea water of the laboratory was at this period about  $13^{\circ}$  C. After equilibrating for half an hour, small quantities of sperm were pipetted into the flasks, and the contents momentarily agitated to ensure even distribution of sperm and eggs.

The rate of development of the fertilized eggs was followed at regular interval by pipetting out a small sample and examining it under the microscope. In the conditions of the experiment outlined above, the rate of development is remarkably regular; no attempt was therefore made to treat the material statistically. The time of the first cleavage was taken as being that where 50% of the eggs in any one sample showed a well-marked cytoplasmic furrow, the remaining 50% being uncleaved. A similar criterion was used for the second and third cleavage stages. In Table I the time intervals between fertilization and the first, second and third cleavages are tabulated to the nearest minute. Any one entry would require the figures  $\pm 2$  to 3 min in order to include practically all of the slowest and fastest cleaving eggs from the batch in question. The figures show that *Psammechinus* is the fastest cleaving species, *Arbacia* and *Sphaerechinus* cleave most slowly and at about the same rate as one another, while *Paracentrotus* falls between the two extremes.

In a short series of subsidiary experiments, *Psammechinus* at  $13^{\circ}$  C was found to cleave at the same rate as *Arbacia* and *Sphaerechinus* at  $18^{\circ}$  C. However, the converse experiment was not possible:  $28^{\circ}$  C proved to be the upper limit of temperature for *Sphaerechinus* egg development, and at no temperature below this limit does *Sphaerechinus* cleave as rapidly as does *Psammechinus* at  $18^{\circ}$  C.

### III. OXYGEN CONSUMPTION DURING CLEAVAGE

GRAY<sup>14</sup> has stated that the rate of cell division during segmentation of the egg of *Echinus miliaris* bears no obvious relationship to the rate of metabolism during this process. Thus, though the early cleavage divisions occur after equal intervals of time, the cleavage rate being therefore constant, the rate of oxygen consumption slowly rises as more and more reserve material is incorporated into the respiring protoplasm.

However, despite the absence of a direct correlation between cleavage and metabolic rate, we are bound to admit that the two processes are very intimately related to some common denominator. This conclusion derives from the experiments of EPHRUSI<sup>15</sup> and TYLER<sup>17</sup> who studied the oxygen consumption and developmental rates of various echinoderm eggs at different temperatures. These authors found that the total quantity of oxygen consumed in reaching a given developmental stage is the same at different temperatures. In other words, the temperature coefficients of cleavage and respiration

are identical, and the two processes cannot be dissociated by varying the culture temperature, provided that this remains within the physiological range.

The following experiment was designed in order to determine whether the relationship between cleavage and metabolic rate within a species established by EPHRUSSI AND TYLER can be extended to cover related species whose cleavage rates differ.

Eggs from a number of females of one of the four sea urchin species were collected. Samples were tested for maturity by trial inseminations, and those eggs batches which passed the test were pooled together, washed in filtered sea water and fertilized in bulk. The fertilized eggs were then thoroughly washed four or five times with fresh sea water and finally concentrated in about 40 ml volume. The concentration was checked roughly by centrifuging a small sample.

The final egg suspension was thoroughly mixed and pipetted, 3 ml at a time, into five WARBURG respirometer vessels. Aliquot volumes of the same suspension were pipetted into three centrifuge tubes and three KJELDAHL flasks.

The central chambers of the WARBURG respirometer vessels were fitted with filter-paper rolls wetted with 6 drops of 5 N potassium hydroxide, and the vessels attached to their respective manometers. They were then placed on rocking racks in a thermostatically-controlled water bath at 18° C and rocked gently during the course of the observations. The first manometer readings were taken two hours after the time of fertilization and continued at ten minute intervals for a further two and a half hours. At the end of each run the eggs were checked for regularity of development. Out of over twenty separate determinations of oxygen consumption, two were rejected on the score of developmental irregularities.

The manometric readings were plotted graphically, after conversion to volumes at N.T.P., as total oxygen consumption against time. The curves so obtained (cf. GRAY<sup>17</sup>) are effectively linear over the time range 2-4 ½ hours after fertilization and were treated as such.

Aliquot volumes of egg suspension pipetted into centrifuge tubes were to serve for dry weight determinations. Since the eggs of sea urchins are surrounded by a mucilaginous jelly-coat which otherwise makes a considerable "dead weight" contribution to dry weight, the jelly-coats were removed by suspending the eggs for fifteen minutes in 0.52 M sodium chloride solution. This treatment, coupled with mechanical agitation, is generally adequate. The stages in the removal of the jelly-coat can be readily observed under the microscope if a small quantity of Indian ink be added to the fluid in which the eggs are lying. I am indebted to Dr. MONROY, of the Stazione Zoologica, for details of this useful technique (cf. MONROY AND RUFFO<sup>18</sup>).

The eggs, freed from their jelly-coats, were again suspended in sea water, and then strongly centrifuged. The supernatant water was removed at the pump and all but the last traces absorbed at the sides of the centrifuge tubes by filter paper. The eggs and tubes were then dried to a constant weight in an oven at 110° C. Owing to the impossibility of removing all sea water before drying the material, the dry weight determinations are slight overestimates of the true dry weight of eggs. However, the salt error involved has been calculated to represent less than 5% of these determinations.

The aliquot volumes of egg suspension in KJELDAHL flasks were also treated so as to remove the jelly-coats from the eggs. Total nitrogen determinations were subsequently made by a micro-KJELDAHL method.

The results of this experiment are given in detail in Table II. It will be seen that

TABLE II

Species	Oxygen consumption per hour ( $\mu$ l)	Aliquot dry weight determinations (mg)	Oxygen consumption per hour per 100 mg dry weight ( $\mu$ l)	Aliquot total nitrogen determinations (mg)	Oxygen consumption per hour per 1 mg total nitrogen ( $\mu$ l)
<i>Psammochinus microtuberculatus</i> (Blainv.)	2.50	44		2.59	
	2.66	46		2.95	
	2.54	50		2.98	
	2.79	52			
	2.37				
Averages	2.57	48	5.35	2.84	0.90
<i>Paracentrotus lividus</i> (Lam.)	3.13	66		4.58	
	3.18	71		4.88	
	3.51	75			
	3.45				
	3.16				
Averages	3.29	71	4.63	4.73	0.70
<i>Arbacia lixula</i> (L.)	3.55	94		6.13	
	3.34	94		6.55	
	3.80	96		6.61	
	3.37	96			
Averages	3.51	95	3.70	6.43	0.55
<i>Sphaerechinus granularis</i> (Lam.)	4.11	94		6.40	
	4.69	101		6.52	
	4.49	102			
	4.43				
Averages	4.43	99	4.47	6.46	0.69

TABLE III

Species	Average time interval between successive cleavages (min)	Oxygen consumption per hour per 100 mg dry weight ( $\mu$ l)	Oxygen consumption between successive cleavages per 100 mg dry weight ( $\mu$ l)
<i>Psammochinus</i> (18° C)	36.1	5.35	3.22
<i>Psammochinus</i> (13° C)	60.0	3.18	3.18
<i>Paracentrotus</i> (18° C)	43.2	4.63	3.34
<i>Arbacia</i> (18° C)	54.6	3.70	3.37
<i>Sphaerechinus</i> (18° C)	56.5	4.45	4.19



*Psammechinus* respire most rapidly of the four species, *Arbacia* most slowly, while *Paracentrotus* and *Sphaerechinus* lie between the two extremes and have equal rates of respiration.

In Table III the cleavage and respiration rates of the four species are set beside one another together with the computed quantities of oxygen consumed between two successive cleavages. The data from a single respiration determination for *Psammechinus* at 13° C is also included. (At this temperature *Psammechinus* cleaves at the same rate as do *Arbacia* and *Sphaerechinus* at 18° C). While *Psammechinus*, *Paracentrotus* and *Arbacia* absorb comparable amounts of oxygen between successive cleavages per unit of dry weight, *Sphaerechinus* is out of line in having a considerably higher oxygen requirement.

The exceptional behaviour of *Sphaerechinus* bears out GRAY's contention that there is no direct and simple correlation between metabolic and cleavage rates. It may be that this species possesses proportionately less non-respiring reserve materials. Nevertheless, the identity in oxygen requirement of the other three species when performing a comparable act of development should not be overlooked since it may indicate that, other things being equal, the metabolic cost of cleavage is proportional to its rate.

#### IV. THE RIBOSE NUCLEIC ACID CONTENT OF SEA URCHIN EGGS

On the basis of HARVEY's experiments with half- and quarter-eggs produced by centrifugal force, MOORE has argued that the mitochondrial granules may represent the "segmentation stuff" of LOEB AND CHAMBERLAIN, whose concentration in the cytoplasm determines cleavage rate. In the introduction to this present paper the logic of MOORE's argument has been questioned: however, since ribose nucleic acid has been shown by BRACHET and by CASPERSSON to be a characteristic component of rapidly dividing tissues, (see BRACHET<sup>19</sup>) and since CLAUDE<sup>20</sup> has put forward the view that the granules rich in ribose nucleic acid are, in fact, the mitochondria of cytologists, I thought it of some interest to determine the relative concentrations of ribose nucleic acid in the virgin eggs of the four neapolitan sea urchin species.

The determinations were made according to the method of BRACHET<sup>22</sup>. Mature virgin eggs were obtained from a number of females. The eggs were thoroughly washed in clean sea-water, and the jelly-coats removed by the method already described. The eggs were then washed once more, concentrated into a small volume of sea water and fixed in a 10% solution of trichloroacetic acid. After prolonged extraction in three changes of this solution the eggs were pipetted on to weighed fragments of coverslips and dried out to constant weight at 110° C. Neutral fats were then removed by extraction in hot ether, and the resultant material dried and weighed.

The weighed samples were then hydrolysed and steam distilled in a sulphuric acid-zinc sulphate-potassium sulphate mixture, as described by BRACHET: the furfural content of the resulting distillate was subsequently determined by means of a PULFRICH photometer, use being made of the coloured product formed between furfural, aniline and acetic acid. Among the various sources of error involved in this technique, the following are noteworthy:

a. Jelly-coats must be completely removed before fixation of the eggs, not only because they contribute to the figures of dry weight, but also because they generate furfural on hydrolysis.

b. Prolonged extraction in trichloro-acetic acid is necessary to remove soluble sugars and glycogen which would otherwise also yield furfural.

c. Failure to extract neutral fat leads to turbidity in the distillate and hence to inaccuracies in the colorimeter readings.

The results of the determinations are given in detail in Table IV. (For the calculation of ribose nucleic acid content, 1303 parts by weight of this substance are taken as yielding 384 parts by weight of furfural).

From these figures it is evident that the content of ribose nucleic acid cannot be the sole or even the overriding determinant of the cleavage interval. This does not exclude the possibility that it plays a role in the whole physiological system on which cleavage depends, but for the solution of this question, interspecific comparisons are not an adequate method.

TABLE IV

Species	Dry weight of ether extracted virgin eggs (mg)	Yield of furfural (%)	Estimated ribose nucleic acid (mg per g)
<i>Psammechinus microtuberculatus</i> (Blainv.)	51.4	19.6	1.30
	41.5	19.2	1.57
	50.1	21.2	1.44
	39.1	16.6	1.44
	37.2	15.1	1.37
			Average 1.42
<i>Paracentrotus lividus</i> (Lam.)	39.6	11.0	0.94
	39.1	9.0	0.78
	38.4	8.5	0.76
	39.7	11.1	0.95
	37.7	9.6	0.86
			Average 0.86
<i>Arbacia lixula</i> (L.)	39.4	11.2	0.97
	40.4	12.4	1.04
	36.5	10.9	1.01
	41.8	12.1	0.98
			Average 1.00
<i>Sphaerechinus granularis</i> (Lam.)	41.0	16.4	1.36
	39.8	14.0	1.20
	51.9	21.2	1.39
	32.6	13.1	1.37
	26.8	10.9	1.38
	27.8	11.8	1.44
			Average 1.36

#### V. THE ORDER OF STRATIFICATION OF THE CYTOPLASMIC COMPONENTS

In the preceding parts of this paper it has been shown that differences in cleavage rate of sea urchin eggs cannot be directly related either to differences in total

metabolic rate or to differences in the initial ribose nucleic acid content prior to cleavage.

There remains, however, another property of these sea urchin eggs which differs in a graded way between the species, and the seriation is in accordance with the seriation of the cleavage rates.

HARVEY<sup>9</sup>, by means of the centrifuge technique, was able to show that the cytoplasmic components of the eggs stratify in different order in different species. I have repeated her experiment and have obtained similar results. The order of stratification is shown diagrammatically in Fig. 1. Working from the centripetal to the centrifugal ends of the eggs, the fast-cleaving *Psammechinus* egg is stratified into oil, yolk (with nucleus), clear zone and mitochondria; the *Paracentrotus* egg into oil, clear zone (with nucleus), yolk and mitochondria; the slow cleaving *Arbacia* and *Sphaerechinus* eggs into

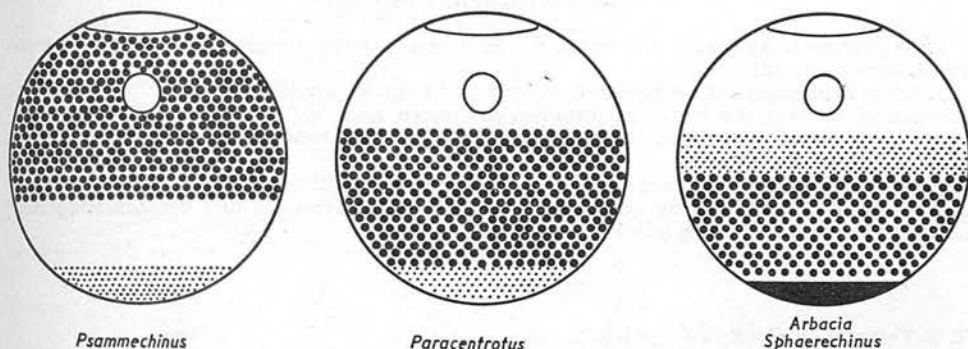


Fig. 1. Diagrammatic representation of the stratified cytoplasmic components of sea urchins' eggs: centripetal pole, with oil cap, is uppermost; coarse granules are yolk, fine granules mitochondria; the black area represents pigment in *Arbacia* and "heavy" clear cytoplasm in *Sphaerechinus*.

oil, clear zone (with nucleus), mitochondria and yolk. Thus the relative density of the yolk granules is least in *Psammechinus*, greatest in *Arbacia* and *Sphaerechinus* with *Paracentrotus* occupying the intermediate position.

It would be entirely premature to discuss this possible correlation, which may be purely fortuitous, before appropriate experiments have been undertaken to test its validity. A direct correlation between relative yolk density and cleavage rate is improbable, considering what is known of the cleavage rates of non-yolky half- and quarter-eggs. However the correlation may well be an indirect one, depending on the density or some linked property of another of the stratifiable cytoplasmic components.

#### Acknowledgements

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#### SUMMARY

1. There are striking differences in the cleavage rates of the eggs of four neapolitan sea urchin species.
2. These cleavage rates are not directly correlated with differences in the rate of oxygen consumption of the eggs during the early cleavage stages, nor

References p. 102.

3. are they correlated with differing ribose nucleic acid concentrations in the virgin eggs.
4. There is a hint that cleavage rate differences may be correlated with variations in the order of stratification of the cytoplasmic constituents when eggs are subjected to centrifugal force.

## RÉSUMÉ

1. Il existe des différences frappantes dans les vitesses de division des œufs de quatre espèces napolitaines d'oursins.
2. Ces différences ne correspondent ni à des différences dans les intensités de consommation d'oxygène au cours des premiers stades de division,
3. ni à des différences dans les concentrations des œufs vierges en acide ribonucléique.
4. L'action de la force centrifuge semble montrer que ces différences de vitesse de division dépendent de variations dans la stratification des constituants cytoplasmiques.

## ZUSAMMENFASSUNG

1. In den Spaltungsgeschwindigkeiten der Eier von vier napolitanischen Seeigelarten treten starke Unterschiede auf.
2. Diese Spaltungsgeschwindigkeiten hängen nicht direkt mit Unterschieden in der Rate des Sauerstoffverbrauchs in den frühen Spaltstadien zusammen, noch
3. sind sie von verschiedenen Ribosenukleinsäurekonzentrationen in den unbefruchteten Eiern abhängig.
4. Es sind Anzeichen vorhanden, dass die Spaltungsgeschwindigkeitsunterschiede von Variationen in der Streckungsordnung der Zytoplasmabestandteile, wenn die Eier der Zentrifugalkraft unterworfen werden, abhängig sein könnten.

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### An Electron Microscope Study of the Nuclear Membrane

THE giant nuclei of full-grown oocytes of Amphibia offer unique opportunities for anatomical and physiological study, on account of the relative ease with which they may be isolated and handled. The material for the present study was obtained from *Triturus cristatus* and *Xenopus laevis*.

Under a low-power binocular, the nuclei are isolated into distilled water with the aid of needle and forceps. In this medium the nucleus rapidly swells, and meanwhile adhering cytoplasmic materials may be pumped away by means of a pipette. The entire nucleus, freed from cytoplasm, is then transferred to fresh distilled water: a copper specimen grid is now placed in the same container, and the nuclear membrane ruptured and stretched out over the grid by means of a pair of fine-pointed tungsten needles. After the stretched membranes have been pumped free from nuclear sap, they may be dried directly or after prior fixation. For the preservation of fine structure, fixation appears to be necessary.

Grids carrying nuclear membranes were examined in a Siemen's electron microscope operating at 52 kV., the magnification of the photographic negative being 12,000–15,000 diameters. The photographs, three of which accompany the present letter, indicate that the nuclear membrane is a compound structure. One component is a porous sheet, the pores being approximately 300 Å. in diameter in hexagonal array, with a repetition distance of about 800 Å. It must be borne in mind, however, that the membranes are readily distorted during preparation, the hexagonal arrangements being frequently disturbed. The other component is a membrane with no evident fine structure. This closely over- or underlies the porous membrane; but the relative positions of the two are not yet known. Under certain as yet ill-defined conditions, the porous membrane disintegrates in the process of specimen preparation, leaving the structureless membrane only.

It may be presumed that the structureless component determines the permeability properties of the nuclear membrane. Other investigations have shown that egg albumen cannot penetrate this membrane,

# PHASMA INTERFERENCE IN DIPLONIC TELEOSTOIDS AND INTERCHANGE SPERMATOCYTES OF TELEOSTOIDS

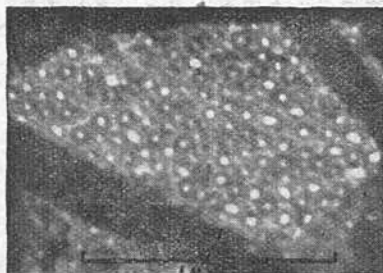


Fig. 1

Fig. 1. *Triturus cristatus*. The porous sheet component of the nuclear membrane after fixation with osmium tetroxide. Magnification 34,000 diameters

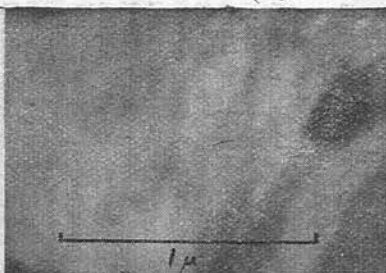


Fig. 2

Fig. 2. *Triturus cristatus*. The structureless sheet component of the nuclear membrane after fixation with osmium tetroxide. Magnification 34,000 diameters

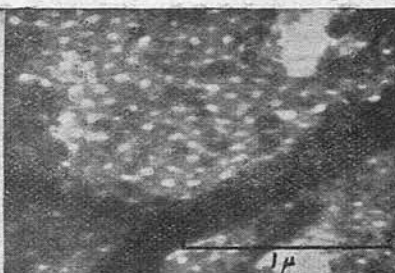


Fig. 3

Fig. 3. *Xenopus laevis*. Portion of nuclear membrane fixed with phosphotungstic acid. Magnification 27,000 diameters

and hence even if a porous structure is present it would be beyond the limits of resolution of the existing microscope. The visibly porous membrane, on the other hand, probably acts merely as a mechanical support for the structureless membrane.

This work is being continued, and a detailed account will be published elsewhere.

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Nov. 12.

# CHIASMA INTERFERENCE IN DIPLOID, TETRAPLOID AND INTERCHANGE SPERMATOCYTES OF THE EARWIG, *FORFICULA AURICULARIA*

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(With Two Text-figures)

## I. INTRODUCTION

During the course of a study of the sex-determining mechanism of *Forficula* (Callan, 1941) some observations on chiasma formation of the autosomes in tetraploid spermatocytes and in spermatocytes where there had been an interchange between two autosome arms were left unpublished. The data obtained were in conflict with the general rule that autotetraploid cells form fewer half-chiasmata per chromosome (Upcott, 1939) than comparable diploid cells, but at the time no explanation of the divergence was apparent. Later work on interference of chiasmata across the centromere in certain other organisms (Pätau, 1941; Callan & Montalenti, 1947; Montalenti, 1946, 1947; Montalenti & Vitagliano, 1946, 1947) has brought to light further rules governing chiasma formation which now enable the earlier *Forficula* data to be interpreted.

The analysis of chiasma data deriving from an island of abnormal cells in an otherwise normal gonad is greatly facilitated by the presence of natural controls adjacent to the 'experimental' material. The present study is of this kind and by virtue of the rigid controls which are available, it is possible to add a further contribution to the causal analysis of meiosis.

The material consists of sectioned testes of the common earwig, *F. auricularia*. The testes were fixed in La Cour's 2BD (La Cour, 1937) and the sections, cut at  $14\mu$ , were stained by Newton's Gentian Violet-Iodine method.

The cytological study of the sex-determining mechanism of *Forficula* was based on the examination of over two hundred individual males. In one testis of one of these males an island of some sixteen tetraploid cells was present which had been fixed at first meiotic metaphase. Of these metaphases a full chiasma analysis of fourteen cells was possible. The presence of multivalent associations in thirteen out of the fourteen is a proof that the tetraploid condition had arisen prior to meiosis, and from the size of the island it is likely that the cause was a failure of anaphase separation in a single spermatogonium four divisions prior to first meiosis.

In a single testis of another earwig an entire cyst of spermatocytes was abnormal in showing a single multivalent autosome association in thirty-six out of the thirty-eight cells which could be analysed. Since all other cysts of the same testis contained typical bivalent autosome associations throughout one must suppose that in the spermatogonium ancestral to the abnormal cyst an exchange of arms or parts of arms between two non-homologous autosomes had taken place.

## II. CHIASMA FORMATION IN DIPLOID CELLS

The autosome complement of *Forficula* consists of eleven pairs of chromosomes. During spermatogonial divisions these chromosomes are highly contracted at metaphase and only with difficulty is it possible to determine that the majority of them possess median or submedian centromeres. At the meiotic metaphases the contraction is even more extreme. However, the diplotene and diakinesis stages fix well and full chiasma counts are therefore possible.

No meiotic bivalent ever forms more than one chiasma within one arm, and in most cases only one arm forms a chiasma; nevertheless, a small proportion of ring bivalents having a single chiasma in each arm are also to be found. It is only at diplotene and diakinesis that 1- and 2-chiasma bivalents can be distinguished with certainty from one another; at metaphase the condensation is so great as to make distinction practically impossible.

Typical diploid chiasma frequencies will be considered alongside the tetraploid and interchange chiasma data. In the present context it should, however, be recorded that the extreme paucity of bivalents with chiasmata in each arm is evidence that in *Forficula*, as in *Culex*, *Dicranomyia*, *Psychoda*, *Simulium*, *Prionotropis*, *Asellus* and *Eisenia*, the formation of a chiasma in one chromosome arm greatly reduces the chances of formation of another chiasma in the other arm across the centromere.

## III. CHIASMA FORMATION IN TETRAPLOID CELLS

Since the tetraploid cells were studied at first meiotic metaphase, the chiasma analysis provides a minimal figure of the number of chiasmata actually formed. This results from the scoring of all bivalents as having formed only a single chiasma: it has been already explained that 1- and 2-chiasma bivalents cannot be distinguished with certainty at metaphase. On the other hand, multivalent associations can be adequately analysed and give a full measure of the chiasmata formed. Table 1 gives the chiasma analysis of the fourteen tetraploid cells which were studied and camera-lucida drawings of two of these are shown in Fig. 1. The majority of the quadrivalents and trivalents are of the linear type, though a few ring quadrivalents are also present. In a single trivalent one chromosome had formed two chiasmata within one arm, the only case which I observed in the whole of the *Forficula* material. The mean chiasma frequency per nucleus in the tetraploid cells is 24.71, the variance of this mean being 0.18.

One hundred and thirty-four diploid cells in neighbouring cysts of the same testis were examined at diakinesis. In these cells the chiasma analysis is a full measure of the chiasmata actually formed: thirteen nuclei each formed twelve chiasmata, the remainder eleven chiasmata. The mean chiasma frequency per nucleus is 11.10, the variance of the mean being 0.0007.

It will be seen that the mean chiasma frequency per tetraploid nucleus is more than twice as great as the mean chiasma frequency per diploid nucleus. To test the significance of this disproportion, a 't' test has been applied to the data

$$t = \frac{\bar{x}_t - 2\bar{x}_d}{\sqrt{(V\bar{x}_t + 4V\bar{x}_d)}}$$

where  $\bar{x}_t$  and  $\bar{x}_d$  are the means, and  $V\bar{x}_t$  and  $V\bar{x}_d$  the variances of the means of the tetraploid and diploid nuclei respectively. The present data give a value  $t = 6.0$ . In considering the significance given by this value, allowance must be made for the marked difference



between the variances of the two means; this contingency is covered by Behrens's test (Fisher & Yates, 1948, table V2) which, with the present data, gives a probability of less than 1 in 500 that  $t$  will exceed 4.0 in random sampling. The mean chiasma frequency per tetraploid nucleus is thus significantly greater than twice that of the comparable diploid.

This disproportionately higher chiasma frequency of the tetraploid cells probably depends on a breakdown of the mechanism which normally determines chiasma interference across the centromere in diploid cells. In the diploid an homologous pair of chromosomes associate at zygotene and when a chiasma forms in one arm it is generally capable of

Table 1. *Chiasma analysis in the tetraploid cells*

Cell no.	No. of								Total chiasmata per nucleus
	Quadri-valents (Chiasmata)	Tri-valents (Chiasmata)	Bi-valents (Chiasmata)	Uni-valents (Chiasmata)					
1	4	(13)	0	(0)	13	(13)	2	(0)	26
2	3	(9)	0	(0)	16	(16)	0	(0)	25
3	3	(9)	2	(4)	12	(12)	2	(0)	25
4	6	(18)	0	(0)	10	(10)	0	(0)	28
5	0	(0)	2	(4)	18	(18)	2	(0)	22
6	2	(6)	2	(5)	14	(14)	2	(0)	25
7	4	(13)	0	(0)	12	(12)	4	(0)	25
8	2	(7)	0	(0)	18	(18)	0	(0)	25
9	4	(13)	0	(0)	13	(13)	2	(0)	26
10	1	(3)	3	(6)	14	(14)	3	(0)	23
11	3	(9)	1	(2)	14	(14)	1	(0)	25
12	2	(6)	1	(2)	16	(16)	1	(0)	24
13	3	(9)	1	(2)	14	(14)	1	(0)	25
14	0	(0)	0	(0)	22	(22)	0	(0)	22

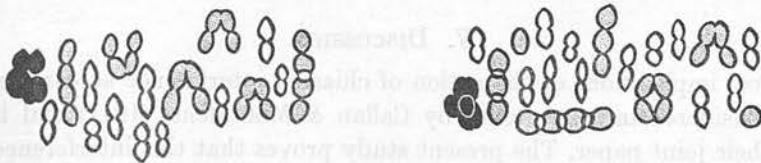


Fig. 1. Two tetraploid spermatocytes at first meiotic metaphase: sex chromosomes solid, bivalents outlined, multivalents and univalent outlined and stippled.

suppressing chiasma formation in the other. In the tetraploid, however, a single chromosome may partner one chromosome in one arm and another chromosome in the other arm. In these conditions of pairing, i.e. where there has been an exchange of partner, a chiasma formed in one arm does not suppress the formation of a second chiasma in the other arm.

#### IV. CHIASMA FORMATION IN 'INTERCHANGE' CELLS

Within the cyst containing interchange cells both diakinesis and metaphase stages are available for study. Three meiotic metaphases are shown in Fig. 2. The chiasma counts, however, have been confined to diakinesis stages where a full analysis is possible. Out of thirty-eight cells which were studied, thirty-five included a single linear quadrivalent, one showed a trivalent and univalent and the remaining two showed bivalents only.

In neighbouring cysts of the same testis ninety-two normal spermatocytes at diakinesis were also studied. The chiasma data from both interchange and normal control cells are given in Table 2. It will be seen that the mean chiasma frequency per nucleus is higher in the interchange than in the normal cells and the value of  $\chi^2$  appended to the table indicates that this difference is highly significant.

The higher chiasma frequency of the interchange cells is entirely attributable to the regular formation of a quadrivalent. This is evidence that pachytene pairing is almost always complete. It also indicates, even more strikingly than does the analysis of the tetraploid cells, that an exchange of chromosome partner in pairing depresses or removes the action of chiasma interference across the centromere.

Whereas in the tetraploid cells a proportion of the quadrivalents formed four chiasmata and thus were present as rings at metaphase, no ring quadrivalents are to be found in the interchange cells. This is readily accounted for if in the interchange association one of the arm pairs is too short to form a chiasma.

Table 2. *Chiasma frequency in interchange and normal diploid cells*

No. of chiasmata ...	11	12	13	Mean chiasmata per cell
No. of cells:				
Normal diploid	87	5	0	11.05
Interchange	3	32	3	12.00

Pooling chiasmata classes 12 and 13,  $\chi^2=94.5$ , which has a probability of less than 1 in 1000.



Fig. 2. Three interchange spermatocytes at first meiotic metaphase: sex chromosomes solid, bivalents outlined, multivalents and univalent outlined and stippled.

## V. DISCUSSION

The theoretical implications of the action of chiasma interference across the centromere have been considered in some detail by Callan & Montalenti (1947) and the reader is referred to their joint paper. The present study proves that this interference is removed or at least depressed if in chromosome pairing an exchange of partner takes place. Chiasma interference must thus be considered as a relational property shared by two partner chromosomes and not transmissible along the length of one of the chromosome threads beyond the point where the pairing relationship has been altered. Theories as to the causal mechanism of chiasma formation which postulate a release of mechanical strain at the point where the chiasma forms (e.g. Darlington, 1935) are thus in keeping with this view.

## VI. SUMMARY

1. The number of chiasmata formed by the bivalents of diploid spermatocytes of *Forficula auricularia* is limited by chiasma interference across the centromere.
2. In tetraploid and interchange spermatocytes the chiasma frequency is raised by the formation of multivalent associations.
3. This indicates that chiasma interference across the centromere is suppressed when there are changes of chromosome partner during pairing.

I wish to thank Prof. Mather and Dr Reeve for advice on the statistical methods employed in this work.

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HYBRIDS BETWEEN SOME MEMBERS OF THE  
RASSENKREIS *Triturus cristatus*

by

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Hybrids between some members of the Rassenkreis  
Triturus cristatus

The great crested newts Triturus cristatus are divided into a number of subspecies inhabiting different geographical areas. Of these we have experimented with three, namely T.c.cristatus (from Chessington, Surrey and the New Forest, Hampshire), T.c.carnifex (from Naples) and T.c.karelinii (from Baku). We have obtained  $F_1$  from karelinii female x cristatus males, carnifex female x karelinii male and carnifex female x cristatus male.

The first of these hybrids was obtained in 1946 and about one in seven of the larvae became exaggeratedly oedematous and died at metamorphosis. There is some evidence that this is not due to their hybrid nature. Only one male and one female have yet bred, though several males have courted. The other two ~~had~~ hybrids, made in 1948, were extremely vigorous, members of each cross breeding when slightly less than a year old. The larval and metamorphic mortality among the back-cross and  $F_2$  animals has been heavy in all three experiments.

The following were the mean chiasmata frequencies per cell in spermatocyte meiosis :

<u>carnifex</u> .....	30.7 - 32.1	(4 specimens)
<u>cristatus</u> .....	36.5 - 38.5	(3 specimens)
<u>karelinii</u> .....	39.5 - 42.2	(2 specimens)
<u>karelinii</u> ♀ x <u>cristatus</u> ♂	.....21.2	(1 specimen)
<u>carnifex</u> ♀ x <u>karelinii</u> ♂	.....15.1 - 21.2	(5 specimens)
<u>carnifex</u> ♀ x <u>cristatus</u> ♂	.....16.1 - 21.9	(4 specimens)

The chiasmata frequency per cell was much more variable within any one hybrid specimen than in the parent races. Whereas the parent races form their chiasmata without much restriction as to position in the chromosomes, the chiasmata formed by the hybrids mostly lie in the terminal regions : they show the same type of localisation as is present in species such as Triturus palmatus and T.vulgaris.

Failure of pairing was very variable both within and between hybrid individuals : on a rough average rather less than half the meioses have all the chromosomes paired. In the two hybrids involving karelinii multivalent chromosome associations were seen, but not in that between carnifex and cristatus. Single bridges and fragments were seen in occasional anaphases

in all the hybrids. There is thus evidence that all three geographical subspecies differ from one another in respect of inversions while karelinii differs from the other two in respect of at least two translocations.

Although groups of hybrid spermatocytes enter first meiotic metaphase in unison, anaphase separation may be delayed for variable periods and thus the normal synchronism is lost in the later spermatogenetic stages. No degeneration takes place before meiosis, but some degree of spermatid degeneration sets in after the second meiotic division. The extent of spermatid degeneration is exceedingly variable as between different hybrid individuals: this variation is not correlated with failure of chromosome pairing at meiosis; as in other Urodele hybrids which have been studied (White, 1946, Benazzi and Lepori, 1949) it is due to physiological unbalance in the diploid, not to abnormal haploid complements resulting from irregularities of segregation.

In the Urodele hybrids which have been investigated by these other authors there has always been male sterility and complete or almost complete sperm degeneration. In our animals, although there is variable spermatid degeneration, some individuals show practically none and all succeed in forming ~~sp~~ mature sperm. The translocations in hybrids involving karelinii would give rise to gametes with duplications or deficiencies even when meiosis was regular, thus accounting for the mortality among larvae of the second generation. The other cross might also be expected to give some aneuploid gametes, but the larval mortality must be due to unbalanced gene combinations.

We believe that this is the first instance in which evolutionary change involving translocations has been demonstrated within a vertebrate species. The failure to demonstrate similar translocations in the hybrids between more distant taxonomic units among the Urodeles is probably because the greater degree of failure of pairing observed in these prevents the formation of multivalents.

We wish to thank Mr L.A. Lantz and Professor G. Montalenti for generous gifts of animals. Fuller details including an account of the genetics of certain characters, will be published elsewhere.

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A Study of Meiosis in inter-racial hybrids  
of the newt, Triturus cristatus.

by

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March, 1950.

## 1. Introduction.

A cytological study of meiosis in hybrids is a useful corollary to a genetical study since it may give some ~~information~~ indication of the overall genetic divergence between the parents. It is also of value in the analysis of chromosome problems per se since, as pointed out by Darlington, 1940, exceptional behaviour of chromosomes sheds light on the sequence of events leading to the normal behaviour.

Newts are very suitable organisms for the investigation of meiosis because of the ~~enormous~~<sup>large</sup> size of the chromosomes and the ease with which they can, at least at metaphase, be fixed and stained. The various geographical races of great crested newts, Triturus cristatus, all possess ~~of~~ chromosome complements of 12 pairs. It is to some extent possible to distinguish the different chromosomes of the haploid set by virtue of size and centromere position, but no attempt has been made to do this in the present work.

The first newt hybrids to have attracted cytological attention were those occurring in nature between Triturus cristatus cristatus and T. marmoratus. Bataillon and Tchou Su (1930, 1932) found failure of chromosome pairing and extensive spermatid degeneration in hybrid males: hybrid females were, however, fertile. White (1946) has reinvestigated hybrids between these two species: the hybrids were, however, bred in the laboratory, and two other geographical races of T. cristatus were involved: T.c.carnifex and T.c.karelinii. The only other newt hybrids which have been examined cytologically were those produced by artificial fertilisation of Triturus cristatus carnifex eggs with T.vulgaris meridionalis sperm (Benazzi and Lepori, 1949). The observations of White and of Benazzi and Lepori are considered in detail later in this paper.

According to Mertens and Muller (1940), Triturus cristatus is divided into five subspecies; T.c.cristatus inhabiting the whole of Europe roughly north of the latitude of the Alps, T.c.carnifex inhabiting Switzerland south of the Alps, Italy, most of Austria and North Jugoslavia, T.c.danubialis



inhabiting the Danube valley from Vienna to Wallachia and Transsylvania, T.c.dobrogicus inhabiting Dobruzha, and T.c.karelinii inhabiting the southern part of the Balkan peninsula, the Crimea, Caucasus, Asia Minor and North Persia.

The zones of overlap of these five forms are typical of the hybrid zones between subspecies as compared with the hybrid zones between species in an Artenkreis. The Austrian population, as we are informed by Mr L.A.Lantz, is a mixed population showing every degree of intermediacy between cristatus, carnifex and danubialis. According to Wolterstorff (1923), who gives a systematic ~~description~~ comparison of all the subspecies except dobrogicus, the Balkan population of karelinii shows resemblances to carnifex which are not shown by the Asiatic members of this subspecies.

Zones of overlap are to be expected in Wallachia between danubialis and dobrogicus and also in Southern Russia between cristatus and karelinii.

According to the generosity of Professor J. Montagu, the karelinii material is now at the disposal of Mr L.A.Lantz. In 1926 J. Montagu gave to Wolterstorff a few specimens, naming the locality as such. In 1927 Mr Lantz raised a pair from eggs of Wolterstorff which bred in 1930, but again only a pair was raised. These bred in 1931, but only a single male reached maturity. This male bred in 1935 and 1937 with another female from Wolterstorff's stock. In 1939 20000 animals were raised, since then a small stock has been established, though a few animals in it have sterility. According to Mr Lantz the immediate surroundings of Lake are unsuitable for growth, and the original specimens must have come either from the mountains of Transylvania, where the species exists in small local populations, or from the northern limit of the range west along the southern shores of the Danube between the mouth of the Duna and Tiber river, where it would be expected to form a continuous population.

These details about the early sterility of the karelinii stock in captivity, and the uncertainty of its locality data must be approached in view of the sterility of its cytology compared with the other two species. The karelinii does for being the hybrid with

## 2. Material

This paper discusses the cytology of the following hybrids : karelinii♀ x cristatus♂♂; carnifex♀ x karelinii♂; and carnifex♀ x cristatus♂. The first hybrid was made in 1946 and the other two in 1948. A description of the appearance and biology of the first and second generation hybrids will be published by one of us ( H.S.) elsewhere. The cristatus males used for making hybrids were collected immediately before mating from a small pond in a bomb crater at Chessington, Surrey, in Southern England. Subspecies cristatus is difficult to keep in a state of sexual excitement in captivity and it is practically impossible to make this subspecies redevelop sexual excitement when this has once been lost : hence the regrettable absence of reciprocal hybrids. The cristatus males used as cytological controls were collected in the New Forest, Hampshire, in Southern England. The carnifex material comes from the neighbourhood of Naples, Italy, and is owed to the generosity of Professor G.Montalenti.

The karelinii material we owe to the generosity of Mr L.A.Lantz. In 1926 P.Kammerer gave to W.Wolterstorff a few specimens, naming the locality as Baku. In 1927 Mr Lantz raised a pair from eggs of these, ~~which~~ which bred in 1930, but again only a pair was raised. These bred in 1933, but only a single male reached maturity. This male bred in 1938 and 1939 with another female from Wolterstorff's stock. In 1939 fifteen animals were raised. Since then a small stock has been established, though a few animals in it seem sterile.

According to Mr Lantz the immediate surroundings of Baku are unsuitable for newts, and the original specimens must have come either from the mountains of Transcaucasia, where the species exists in small isolated populations, or from the northern limit of the damp belt along the southern shore of the Caspian between the mouth of the Kura and Bandar Shar, where it would be expected to form a continuous population.

These details about the early sterility of the karelinii stock in captivity, and the ambiguity of its locality data must be emphasised in view of the peculiarity of its cytology compared with the other two subspecies. The karelinii used for making the hybrids with

T. marmoratus examined by White (1946) also came from this source.

The localities from which the members of the three subspecies were obtained show that they were well away from possible zones of overlap, and from this point of view the animals can thus be regarded as typical.

Chimpanzees were therefore studied in "squash" preparations of the brain. The brain was removed in toto, embedded in paraffin wax, and cut into sections in the coronal plane. The sections were stained with hematoxylin and eosin and mounted on glass slides. In some cases, the sections were stained with cresyl violet or fast blue FCF. The sections were then mounted on glass slides and covered with a cover slip.

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### 3. Cytological methods employed.

In order to study the distribution and frequency of chiasmata in newt meiosis, sections are entirely useless. As has been pointed out by White (1946), this is due to the fact that although the chromosomes are relatively large, the spermatocyte cells are not unduly so and the bivalents are thus crowded in or around a very narrow spindle.

Chiasmata were therefore studied in "squash" preparations stained in iron aceto-carmin. The newts were anaesthetised and the testes exposed by making a small longitudinal incision in the abdominal body wall of each. Parts of the testes were excised unilaterally and fixed in Carnoy's 3 : 1 alcohol-acetic acid mixture (for squash preparations) or in San Felice's fluid (for sections). The newts were meanwhile sewn up with gut and allowed to recover. In all but one of the experimental animals the wounds subsequently healed satisfactorily so that they could be used for breeding at a later date.

Material for squash preparations was left to fix for half an hour to two hours. Fragments were taken and tapped out on a slide in aceto-carmin by means of a bone instrument handle. No attempt was made to stain intensely at this stage, the material being immediately covered with a well-cleaned coverslip and pressed out between filter papers by light finger pressure. If cursory examination showed the presence of meiotic divisions on the slide, the slide was inverted in 10% acetic acid in a ridged dish for the coverslip to separate. After separation the cover slip was stained for 5 - 10 minutes in a tube containing 0.5% carmin in 45% acetic acid ; sufficient ferric acetate crystals had been added to the stain to darken it without causing precipitation. The coverslip was subsequently passed through 3 : 1 alcohol-acetic acid, alcohol and xylol and mounted in D.P.X. In squash preparations made in this fashion the chromosomes are nicely spread out in one plane, thus making analysis and photography relatively simple matters.

Sections cut at 25 $\mu$  and stained according to Newton's Gentian Violet technique were used for studying the gross outlines of spermatogenesis and for the detection of anaphase bridges.

With the exception of the preparation of T.c.carnifex, all the fixations were made early in September, 1949. All but one of the hybrid newts contained testes with abundant meiotic divisions and all stages of spermatogenesis up to mature sperm bundles: the testes of one of the two specimens of karelinii ~~♀~~ crisatus ♂ (individual 8) contained only spermatogonia and mature sperm. The testes of the controls T.c.crisatus and T.c.karelinii likewise contained the requisite meiotic stages and all subsequent stages leading to mature sperm.

The individuals of T.c.carnifex were collected during the winter 1944/5 and meiosis was induced artificially in February 1945 by placing four males for one month in a thermostatically controlled incubator set at 25°C. They had previously been held at a temperature of ca. 12°C. The temperature control of spermatogenesis in Triturus has been extensively investigated by Galgano (vide Galgano and Falchetti, 1940).

#### 4. Meiosis in the parent races.

Whereas in the common British newts Triturus vulgaris and T. palmatus the chiasmata are confined to the end regions of the chromosomes (terminal localisation), the three races of T. cristatus form their chiasmata in a much less restricted fashion. Chiasmata were counted in 10 cells of each individual: the data is compiled as a frequency distribution in table 1. The races differ significantly in chiasma frequency. The mean chiasma frequencies per cell are as follows :-

T.c. carnifex:  $30.7 \pm 0.6$ ,  $32.1 \pm 0.9$ ,  $30.7 \pm 0.6$ ,  $31.2 \pm 1.0$ .

T.c. cristatus:  $37.2 \pm 0.7$ ,  $38.5 \pm 0.5$ ,  $36.5 \pm 0.3$ .

T.c. karelinii:  $39.5 \pm 0.8$ ,  $42.2 \pm 0.4$ .

T.c. carnifex never forms more than two chiasmata within a single chromosome arm; T.c. cristatus occasionally forms three chiasmata within an arm; in T.c. karelinii this is a common occurrence. In all three races, when a bivalent forms less than its total possible chiasmata, those which fail to form are in the proximal regions; the bivalent then takes on the aspect of that of a species with terminal localisation. It is very rare to find a bivalent which has formed proximal chiasmata but failed distally. Complete failure to form chiasmata is of even rarer occurrence: this has been observed twice in T.c. carnifex, once in T.c. cristatus and not at all in T.c. karelinii during the routine examination of some hundreds of meiotic divisions in the three races. Typical first meiotic metaphases are shown in text figure 1 and plate 1.

Spermatogenesis follows an entirely normal course in all three races and requires no special description. In keeping with the observations of White and others, no inversion bridges were seen despite a careful search. Groups of cells enter meiosis in synchronism and remain in step throughout the later stages, a point to be remembered when we come to consider the inter-racial hybrids.

5. Meiosis in the inter-racial hybrids.

In striking contrast to the parental races, the hybrids form, on a rough average, about half as many chiasmata per cell. Chiasma frequencies per cell are set out as a frequency distribution in table 2, 20 cells from each individual having been analysed.

The mean chiasma frequencies per cell are as follows :-

<u>karelinii</u> ♀ x <u>cristatus</u> ♂	21.2 ± 1.0
<u>carnifex</u> ♀ x <u>karelinii</u> ♂	15.1 ± 0.7, 19.0 ± 0.9, 21.2 ± 0.9, 19.0 ± 0.7, 16.6 ± 0.8.
<u>carnifex</u> ♀ x <u>cristatus</u> ♂	21.9 ± 0.7, 17.2 ± 0.6, 21.2 ± 0.6 16.1 ± 0.9.

There are no noticeable differences between the three types of hybrid as regards chiasma frequency.

Not only are the chiasma frequencies lower than those of the parent races : the bivalents are paired very irregularly and univalents are of common occurrence. A frequency distribution of univalent formation is shown in table 3. It will be seen that there is a wide range of variation between individuals, mean univalent frequency being highly correlated with mean chiasma frequency as is to be expected since both are derived from the same cell counts.

There is also a high degree of variance between cells both for chiasma and univalent number. As can be seen in table 2, the variances of chiasma number are nearly as large as are the means per cell. This is readily explicable since it is rare for more than one chiasma to form in one chromosome arm and thus chiasma interference must be almost absent (cf. Haldane, 1931).

The variances of the number of pairs of univalents are also nearly as large as their respective means. In the case of the carnifex x cristatus hybrids, for example, where the situation does not appear to be complicated by translocations and consequent multi-valent formation, we have :-

Individual a,	Mean	0.55,	Variance	0.47	
"	c,	"	1.05,	"	0.58
"	d,	"	0.45,	"	0.47
"	e,	"	1.60,	"	1.20.

If the variances were in fact equal to the means, this would suggest a Poisson distribution where each

chromosome pair was equally likely to fail to form chiasmata. The values found suggest that this is true at least for many of the chromosome pairs.

The chiasmata which are formed lie in the end regions of the chromosomes. Chiasmata are, indeed, as localised in the hybrids as are those of a species such as T. palmatus. Typical first meiotic metaphases are shown in text figure 2 and plate 1.

As White pointed out in his study of inter-specific hybrids, failure to form bivalents appears to disturb the mechanism normally responsible for anaphase separation at the first meiotic division: metaphases accumulate, anaphases being delayed. This is to be seen very strikingly in the sectioned material. Groups of cells pass in perfect synchronism to the formation of the first meiotic spindle. Anaphase, however, sets in irregularly so that it is not uncommon to find groups of cells where neighbours may be at any stage from first metaphase to second anaphase. The loss of synchronism at first meiosis is reflected on all subsequent stages. It varies somewhat from tubule to tubule within the same testis, and also between different individuals.

The univalents fail to co-orientate with their homologues at first metaphase, as is invariably the rule after total chiasma failure. They do not, however, self-orientate and divide, as do those in the Triticum-Aegilops hybrids studied by Kihara, 1931. They appear to remain entirely passive during the anaphase and are included by accident of location in the daughter nuclei. We may recognise three different degrees of variation in univalent behaviour at meiosis:

1. delayed self-orientation followed by equational division, as in Triticum-Aegilops hybrids.
2. delayed self-orientation not followed by division, as in the trisomic Mecostethus (Callan, 1941).
3. neither self-orientation nor division, as in the present newt hybrids.

The distribution of the univalents between daughter nuclei does not appear to be altogether at random. Homologous pairs of univalents frequently lie close together during the first anaphase, possibly because they underwent some slight degree of pachytene pairing,



though insufficient for the formation of chiasmata. This gives rise to even less regular segregation than would occur with random distribution. The point at issue has, however, not been studied in detail.

Multivalent chromosome associations have been observed in certain of the hybrid newts. In karelinii ♀ x cristatus ♂, individual 9, two trivalents and two quadrivalents were found in the 20 cells analysed. In the 20 cells each of the hybrids carnifex ♀ x karelinii ♂, individual c formed one ~~xxxxxxx~~ trivalent and one quadrivalent, d formed two quadrivalents, e formed one quadrivalent and f two trivalents. Individual b of this cross formed no multivalents, neither did any individuals of the hybrid carnifex ♀ x cristatus ♂. Two drawings of metaphase plates containing multivalents are shown in text figure 3. It should be noticed that one of these cells, derived from karelinii ♀ x cristatus ♂, individual 9, itself contains two multivalents. The formation of multivalents proves that chromosomal translocations differentiate T.c. karelinii from the other two subspecies. It is therefore evident that even when meiosis of a hybrid involving T.c. karelinii is apparently entirely regular, the products of segregation are likely to contain genetical deficiencies and duplications.

As might be expected, multivalent chromosome associations have not been observed in the parental races.

Anaphase bridges have also been observed in a small proportion of cells in all the hybrid newts. Sometimes they occur with lagging acentric fragments, in which case they must represent the consequences of inversion cross-over at the preceding diplotene. More frequently, however, they are not associated with visible fragments. In normal circumstances this might be interpreted as "stickiness" without evident explanation. However, since only single bridges occur in any one anaphase, and since the bridges have all the appearance of stretched continuous chromatids, we are inclined to describe these also as the products of inversion cross-over, the inversions involved being small, terminal, and the chiasmata likewise terminal or nearly so. In these circumstances the fragment

produced by inversion cross-over might well be outside the range of optical resolution. Four examples are illustrated in text figure 4. Whether or not the bridges without fragments are conceded to be due to inversion cross-over, those with fragments prove the existence of a further form of structural heterozygosity in the chromosomes of the inter-racial hybrids. As has already been mentioned, no bridges have been observed in the parent races.

Despite the variable delays in anaphase ~~separation~~ separation in different spermatocytes of the newt hybrids, separation is always ultimately accomplished. No restitution nuclei are formed: it would appear that the spindle never collapses before anaphase separation has occurred: after separation the spindle disintegrates and then, and only then, can the daughter chromosomes relapse into resting nuclei.

A notable feature of the spermatogenesis of the hybrid newts is the degeneration of many of the spermatid nuclei resulting as daughters from the second meiotic division. There is a very wide range of variability in the extent to which degeneration occurs and the newts must be considered individually. There are no signs of degeneration of spermatids in the parental races, neither are there the slightest signs of degeneration of spermatocytes in the hybrids. In the hybrids, degeneration is strictly a post-meiotic phenomenon.

T.c.karelinii ♀ x T.c.cristatus ♂♂. Individual 9 showed about 5 - 20% of the spermatids in degeneration, the proportions varying from follicle to follicle. There was no evident reduction of sperm in those parts of the testis filled with compact sperm bundles (see plate 2, figure 2). Individual 8 was not available as sectioned material, but the fact that the "squash" preparations contained practically nothing but sperm may be taken to indicate that this specimen was not unlike individual 9.

T.c.carnifex ♀ x T.c.karelinii ♂. Individual b showed the highest proportion of degenerating spermatids, from roughly 70 - 100%, with variation from follicle to follicle. Only a small proportion of follicles produced reasonably compact sperm bundles, the rest forming sperm scattered in irregular fashion among the products of

degeneration (see plate 2, figure 3). Individuals c and f showed about half the spermatids in degeneration, most of the sperm bundles being regular and compact. Individuals d and e showed the least degeneration, of the order 20 - 30% of the spermatids. In most instances sperm bundles were not recognisably smaller than those of the parent races.

T.c.carnifex ♀ x T.c.cristatus ♂. Individual e showed the highest proportion of degenerating spermatids of any of the hybrids, from 90 - 100%. This specimen formed practically no sperm at all (see plate 2, figure 4). Individual c showed great variation from follicle to follicle: in some, degeneration was as high as 50%, in others it was negligible. Individual d was practically free from spermatid degeneration in any of the follicles and formed sperm bundles as large and as compact as those of the parent races.

Secondly, there are the hybrids between T. cristatus and T. maculatus studied by White (1946). The hybrids were obtained in captivity by natural fertilization, the parent species being closely allied though taxonomically distinct. Hybrid females are fertile, the males, which were F<sub>1</sub> and "double back-cross" individuals, showed normal spermatogenesis as far as the first meiotic prophase. First meiotic metaphase is characterized by a large reduction in chiasma frequency as compared with the parents, the chiasmata being restricted to the chromosome ends, and failure of bivalent formation on a large scale. Only 2-3% of the first meiotic divisions are free from univalents. There is an arrest of development at first metaphase and the subsequent anaphase, in which some diploid interkinetic nuclei are formed. The interkinetic nuclei pass to a second meiotic division characterized by extreme variability of chromosome number. With the exception of a few cells in one individual only, all the spermatid nuclei formed during the second meiotic division degenerate.

White, in his study of the hybrids of T. cristatus and T. maculatus, in 1946, found that the hybrids are fertile and produce viable offspring. Degeneration is entirely post-meiotic, and all the hybrid sperm are of normal size and structure.

Any attempt at a cytological evaluation of the

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## 6. Discussion

Three types of newt hybrids have now been examined cytologically. Firstly we have the hybrids T.cristatus carnifex x T.vulgaris meridionalis studied by Benazzi and Lepori (1949). The parent species are widely separated taxonomically and fertilisation can only be brought about by artificial means. The hybrids are completely sterile and form no sperm: extensive degeneration sets in among early spermatogonia so that most testis tubules are completely devoid of germ cells. When germ cells are present they undergo arrest at first meiotic metaphase, where the majority of the chromosomes are present as unpaired univalents. Degeneration either sets in at this stage or, very rarely, after an irregular second division has been accomplished.

Secondly there are the hybrids between T.cristatus and T.marmoratus studied by White (1946). The hybrids were obtained in captivity by natural fertilisation, the parent species being closely allied though taxonomically distinct. Hybrid females are fertile, the males, which were  $F_1$  and "double back-cross" individuals, showed normal spermatogenesis as far as the first meiotic prophase. First meiotic metaphase is characterised by a large reduction in chiasma frequency as compared with the parents, the chiasmata being restricted to the chromosome ends, and failure of bivalent formation on a large scale. Only 2-3% of the first meiotic divisions are free from univalents. There is an arrest of development at first metaphase and the anaphase sometimes fails, in which case diploid interkinetic nuclei are formed. The interkinetic nuclei pass to a second meiotic division characterised by extreme variability of chromosome number. With the exception of a few cells in one individual only, all the spermatid nuclei deriving from the second meiotic division degenerate without forming sperm.

Thirdly, we have the inter-racial hybrids of T.cristatus described in the present paper, in which the upsets to spermatogenesis are on a much smaller scale. Degeneration is entirely post-meiotic, and all the hybrids form sperm to a greater or lesser extent.

Any attempt at a statistical evaluation of the

gap of belief.

fertility of newts is impossible. The fertility of a male can be evaluated only by the cooperation of a fertile female. The fertility of a female is similarly dependent on the cooperation of a fertile male : a female rarely spawns unless she has been courted ; if the courtship was insufficient to stimulate her to pick up a spermatophore, she consequently produces infertile eggs or eggs fertilised by a long previous mating. To add to the complexity, numerous psychosomatic stimuli disturb a newt during the breeding season.

Of the 9 females of karelinii ♀ x cristatus ♂ born in 1946, no animal spawned before 1949, when only one, successfully mated with her brother, spawned. One of the others was with a karelinii ♂ and one with a cristatus ♂. The remainder were with brothers showing various degrees of sexual development. It is clear that this hybrid shows much of the laboratory sterility characteristic of subspecies cristatus.

Of the 4 females of the 1948 family carnifex ♀ x karelinii ♂ mated under observation, three spawned in 1949. One F<sub>1</sub> pair and one backcross to carnifex ♂ were fertile : one F<sub>1</sub> pair was sterile and a similar attempted backcross produced no spawn. The 32 animals of this F<sub>1</sub> which were reared at 20°C. were all nubile at a year old and could have bred given optimal conditions both psychological and physical.

7 females of carnifex ♀ x cristatus ♂ born in 1948 spawned in 1949 and others may have done so in the community tank for females. It seems likely that all females would have been mature at a year old, but for epidemics in various colonies. One fertile F<sub>2</sub> and three backcrosses to carnifex ♂ were obtained. Only 2 males out of about 25 showed reasonable secondary sexual development, the others showing the arrested secondary sexual characters typical of subspecies cristatus in confinement.

This is the first record of crested newts breeding at one year old : it is due to their having been kept at 20°C. throughout the winter. 21 animals of the cross carnifex ♀ x karelinii ♂ kept in unheated rooms showed no secondary sexual characters until September 1949 but by November were indistinguishable from their sibs which had bred in the spring. The same developmental timing has been observed in colonies of T.c.carnifex.

The three 1949  $F_2$  families, in the order referred to above, contained 4, 24 and 4 young counted as fortnight old larvae. None of these survived metamorphosis. The four 1949 backcross families, in comparable order, contained 32, 49, 24 and 3 larvae at the age of a fortnight, of which 10, 19, 2 and 1 metamorphosed. These heterogeneous figures give an overall mortality of 79%. The small numbers in some families are characteristic of the first spawning of newts.

The only other families reared under similar conditions were the two 1948  $F_1$  families, where out of 55 and 63 young larvae 53 and 53 respectively metamorphosed; and 1949 carnifex ♀ x danubialis ♂ and carnifex ♀ x ♂, both from two year old mothers spawning for the first time, in which 14 out of 14 and 3 out of 8 metamorphosed; an overall mortality of 12%.

In the absence of controls it is not possible to state that  $F_1$  families are exceptionally viable. It is also ~~questionable~~ <sup>uncertain</sup> whether backcross families are exceptionally inviable: as might be expected, they show a mortality approaching that found in the  $F_2$ . From earlier data which is less directly comparable we consider that both crosses show a genuine divergence from the typical picture of larval and metamorphic ~~mortality~~ viability of this and other species of newts.

It should be emphasised that no fertile matings have yet been made between  $F_1$  males and females of a ~~species~~ species. The backcrosses may be taken as measuring the viability of female pronuclei formed by hybrid females. On the existing data it is possible, either that ~~backcross~~ backcrosses of hybrid males to the pure species would show about the same low viability as the backcrosses of hybrid females, or that they would be completely inviable.

The complete mortality of the  $F_2$  family from karelinii ♀ x carnifex ♂ is significant. It is what might be expected from the observations on spermatogenesis. It is probable that larval mortality takes the place of hybrid sterility as a barrier to subspecies crossing, as embryonic mortality does in the hybrids between members of the Artenkreis Pleurodeles (Steiner, 1942 and 1945)

At first sight one might be tempted to imagine that the spermatid degeneration of the inter-racial

hybrids is the direct result of genic unbalance in the haploid cells resulting from irregularities of ~~xx~~ segregation at meiosis. This is certainly not the case. There is no correlation between frequency of univalent formation and degree of spermatid degeneration, a correlation which would be expected were genic unbalance in the haploid the cause of degeneration. White arrived at the same conclusion as regards his interspecific hybrids.

The interspecific and inter-racial newt hybrids in fact manifest three grades of spermatogenetic upset which are accountable to differences of degree in the genetic unbalance of diploids : the wider the cross, the earlier the onset of degeneration and the greater the degree of sterility.

Translocations and inversions are detectable in those parts of the chromosomes of the inter-racial hybrids which succeed in forming chiasmata. These will contribute their quota to the general reduction of fertility in the hybrids. What is the situation in those parts of the chromosomes where chiasmata fail to form? It might be argued that the middle regions of the chromosomes of the hybrids are so unlike in structure that pairing fails for this very reason. However we must beware of making this deduction : T.palmatus has an even greater degree of terminal chiasma localisation than have the hybrid newts, and this within the normal complement of the species ! It therefore seems more likely that a "time limit" in the sense of the term used by Darlington (1935) is operative during the pairing of the chromosomes in the hybrids. It is significant that the type of chiasma localisation occurring in the hybrids conforms to that present in T.palmatus and T.vulgaris. Evidently terminal initiation of pairing is the rule in the genus Triturus.

Can the presence of translocations in T.c.karelinii compared with the other two subspecies be considered a taxonomic character ? As Professor Sewall Wright emphasises, it is difficult to imagine, on theoretical grounds, how a translocation arising in one animal in a large mating population could survive, let alone spread, so as to become characteristic of the population : the selection against heterozygotes would be so great.

Translocations could survive in populations subject to intense inbreeding in the first few generations after their origin, e.g. if an animal carrying the translocation was one of the first pair to colonise a pond or similar discrete habitat. The early history of what is known to European herpetologists as Stamm Kammerer, especially Mr Lantz's colony, is diagrammatically the history of such a pattern of population growth and the translocations may be something peculiar to it. It would be reproductively isolated from all wild T.c. karelinii in the same way as it is isolated from wild T.c. cristatus and T.c. carnifex and must be considered a domestic animal. The sporadic occurrence in Stamm Kammerer of animals with apparent gonadic sterility suggests that the stock is not quite normal.

We reject this hypothesis because in one cell of karelinii ♀ x cristatus ♂<sup>♂</sup>, individual 9, two multivalents were observed. Translocations cannot be common among newts since they have never been recorded in wild animals. The coincidence, that an exceptional animal containing a translocation was given a chance to be fertile solely by falling ~~into~~ into the care of a herpetologist is great. If two translocations are present it becomes too great to be considered seriously.

It therefore seems unlikely that subspecies karelinii, at least, arose by a large population becoming geographically isolated, perhaps by one of the ice-sheets, and thus reproductively isolated, so that genetic divergence occurred. There is an alternative. Since almost all of the Asiatic range of T.c. karelinii was under some ice sheet during the Pleistocene, it is possible that this ~~range~~<sup>area</sup> was colonised comparatively recently by the descendants of a few individuals who may have contained the translocations, and that the subspecies only subsequently spread into Europe making its range continuous with those of T.c. carnifex and T.c. danubialis. Dr B. Rensch considers that the Caucasian ice sheet merely displaced the fauna from the foot and slopes of the mountains: the fauna moved away and returned on the retreat of the glaciation. A post-glacial colonisation by a few individuals is improbable on this view.

Whatever the mechanism involved, the geographical



barrier was removed before a complete sterility barrier had arisen, thereby permitting zones of genetic interchange to be re-established. Zones of intermediacy seem objective and they cover large geographical areas. The magnitude of the sterility barriers, both gametic and developmental, which we have found, therefore seem surprising.

Finally, it is important to emphasise that Kammerer's animals may have been captured in an area of isolated populations, where the newts of one pond could have a chromosomal arrangement radically different from those of the next, and without loss of fertility. In view of the possible existence of chromosomal polytypicism it is important that wild T.c.karelinii, comparable with the T.c.cristatus and T.c.carnifex ~~material~~ <sup>material</sup> should be recaptured. It is similarly important that animals from different areas within the range of a single subspecies should be investigated.

We wish to thank Professor J.B.S.Haldane for a number of useful suggestions incorporated in this paper.

## 7. Summary.

The spermatocytes of three geographical races of the newt Triturus cristatus, T.c.cristatus, T.c.carnifex and T.c.karelinii form many chiasmata whose positions in the bivalents are not markedly localised. Mean chiasma frequencies per cell range from 30 - 40, T.c.carnifex forming the fewest and T.c.karelinii the most chiasmata.

The three kinds of inter-racial hybrids which have been studied, karelinii ♀ x cristatus ♂♂, carnifex ♀ x cristatus ♂ and carnifex ♀ x karelinii ♂ are similar to one another in forming about half as many chiasmata per spermatocyte as are formed by the parent races. Mean chiasma frequencies per cell range from 15 - 21. The chiasmata which are formed lie for the most part in the terminal regions of the chromosomes. Univalent chromosomes are found to a varying extent in all the hybrids, these resulting from total chiasma failure.

Multivalent chromosome associations occur in the hybrids involving T.c.karelinii. The chromosome complement of this subspecies differs from that of T.c.carnifex by at least one translocation and from that of T.c.cristatus by at least two. These translocations give rise to genetic duplications and deficiencies on segregation at meiosis. The races also differ by virtue of inversions, some of which may be small in size and terminal in position.

The presence of univalents which fail to co-orientate at meiosis delays to varying degrees the completion of the first anaphase; the normal follicular synchronism is thereby lost.

Spermatid degeneration may set in after the second meiotic division: the extent to which it occurs is exceedingly variable from one individual to another and from follicle to follicle within a single individual: it bears no relationship to the frequency of univalent chromosomes at meiosis. To varying degrees all the hybrids form sperm.

The F<sub>1</sub> hybrids are vigorous; F<sub>2</sub> and backcross hybrids, on the other hand, have a high larval and metamorphic mortality. In view of the magnitude of the sterility barriers to hybridisation between the races, it is surprising that large zones of intermediacy occur in nature.

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TABLE 1.

## FREQUENCY DISTRIBUTION OF TOTAL CHIASMATA PER CELL IN THE PARENT RACES.

Newt	Chiasmata per cell														Mean chiasmata per cell	Variance	Stanford error $\pm$ *					
	26	27	28	29	30	31	32	33	34	35	36	37	38	39				40	41	42	43	44
<i>Triturus c. carnifex</i> 1.		1		1	4		2	1	1											30.7	4.2	0.6
" " 2.				2	1	2	1	1	2			1								32.1	7.7	0.9
" " 3.			1	1	3	3		1	1											30.7	3.1	0.6
" " 4.	1	1		1	1		2	1	2	1										31.2	9.5	1.0
<i>Triturus c. cristatus</i> a.								2	1		2	1	3	1						37.2	4.8	0.7
" " b.										2	2		3	3						38.5	2.5	0.5
" " e.								2	3	3	2									36.5	1.2	0.3
<i>Triturus c. karelinii</i> a.														1	2	3	2	2		42.2	1.7	0.4
" " b.										1	2	1	1	1	1	2	1			39.5	6.1	n 0.8
										1	1	1	1	1	1	1	1			37.5	6.1	0.8

TABLE 2.

## FREQUENCY DISTRIBUTION OF TOTAL CHIASMATA PER CELL IN THE INTER-RACIAL HYBRIDS

Newt	Chiasmata per cell																								Mean chiasmata per cell	Variance	Standard Error $\pm$	
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32				
T.c.karelinii x T.c.cristatus	9.			1	1				2		3	2		4			4	1	2							21.2	18.7	1.0
T.c.carnifex x T.c.cristatus	a.								1	1	1		5		5	1	3		1	1		1				21.9	10.5	0.7
"	"	c.				1	2	2	5	1	4	1	1	2		1										17.2	6.9	0.6
"	"	d.						1		1	2	1	2	2	2	6		2					1			21.2	8.0	0.6
"	"	e.	2	1	1	2	2		3	2	1	1	1	2	2											16.1	15.4	0.9
T.c.carnifex x T.c.karelinii	b.		2		2	2	3	2	3	2		2	2													15.1	9.0	0.7
"	"	c.	1				1	1	3	1	2	2	2	2	1	1	1	1	1							19.0	15.8	0.9
"	"	d.						2	2		1	3		2	2	1	2	2	2	1						21.2	15.0	0.9
"	"	e.			1	1		1		3	1	2	2	7	1	1										19.0	8.9	0.7
"	"	f.	1	1			1	1	3	3	2	3	1	1	1	2										16.6	12.1	0.8

TABLE 3.

FREQUENCY DISTRIBUTION OF UNIVALENTS IN THE INTER-RACIAL HYBRIDS

Newt	Number of univalents per cell													Mean univalents per cell		
	0	1	2	3	4	5	6	7	8	9	10	11	12			
T.c.karelinii x T.c.cristatus	9.	9		5	2	3							1			1.9
T.c.carnifex x T.c.cristatus	a.	11		7		2										1.1
" "	c.	4		12		3		1								2.1
" "	d.	13		5		2										0.9
" "	e.	3		8		3		6								3.2
T.c.carnifex x T.c.karelinii	b.			6		7		5		2						4.3
" "	c.	6		8	1	2		2			1					2.45
" "	d.	10		3		5		2n								1.9
" "	e.	5		9		5					1					2.4
" "	f.	1		5	1	9		1	1	1				1		4.1

## LEGENDS TO TEXT FIGURES

### Text Fig. 1.

First meiotic metaphase chromosomes in three geographical races of the newt Triturus cristatus :

- (a) Triturus cristatus carnifex; 35 chiasmata; individual 2.
- (b) T.c.carnifex; 30 chiasmata; individual 2.
- (c) T.c.cristatus; 34 chiasmata; individual e.
- (d) T.c.cristatus; 39 chiasmata; individual e.
- (e) T.c.karelinii; 44 chiasmata; individual a.
- (f) T.c.karelinii; 39 chiasmata; individual b.

From squash preparations fixed in 3:1 Alcohol-Acetic Acid and stained in Iron Aceto-Carmine.

### Text Fig. 2.

First meiotic metaphase chromosomes in inter-racial hybrids of the newt Triturus cristatus :

- (a) T.c.karelinii x T.c.cristatus; 25 chiasmata, no univalents; individual 9.
- (b) T.c.karelinii x T.c.cristatus; 16 chiasmata, 2 univalents; individual 9.
- (c) T.c.carnifex x T.c.karelinii; 15 chiasmata, 2 univalents; individual 6.
- (d) T.c.carnifex x T.c.karelinii; 17 chiasmata, 2 univalents; individual 6.
- (e) T.c.carnifex x T.c.cristatus; 14 chiasmata, 4 univalents; individual c.
- (f) T.c.carnifex x T.c.cristatus; 10 chiasmata, 10 univalents; individual e.

From squash preparations fixed in 3:1 Alcohol-Acetic Acid and stained in Iron Aceto-Carmine.

### Text Fig. 3.

Multivalent chromosome associations at first meiotic metaphase in newt inter-racial hybrids :

- (a) T.c.karelinii x T.c.cristatus; 17 chiasmata, 2 quadrivalents, 2 univalents; individual 9.
- (b) T.c.carnifex x T.c.karelinii; 16 chiasmata, 1 trivalent, 3 univalents; individual c.

From squash preparations fixed in 3:1 Alcohol-Acetic Acid and stained in Iron Aceto-Carmine.



Text Fig. 4.

Late anaphase of first meiosis in newt inter-racial hybrids, showing bridges ;

- (a) T.c.carnifex x T.c.cristatus; inversion bridge and fragment; individual c.
- (b) T.c.carnifex x T.c.karelinii; inversion bridge and fragment; individual e.
- (c) T.c.carnifex x T.c.karelinii; bridge and no visible fragment; individual e.
- (d) T.c.carnifex x T.c.karelinii; bridge and no visible fragment; individual d.

From sections fixed in San Felice's fluid and stained in Gentian Violet.

Plate 2.

Sections of newt testes, fixed in San Felice's fluid and stained in Gentian violet, selected to show tubules with various spermatocytes. All x 50.

1. T.c.karelinii; note the large sperm bundles; individual d.
2. T.c.karelinii x T.c.cristatus; sperm bundles were less as large as those of T.c.karelinii; individual e.
3. T.c.karelinii x T.c.carnifex; tubules mostly filled with sperm cells in spermatids and a few small bundles of sperm; individual e.
4. T.c.carnifex x T.c.cristatus; tubules contain even fewer sperm than in 3; individual e.

## LEGENDS TO PLATES

### Plate 1.

"Squash" preparations of newt first meiotic metaphase, fixed in 3:1 Alcohol-Acetic Acid and stained in Iron Aceto-Carmine. All x 750.

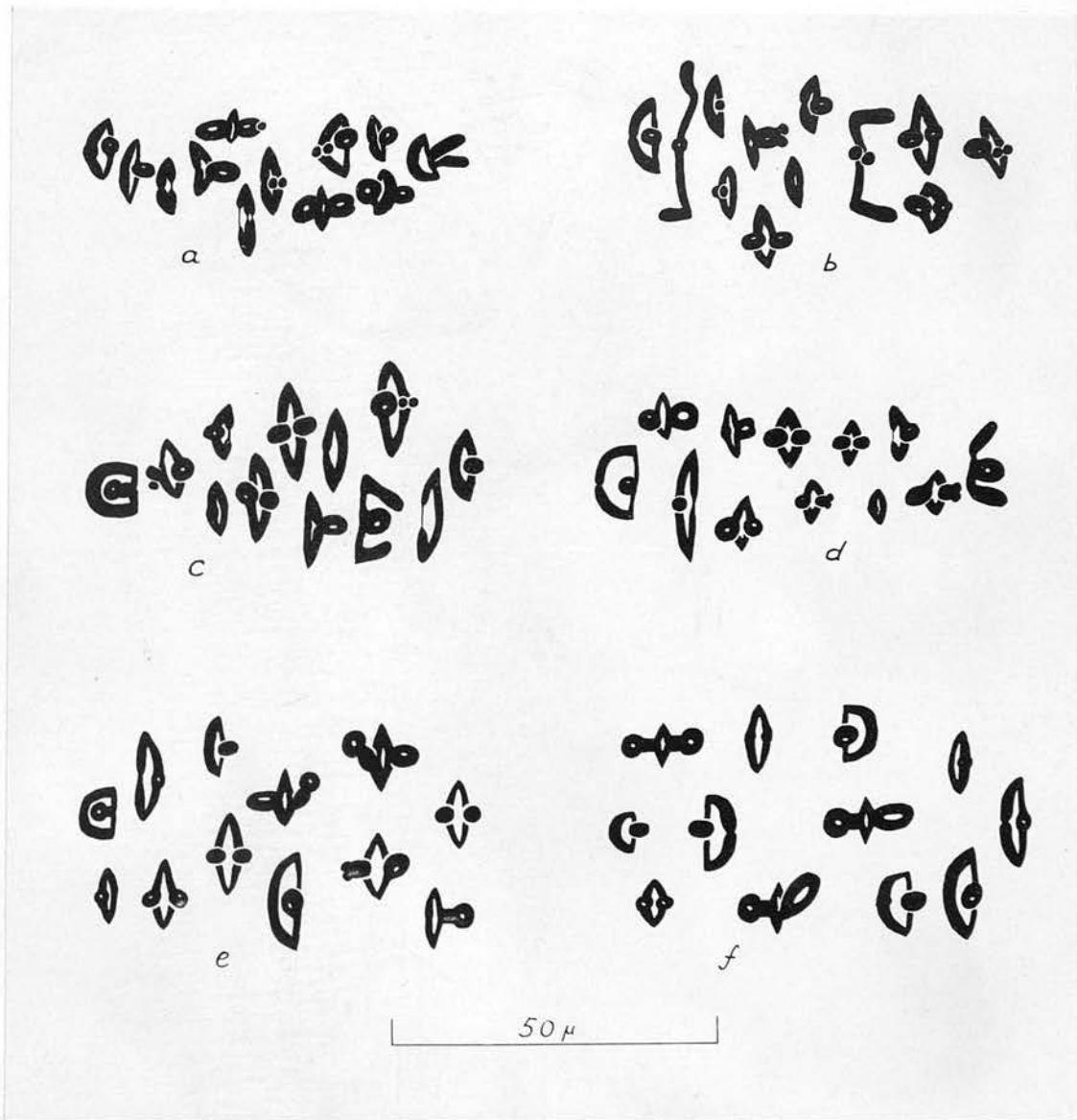
1. T.c.carnifex; 32 chiasmata; individual 3.
2. T.c.cristatus; 34 chiasmata; individual e.
3. T.c.karelinii; 40 chiasmata; individual a.
4. T.c.carnifex x T.c.cristatus; 15 chiasmata, 2 univalents; individual c.
5. T.c.carnifex x T.c.karelinii; 13 chiasmata, 4 univalents; individual b.
6. T.c.karelinii x T.c.cristatus; 24 chiasmata; individual 9.

### Plate 2.

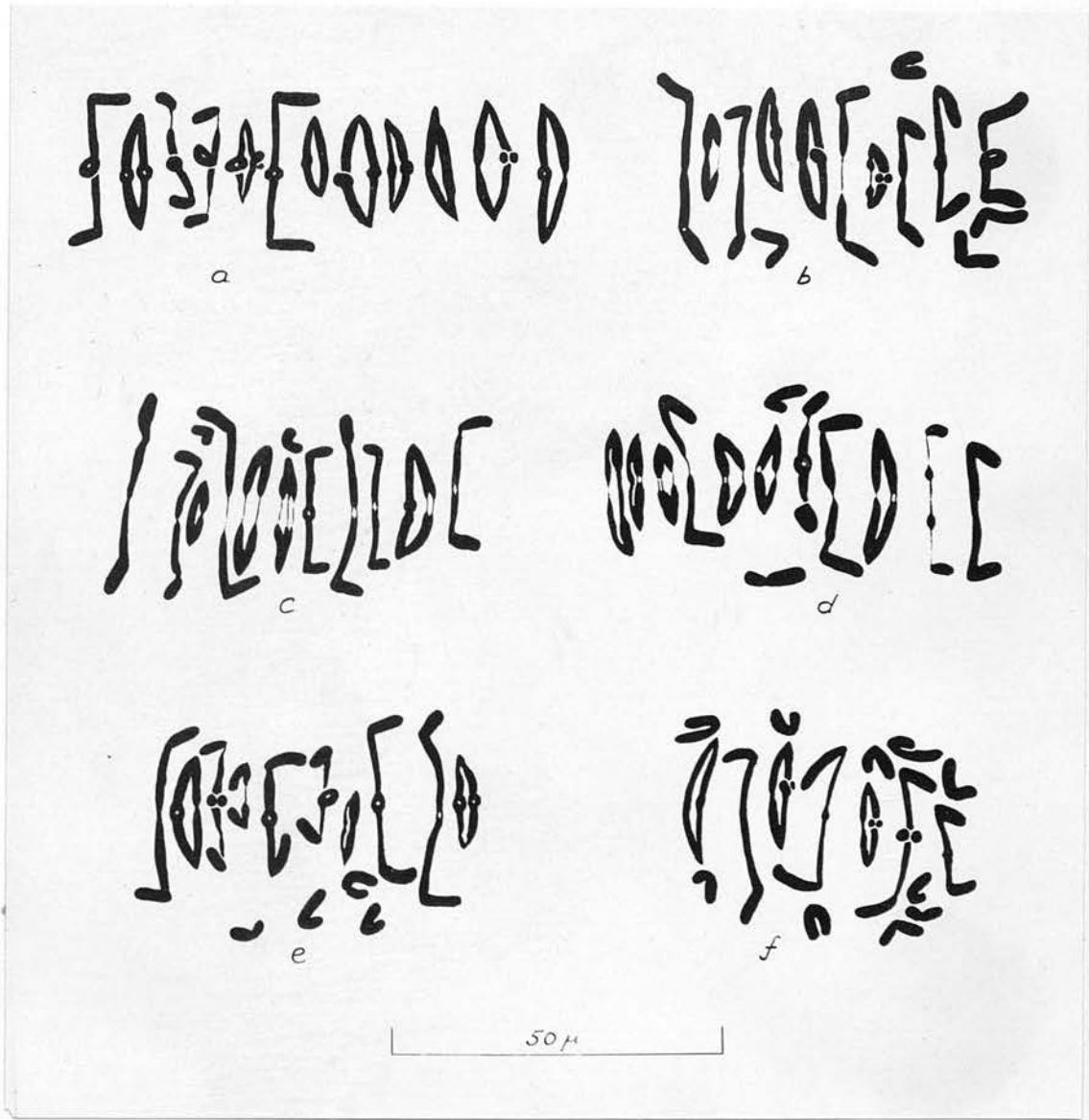
Sections of newt testes, fixed in San Felice's Fluid and stained in Gentian Violet, selected to show tubules with maturing spermatozoa. All x 60.

1. T.c.karelinii; note the massive sperm bundles; individual a.
2. T.c.karelinii x T.c.cristatus; sperm bundles more or less as large as those of T.c.karelinii; individual 9.
3. T.c.carnifex x T.c.karelinii; tubules loosely filled with degenerating spermatids and a few small bundles of sperm; individual b.
4. T.c.carnifex x T.c.cristatus; tubules contain even fewer sperm than in 3; individual e.

TEXT FIGURE 1



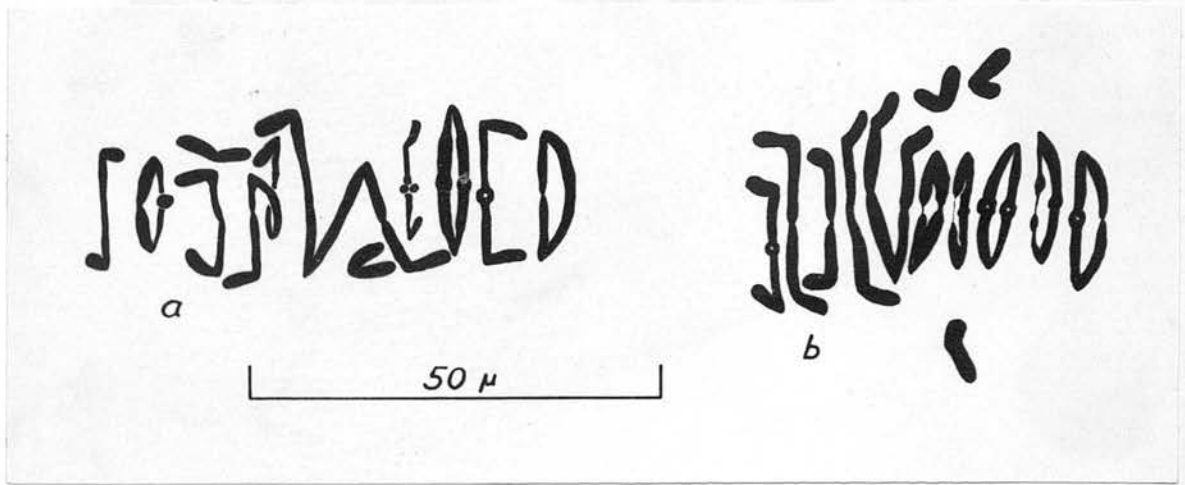
TEXT FIGURE 1.



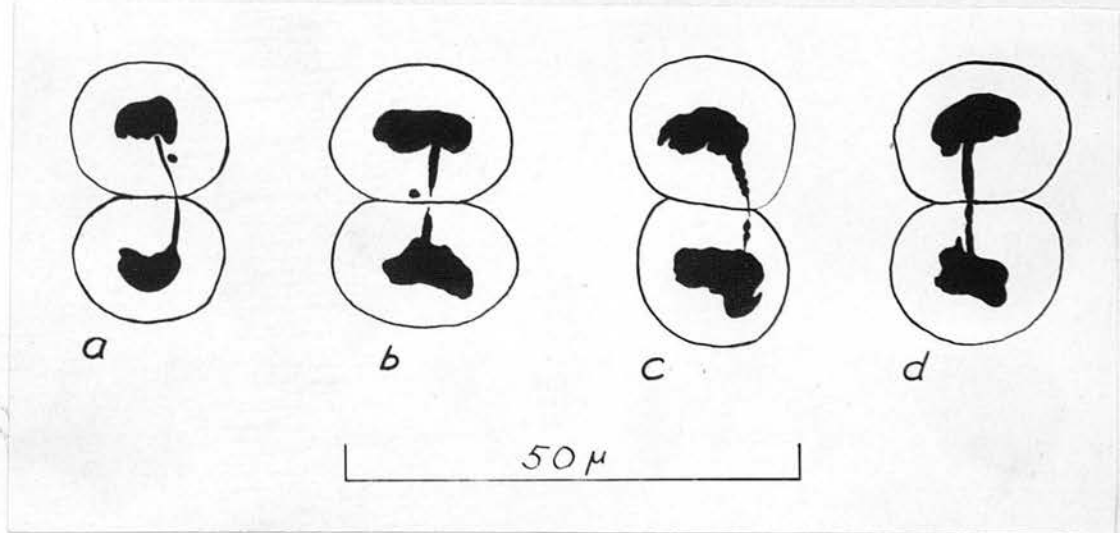
TEXT FIGURE 2.

TEXT FIGURE 2

PLATE 1



TEXT FIGURE 3.



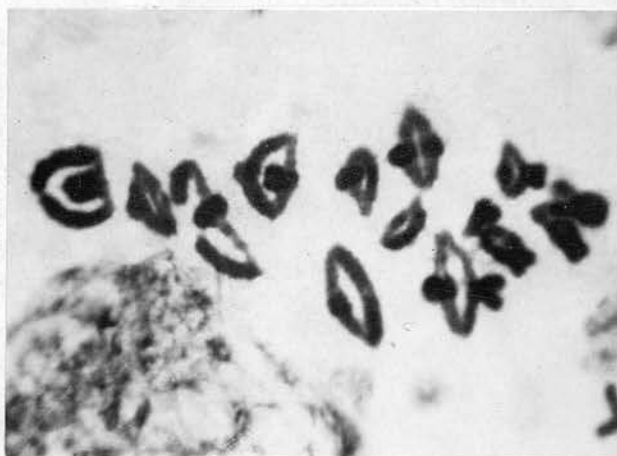
TEXT FIGURE 4.

PLATE I.

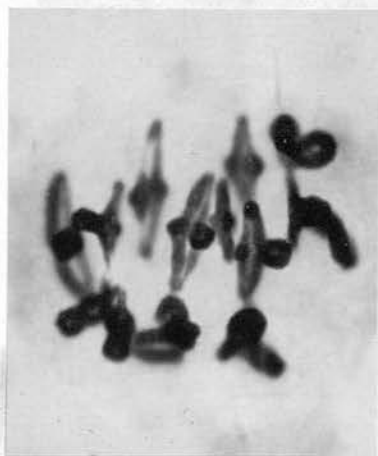
1.



2.



3.



4.



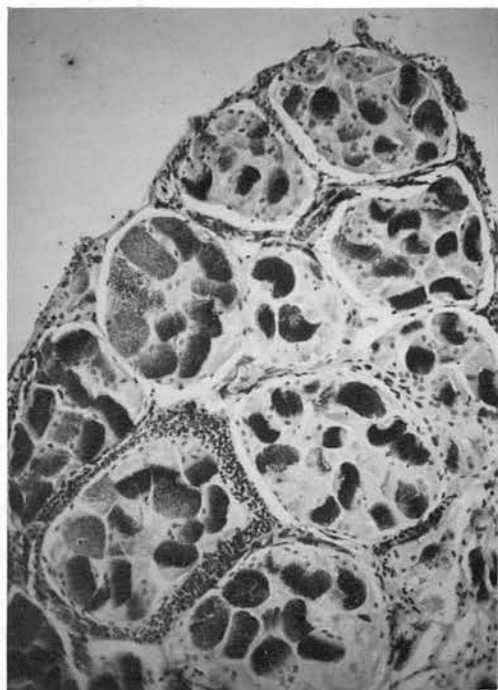
5.



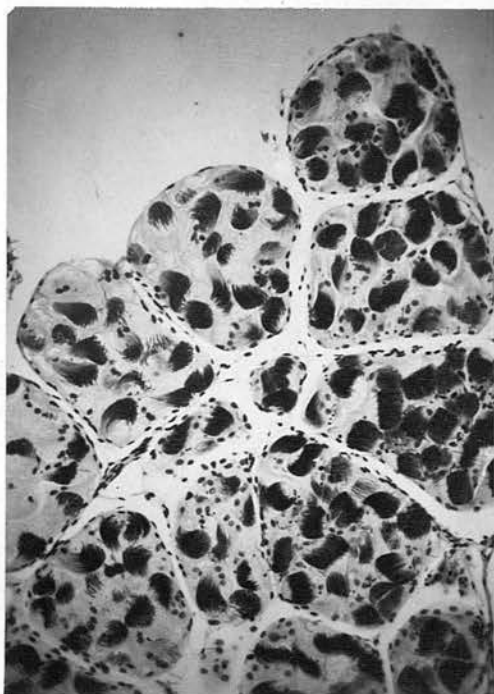
6.



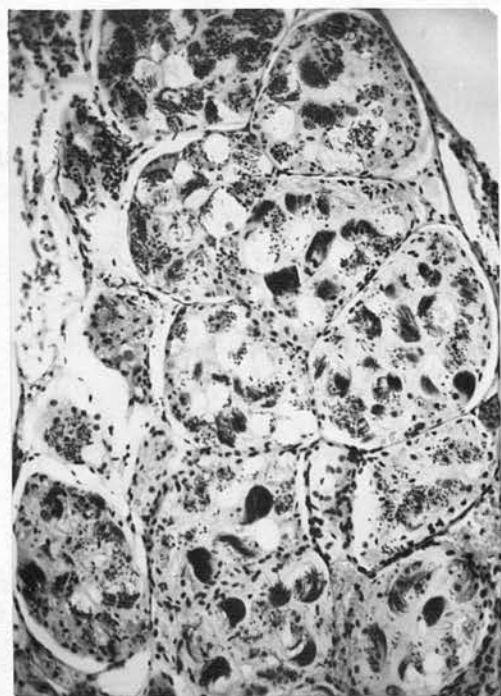
PLATE 2.



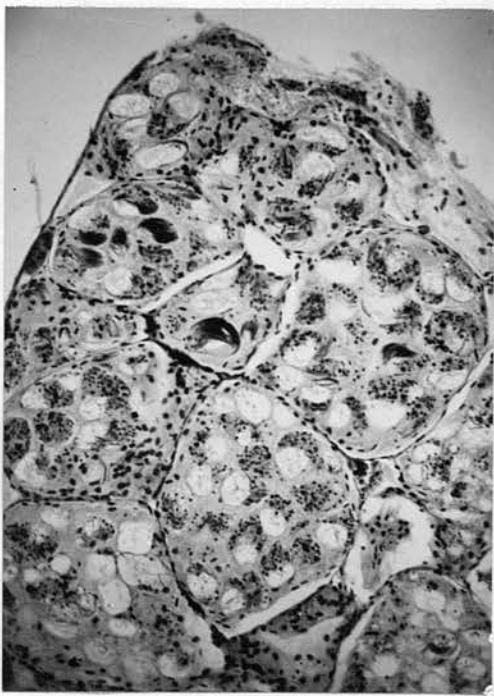
1.



2.



3.



4.

In the past few years there has been considerable interest in the chemical nature of the sap of the nuclear sapwood, particularly in the case of the Douglas fir. This interest has been stimulated by the discovery that the sap of this tree contains a substance which is highly resistant to chemical degradation, and which has been shown to be a complex of the products of the action of the enzyme ligninase on lignin.

THE CHEMICAL NATURE OF NUCLEAR SAP

by

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The nuclear sap of Douglas fir (Picea sitchensis) was prepared by the method of Callan and Leaf (1949). The sap was obtained from the nuclear sapwood of trees which had been cut in the autumn and stored in a cool, dark place for several months. The sap was then extracted from the wood by the method of Callan and Leaf (1949). The sap was found to be a complex of the products of the action of the enzyme ligninase on lignin. The chemical nature of the nuclear sap was investigated by the method of Callan and Leaf (1949). The results of the investigation are given in the following table.

Element	Percentage
Carbon	50.0
Hydrogen	6.0
Oxygen	44.0

The nuclear sap was found to be a complex of the products of the action of the enzyme ligninase on lignin. The chemical nature of the nuclear sap was investigated by the method of Callan and Leaf (1949). The results of the investigation are given in the following table.



THE CHEMICAL NATURE OF NUCLEAR SAP.

In the past few years there has been considerable argument as to the disposition within cell nuclei of the chemical substances, predominantly proteins, histones and nucleic acids, which can be extracted from isolated nuclei by chemical procedures. (1,2,3,4) It is the purpose of the present communication to describe two preliminary experiments which provide information as to the chemical constitution of nuclear sap.

The first experiment has established the ultra-violet absorption curve of a bulk preparation of nuclear sap from mature oocytes of Xenopus laevis. 100 nuclei were isolated and cleaned in 0.2 M potassium hydrogen phosphate saturated with magnesium hydrogen phosphate, at pH 6.4. In this medium the disperse phase of the nuclear sap colloids distends on isolation: the structural phase, on the other hand, remains unchanged in size and maintains the disposition of chromosomes and nucleoli as in life. The nuclei were subsequently suspended in phosphate at pH 7.2, when the structural phase loses its rigidity and passes into colloidal solution. The nuclear membranes were now broken by bubbling a gentle stream of air through the suspension. The membranes, which are hydrophobic, come to lie at the air-water interface or stick to the glass walls of the container. The container was now lightly centrifuged, the nucleoli and chromosomes collecting at the bottom of the tube. The supernatant, which consists of a solution of nuclear sap, was pipetted off into a quartz cell 20mm <sup>x 10 mm</sup> x 2.5mm. The tube was rinsed once with phosphate, recentrifuged and the second supernatant added to the first. The total volume of collected fluid was 0.25ml. The ultra-violet absorption curve (fig 1.) was obtained by means of a Beckmann spectrophotometer, the control fluid being phosphate solution without nuclear sap.

It may be concluded from the shape of the absorption curve that the nuclear sap contains little or no purines or pyrimidines, the broad peak at 2700-2800A being characteristic of cyclic amino-acids. This result confirms the observations on the absorption of nuclear sap determined by means of <sup>the</sup> ultra-violet microscope and circumvents some of the criticisms which have been levelled at the latter method.

The second experiment consists in a preliminary amino-acid determination by paper chromatography of the acid hydrolysate of nuclear sap. 500 nuclei from mature oocytes of Triturus cristatus were isolated and cleaned in an aqueous unbuffered solution consisting of 19 parts 0.2M potassium chloride and 1 part 0.2M magnesium chloride. After collection of the nuclei, the membranes were broken and discarded as described above and the suspension lightly centrifuged. The supernatant was pipetted off and dried over a water bath at 100°C. The material was stored in a vacuum dessicator. Prior to chromatographic runs, the dry material was taken up in the minimum quantity of water (0.5ml.) and the proteinaceous matter precipitated by the addition of 0.05ml. of 90% trichloroacetic acid. The precipitate was collected by centrifugation and washed with 0.5ml. of 10% trichloroacetic acid, the supernatants being discarded. (The supernatants gave a negative ninhydrin reaction). The precipitate was now hydrolysed with 6 N hydrochloric acid at 105°C for 16 hours. The resultant hydrolysate was repeatedly evaporated and the residue, which contained a certain amount of salt, was extracted with 95% alcohol, the extract being subjected to two-dimensional chromatography on Whatman No.1 paper with phenol/water/0.1% ammonia followed by n-butanol/acetic acid/water.

A tracing of the developed chromatogram is shown in fig.2. The chromatogram was not ideal as it was overloaded and suffered some distortion, probably owing to salt. However, the result suggests that the nuclear sap is complex in its amino-acid composition. Tryptophan is unfortunately destroyed by acid hydrolysis so that we cannot say whether or not it is a constituent of nuclear sap. It was also impossible with the present technique to determine the presence or absence of cystine and methionine. These questions will be examined at a later date. It is difficult to judge the relative quantities of the various amino-acids but the amount of arginine plus lysine plus histidine does not appear to be nearly as great as would be expected were basic proteins such as histone present in considerable proportion. Moreover the nuclear sap contains glycine and Threonine these acids have not been found in calf thymus and rat liver preparations. Finally, as a check on the ultra-violet absorption curve, the chromatogram was photographed in ultra-violet light. The photographs were a complete blank though control spots of purines and pyrimidines under the same conditions and containing 10µg. were readily

detectable.

It may be concluded from these experiments, which are of a preliminary nature only, that the nuclear sap of amphibian oocytes nuclei contains no detectable nucleic acid nor does it contain a large proportion of basic proteins. We would emphasise that these conclusions apply only to the nuclei which we have studied : these have been selected on account of the ease with which they may be isolated and fractionated. It may be that the nuclear sap of other nuclei has other constituents.

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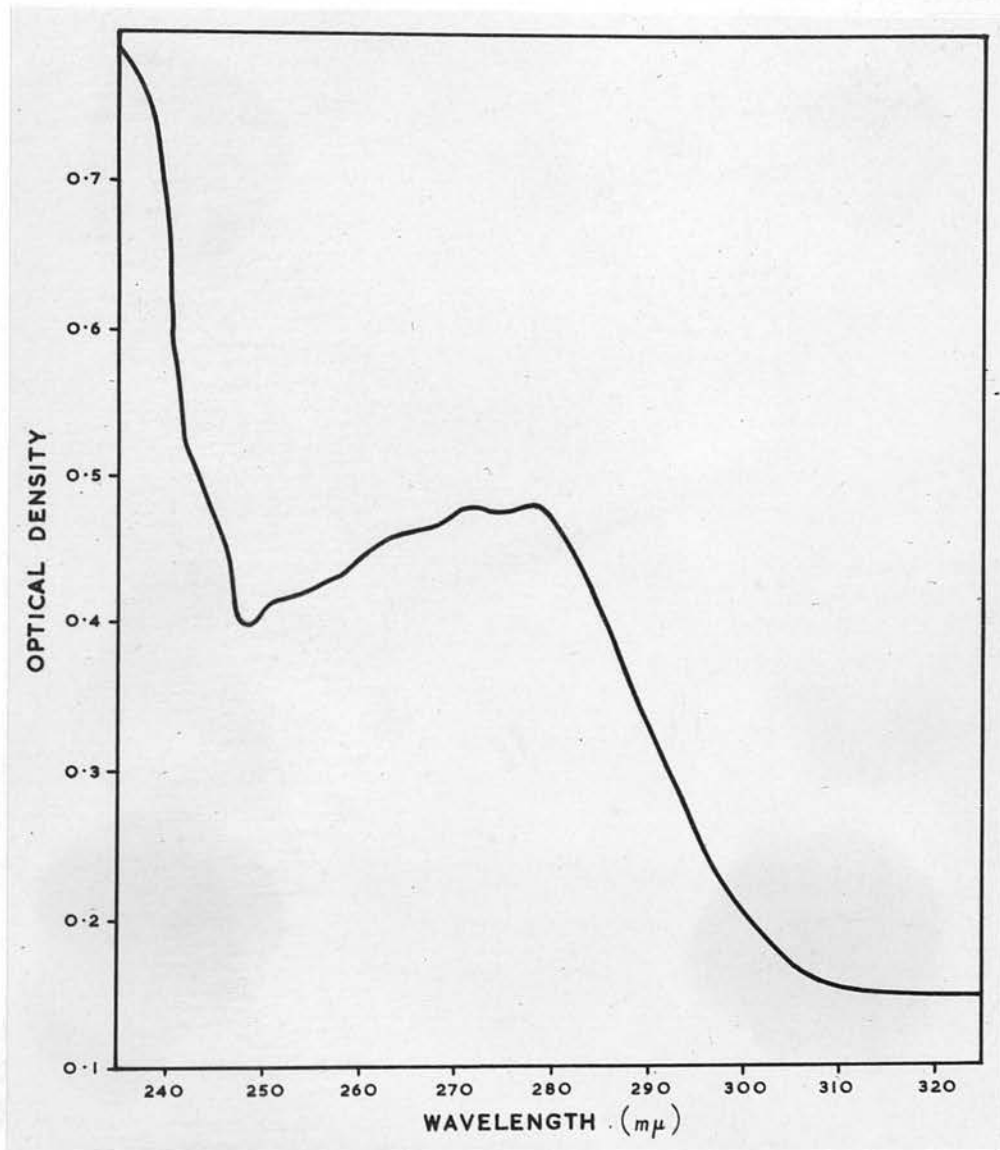


Fig.1. Ultra-violet absorption spectrum of nuclear sap.

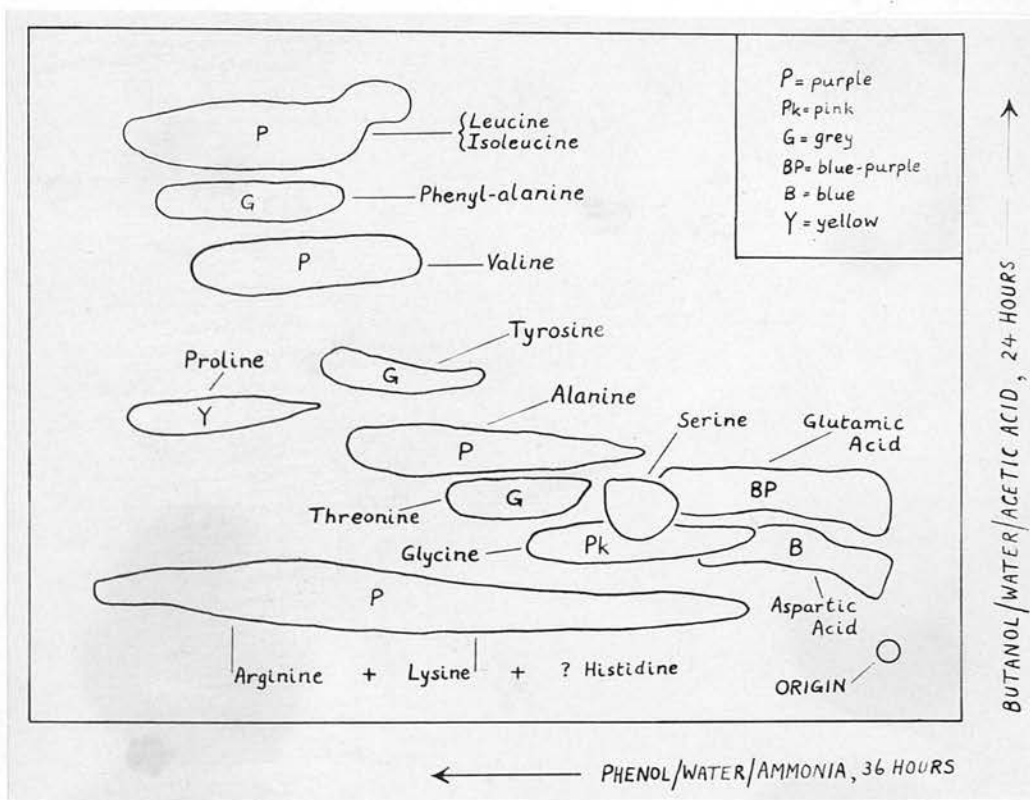


Fig.2. Tracing of paper chromatogram of acid hydrolysate of nuclear sap. The colour differentiation was produced by 5% collidine in the ninhydrin spray.

SUMMARY

The membrane surrounding the nucleus of the oocytes of two species of amphibians, *Rana lessonae* and *Rana temporaria*, has been studied by electron microscopy. The membrane is shown to consist of two layers, an outer layer of which is approximately 100 Å thick and an inner layer which is about twice as thick as the outer layer. The inner membrane is shown to be continuous with the nuclear envelope. The pores of the outer membrane are approximately 100 Å in diameter and are distributed in a regular pattern.

EXPERIMENTAL STUDIES ON AMPHIBIAN OOCYTE NUCLEI.

1. INVESTIGATION OF THE STRUCTURE OF THE NUCLEAR MEMBRANE BY MEANS OF THE ELECTRON MICROSCOPE.

by

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Manuscript submitted to Proceedings of the Royal  
Society, January, 1950.

## SUMMARY

The membrane surrounding the nucleus of the oocytes of two species of amphibian is shown to consist of two structures, an outer porous layer and an inner layer which is apparently continuous. The porous layer is about twice as thick as the inner layer, the dried membrane as a whole having a thickness of approximately 500 A. The pores are of regular size and arrangement in the outer layer: pore diameter is approximately 400 A, the separation distance between pore centres 1000 A. Both layers consist of relatively insoluble protein materials, the porous layer also containing some lipid.

As recently as 1937, Paschinger has denied the existence of an organized membrane surrounding the nucleus. He regards the apparent membrane as a physical interface, and nothing more. Yet the simplest experiments in which a number of a wide variety of cells supply convincing evidence that the nuclear membrane is an organized structure with detectable mechanical strength. If, by means of a micro-manipulator, one attempts to puncture a cell such as an amoeba or salivary gland cell of *Drosophila*, the needle meets resistance at the plasma membrane, having overcome this resistance, the needle moves relatively freely in the cell interior until it reaches the nuclear membrane. Here further resistance is encountered, the nuclear membrane has been punctured, the needle again moves relatively freely inside the nucleus.

Paul (1943) has reviewed the evidence for the existence of a nuclear membrane in all cells containing nuclei. He points out that whereas in certain cells the membrane is clearly visible in the living state, in other cells its existence can only be inferred by indirect means.

During most biological processes, the nuclear membrane is a highly permeable structure which divides the cell into two environments differing from one another both chemically and physically. Across this

## 1. INTRODUCTION

Although the nuclear membrane is a characteristic feature in the structural organisation of the vast majority of plant and animal cells, comparatively little attention has been paid to its nature and properties. The reasons for the neglect are not hard to seek: most cells are small objects, nuclei still smaller. Physiologists have devoted a great deal of attention to the plasma membrane and the cell cytoplasm, considerably less to the cell nucleus. Cytologists have devoted more of their attention to the cell nucleus, less to the cell cytoplasm: the nuclear membrane, which does not normally undergo spectacular changes during the life of the cell, is generally dismissed as either present or absent according to the stage in the cycle of cell division which is under examination.

As recently as 1937, Fischinger has denied the existence of an organised membrane surrounding the nucleus. He regards the apparent membrane as a physical interface, and nothing more. Yet the simplest experiments in micro-dissection of a wide variety of cells supply convincing evidence that the nuclear membrane is an organised structure with detectable mechanical strength. If, by means of a micro-manipulator, one attempts to puncture a cell such as an Amoeba or salivary gland cell of Drosophila, the needle meets resistance at the plasma membrane: having overcome this resistance, the needle moves relatively freely in the cell interior until it meets the nuclear membrane. Here further resistance is encountered: once the nuclear membrane has been punctured, the needle again moves relatively freely inside the nucleus.

Baud (1948) has reviewed the evidence for the existence of a nuclear membrane in all cells containing nuclei. He points out that whereas in certain cells the membrane is clearly visible in the living state, in other cells its existence can only be inferred by indirect means.

Unlike most biological membranes, the nuclear membrane is a wholly intra-cellular structure which divides the cell into two environments differing from one another both chemically and physically. Across this



membrane there are movements of metabolites, of special interest being those associated with gene activity. It is therefore evident that the structure and properties of the nuclear membrane merit experimental study.

The small size of most nuclei has placed them outside the direct reach of the experimenter by techniques which do not involve micromanipulation. There are, however, various animal cells, themselves of large size, which contain nuclei sufficiently large to be dealt with free-hand. The nuclei (germinal vesicles) of maturing amphibian oocytes may attain diameters approaching one millimetre and are particularly suitable for study. These nuclei have been used as material for wide variety of investigations by Brachet (1938-'40), Duryee (1937, '38, '41, '42), Gesch (1940) and Waddington (1938). Although these authors have made occasional references to the membrane, none have been concerned with a study of its structure.

The present paper is one of a series which will describe experiments with oocyte nuclei of amphibians. The aim of the experiments is to exploit these giant nuclei systematically in order to obtain information which, with certain reservations, may have general application to nuclei less suited to experimental study.

## 2. MATERIAL AND GENERAL METHODS.

Two species of amphibia have been used for the present study; Triturus cristatus cristatus and Xenopus laevis. In most experiments the newt or toad was anaesthetised with ether, the abdominal body wall cut longitudinally for about one centimetre to one side of the mid-ventral line, and part of the ovary excised. The wound was subsequently sutured with two or three stitches of gut and the animal allowed to recover. Regeneration of a continuous body wall normally occurs within one month from the time of operation.

In early experiments the ovarian fragments were placed in amphibian Ringer solutions: the oocytes distend in such media and rapidly undergo irreversible damage: they maintain condition for somewhat longer periods in Ringer solutions containing 0.5% gelatine, but ultimately it was found preferable to dispense with aqueous solutions entirely. Excised fragments were placed in dry embryo-cups covered by glass plates sealed with liquid paraffin,

or they were totally immersed in liquid paraffin. Where slight contamination with paraffin is of no disadvantage, the latter method is excellent: the majority of the oocytes remain bathed in the body fluid of the animal from which they were removed and they do not come into direct contact with the paraffin, which nevertheless seals the preparation from ~~direct contact with~~ the atmosphere. Although fresh material was used throughout most of these experiments, it may be worthwhile to record that ovarian fragments immersed in paraffin and held at a temperature of 3°-6° C. maintain splendid condition for over 24 hours.

The young oocytes are transparent and their nuclei are readily visible as "granular" spheres, the granulations being in fact large numbers of nucleoli which lie adjacent to the inner surface of the nuclear membrane. In older oocytes the yolky cytoplasm is opaque and the nuclei are not visible from the outside. In order to isolate nuclei from full grown oocytes, the following procedure is adopted. A fragment of ovary bearing a few large oocytes is rinsed several times in the medium intended for isolation and placed, in its container, on the stage of a low-power binocular microscope (magnification X 16). The stalk of attachment of a full-grown oocyte is now seized with watch-makers forceps and the follicular envelope surrounding the oocyte punctured with a shallow, almost tangential, prick from a sharp needle. In the case of newt oocytes, provided the isolating medium does not coagulate the yolky cytoplasm, the latter flows out in a steady stream from the puncture when slight pressure is applied by the needle to the oocyte's surface. It is generally possible to see the position of the nucleus, which stands out as a swelling on the extruded ribbon of cytoplasm. The nucleus can be removed from its adherent cytoplasm by the gentle pumping action of a pipette whose orifice is somewhat wider than the cross-section of the nucleus. The orifice of the pipette should have been smoothed off in a small gas flame prior to use.

In the case of Xenopus oocytes, the position of the nucleus can be judged from the outside; it lies immediately below the surface at the pigmented pole. The yolky cytoplasm of Xenopus oocytes is very much stiffer in consistency than that of the newt. With practice it is possible to tear the follicular envelope immediately overlying the nucleus, in which case the nucleus often pops

out from the oocyte already free from adhering cytoplasm. More frequently, because of an awkward disposition of the stalk with respect to the pigmented pole, the oocyte must be punctured elsewhere. In this case the oocyte must be torn open, exerting as little pressure on the stiff cytoplasm as possible. If a Xenopus oocyte is punctured and squeezed, the nuclear sap generally bursts out through a small hole in the nuclear membrane and the nuclear isolation fails.

The nuclei from full-grown oocytes of Triturus and Xenopus contain numbers of nucleoli which form a small central mass surrounding the chromosomes. In Xenopus, and in Triturus nuclei which are not fully mature, there are also peripheral nuclei adjacent the nuclear membrane. Lying between the group of central nucleoli and the membrane is a wide layer of nuclear sap. Triturus nuclei are spheres or slightly prolate spheroids with a regular outline. Xenopus nuclei, on the other hand, though being spherical, have a very uneven outline. The membrane is sacculated and if the nucleus distends, as it does if isolated into a non-coagulating colloid-free medium, the sacculations at first project stiffly from a taut sphere: if the nucleus distends to an extreme degree the areas of membrane between sacculations expand, taking up the strain until this nucleus too becomes a sphere of even outline.

For most purposes the easily isolated, larger and smooth outlined nuclei of Triturus are preferable to those of Xenopus. However they can only be obtained for a limited period during the year, unlike those of Xenopus which are available throughout the year. Most of the work with the electron microscope has been carried out on Xenopus nuclei the Triturus material being in the main reserved for experiments where measurements of surface area were involved. However sufficient Triturus material was studied to show that it does not differ materially from Xenopus so far as the fine structure of the nuclear membrane is concerned.

Electron micrographs were obtained using a Siemens' Electron Microscope operating in all cases at 52KV and at a magnification of 13,000 times. The plates used were either Barnet Process or Ilford Rapid Process, Experimental and they were developed in undiluted Kodak D.8 developer. Exposure times were two to four seconds.

Most of the preparation were mounted on conventional copper grids with or without supporting films of Formvar (Polyvinyl Formal). A few were mounted on platinum and

a further few on stainless steel grids in order to avoid the effects of metal ions during chemical <sup>a</sup>treatment of the mounted specimens.

### 3. OBSERVATIONS<sup>1</sup>

The first isolations of nuclei (Triturus) for examination of membranes in the electron microscope were made in unbuffered solutions of sodium chloride at 0.8% concentration. This solution is specifically not an adequate physiological medium for nuclear isolation. The nuclei distend to about one and a half times their original diameter<sup>er</sup> owing to the absence from the medium of a non-<sup>or</sup>penetrating colloid balancing the osmotic pressure of nuclear sap colloids. This is no disadvantage<sup>A</sup> for the examination of the membrane, since one thereby starts with a nucleus larger than life and whose membrane is stretched free from wrinkles and minor irregularities of contour. So far as electrolytes alone are concerned, sodium chloride solution is not a suitable medium for the preservation of nuclear sap and nucleoli; however the fine structure of the nuclear membrane seems to be as well<sup>Preserved</sup> in sodium chloride solution as in more complex media designed to maintain the nuclear colloids in lifelike condition.

Nuclei were allowed to distend after isolation and their<sup>ir</sup> membrane were then ruptured and stretched over the central squares of copper mounting-grids by means of fine-pointed tungsten needles. These needles were made by the method recommended by Pantin (1946). Fortunately the membrane is adhesive when punctured: it is therefore not unduly difficult to obtain a flat preparation lying with its inner surface directed upwards and its outer surface in contact with the grid. Some of the preparations made in this way were washed thoroughly in sodium chloride solution followed by distilled water and dried off in a vacuum dessicator without prior fixation. Such preparations were found to be relatively free from foreign matter when examined in the electron microscope; they were, however, very uneven in texture, a condition apparently resulting from stresses set up during dessication. They gave the appearance of an irregular meshwork supporting hosts of small annuli; in favourable patches which by accident had been exposed to less severe stresses during

dessication, as happens, for example, in areas circumscribed by folded membrane, the annuli were replaced by a continuous sheet bearing pores of approximately the same size as those within annuli.

Later preparations, mostly of Xenopus, mounted direct on grids, were handled in a similar manner but slight modifications in procedure were adopted. Xenopus nuclei were isolated in distilled water rather than in sodium chloride in order that the membrane should be stretched free from most sacculations before being mounted. When the nuclei are isolated in distilled water they stretch beyond the normal limit of one and a half times the initial diameter (this happens also to be the limit of elastic expansion below which no permanent deformation is produced). If left unchecked, the nuclei will continue to distend until they burst, the nucleoli and other nuclear contents all going into a homogeneous solution a few seconds after isolation and developing an excessively high osmotic pressure in the process. However the nuclei were punctured and the membranes stretched over grids before extreme distension had occurred.

Membranes isolated in distilled water were for the most part fixed in 2% or 0.1% phosphotungstic acid or in osmic acid vapour. A few membranes were washed in 10% sodium chloride solution after mounting and before fixation, the aim being to effect the complete removal of contaminating cytoplasmic and nuclear sap materials, following the principles advocated by Bensley (1938). Clean preparations were obtained in this way without disturbance of the membrane structure; the insolubility of the nuclear membrane <sup>in 10% Sodium Chloride</sup> has already been noted by Bensley.

Preparations made as described above enabled one component of the membrane structure to be identified with certainty. This component is a sheet of material with pores evenly spaced out over its area. It will be referred to as the a-layer. In these preparations the pore diameter is approximately 300A, the distance between pore centres 800A. A typical area is shown in plate 1, figure 1.

It is of the utmost importance to recognise that the methods of preparation of biological structures for examination in the electron microscope are likely to give rise to artifacts of various kinds. There are four stages at which such artifacts can occur: a. in the course of manipulating the fresh material; b. in the process of fixation; c. as a result of dessication, which is a prerequisite for examination in the electron microscope; and d. as a result of the

heating of the preparation while it is lying in the electron beam.

Artifacts originating from sources a. and b. can be guarded against by comparing the results obtained with a variety of experimental procedures. The structure of the a-layer is repeatedly observed whether the membranes are isolated in distilled water or in physiological salines and, within reasonable limits, it does not vary with the time interval between isolation and fixation. Moreover the same structure is visible whether the membranes are fixed in phosphotungstic acid, osmic acid, trichloroacetic acid or alcohol (provided, in the latter case, as will be seen later, extraction of lipoids is prevented). As has already been stated, the structure is sufficiently stable to be readily recognisable even in unfixed preparations. A further argument against the structure of the a-layer being an artifact ~~in the observation~~ lies in the observation that pore size and distribution are of great regularity. Were this structure an artifact one would expect considerable variation both in pore size and distribution, especially when preparations made by different techniques are compared. This is decidedly not the case.

Artifacts arising from source c. are to some extent linked with those from source b. : the less water a structure contains, the more likely is it to fix and dry without distortion. Compared with other biological structures, the nuclear membrane is a strikingly dense object and it shows no evidence of hydrating on isolation. However it is probable <sup>that</sup> the dimensions <sup>of</sup> pore diameter and pore separation distance already quoted are somewhat underestimated of the dimensions in life since nuclear membranes which are not supported undergo shrinkage on fixation and drying. They should therefore be accepted as minimal figures only.

Artifacts arising from source d. can be checked by comparing the structure of ~~preparation~~ a preparation when first examined in the microscope with that after a lengthy exposure. During examination in the electron beam the sheets of membrane often contract and split and curl upon themselves. This gross mechanical effect does not disturb the fine structure of the membrane except in so far as it may add to an overall underestimate of dimensions.

By accident of fixation which we are not able to define precisely, some preparations do show a readily recognisable artifact. Instead of a porous membrane these preparations consist of sheets of annuli which are strung together in irregular lines ( plate 2 , figure 3 ). It is immediately evident on examination of photographs of preparations such as these that neighbouring groups of annuli fit together as in a jig-saw puzzle : they are , in fact , derived by the mechanical breakdown of <sup>the</sup> porous sheets which are seen in the majority of preparations. A noteworthy point established by " annular " preparations is that the regions of mechanical weakness in the porous membrane lie between pores rather than across them. It may be that the rims of the pores are built up above the general level of the membrane ; indeed some photographs give a distinct impression that this is the case.

A question is raised by the " annular " preparations ; the groups of annuli are often well separated from their neighbours and yet remain in the form of a sheet : they must therefore be supported by <sup>a</sup> further structure. This second component is a continuous membrane , which will in future be referred to as the b-layer. It was first seen in some of the early preparations of Triturus which had been made from oocytes excised 24 hours before isolation of the nuclei and held at room temperature. Preparations of such nuclei showed no traces of the a-layer ; instead they showed a continuous membrane covered with unidentifiable debris. The a-layer had evidently broken down by autolysis during the interval between excision of the oocytes and the mounting of the membrane . Similarly other early preparations which had <sup>had</sup> a very extended washing in distilled water after mounting and before fixation showed no signs of structure of the a-layer but again suggested the existence of a continuous layer supporting debris.

It was later found that the a-layer could be destroyed at will . Nuclei were isolated in 0.2M potassium phosphate solution containing magnesium phosphate at saturation. At pH ranging from 6.6 to 6.8 ( determined by glass electrode measurements ) the nuclear sap components have a very life - like appearance apart from the distension which normally occurs

in colloid free media. The external surfaces of nuclear membranes are remarkably sticky when isolations are made in phosphate. It was felt that this property could be used to advantage in a mounting technique not requiring the use of needles. Nuclei were isolated in phosphate solutions and pipetted on to cover-slips previously coated with films of formvar. On removal of the bulk of the liquid, the nuclei flattened down and their lower surfaces become rigidly attached to the formvar. If a single drop of liquid is now released from a pipette held vertically above the flattened nucleus, in most instances the membrane ruptures in a ring around the area adhering to the formvar leaving thereby a flat area of membrane whose inner surface is directed upwards. The membrane can be fixed and the film plus membrane subsequently separated from the glass coverslip and together mounted on a copper grid ready for examination. Technical details of this mounting technique, which has been developed for the study of tissue culture cells, are given by Martin and Tomlin, 1950. Preparations made in this way show the b-layer lying on the formvar film: of the a-layer there is no trace, but irregular scattered debris probably represents the products of its breakdown. The disruption of the a-layer is effected specifically by the phosphate ion and it occurs over a wide pH range. In order to study the structure of the b-layer membranes from nuclei isolated in phosphate were stretched directly over copper grids without a supporting formvar film. Figure 4, plate 2, is a photograph of a preparation of this kind. It shows the b-layer, without evident fine structure, bearing debris derived from the a-layer.

The a-layer can also be disrupted mechanically. It is a property of the nuclear membrane to lie at an air-water interface in preference to total immersion in water. Gross 1916, made a similar observation when working with the nuclei of molluscan oocytes. Though this property is particularly striking when nuclei are isolated in phosphate solutions, the direct effects of which (just have) been noted, it also occurs to a lesser extent with nuclei isolated in distilled water or in chloride solutions. It is possible to pick up the part of a membrane lying in the air-water inter-face directly on a grid or on to formvar which can subsequently be mounted on a grid. Preparations made



on a grid or on to formvar which can subsequently be mounted on a grid. Preparations made in this way show the b-layer covered with débris from the a-layer but ~~we~~ very little trace of the latter's structure remains. With this effect in mind, it is small wonder that the first unfixed membranes should have been uneven in texture, since on drying out they too were exposed to surface tension forces. It is remarkable that any trace of the structure of the a-layer should have remained at all !

It was now necessary to determine the topographical relationship of the a-layer and b-layer. Proofs negative and positive were obtained that the a-layer lies on the outside of the nucleus. Nuclei of Xenopus were isolated in distilled water and pipetted on to coverslips bearing a formvar film. The same technique was adopted as with nuclei isolated in phosphate solution; water was abstracted and the nuclei flattened and then a drop of water pipetted from above directly over the area of membrane lying in the air-water interface. The membranes in distilled water are not noticeably adhesive and it is only in a small proportion of cases that a piece of membrane is left adhering to the formvar after rupture of the nucleus has occurred. In the few successful cases the membrane adhering to formvar (which was not exposed to the surface tension forces at the interface prior to fixation) was subsequently fixed in 0.1% phosphotungstic acid solution and, after washing in distilled water, mounted together with the formvar film on a copper grid. A photograph of a typical preparation of this kind is shown in plate 1, figure 2. It will be seen that the a-layer is beautifully preserved in all its regularity: photographic contrast is, however, less than in preparations mounted direct on grids owing to the presence of the continuous film of formvar as well as the nuclear membrane itself. Pore diameter is 500 Å, the distance between pore centres 1300 Å.

(same para.) It is likely that these dimensions are somewhat larger than those existing in life, since the membranes were ~~expanded~~ expanded by osmotic pressure before fixation to the formvar.

Preparations made in the way just described were shadowed in vacuo with palladium (Williams and Wyckoff, 1944). If the a-layer lies free at the surface exposed to shadowing, then the pore rims should cast shadows over the pores themselves. However this was not the condition which we observed in the photographs. The effect of shadowing was merely to decrease still ~~further~~ the photographic contrast, though odd fragments of débris lying on the upper surface threw shadows in the normal way. This negative result indicated that the a-layer is not lying exposed at the surface ; it is covered by the uniform b-layer ( see text- figure ~~at~~ 1a). This means that in the ~~uniform~~ case of the intact nucleus the a-layer lies outside, the b-layer inside.

Proof positive of the topographical relationship was also established. Xenopus nuclei were isolated in distilled water and mounted direct on copper grids by means of tungsten needles. They were then fixed in 0.1% phosphotungstic acid solution. In these preparations the outer layer of the membrane is in contact with the grid, the inner layer directed upwards. Free sheets of formvar were now prepared on the air-water interface and the grids carrying membranes brought up to the surface so that the inner membrane of the nucleus was overlaid by formvar. On removing the grid from the water the outer surface of the membrane ( see text-figure 1b) was exposed to the forces of the air-water interface with the result that the a-layer suffered considerable disruption but this was of some advantage in establishing the point at issue. After drying , the preparations were inverted, set up in vacuo and shadowed with palladium. In these preparations, as will be evident from text-figure 1b, the outer layer of the nuclear membrane is directly exposed to the metallic particles where it is not itself in the shadows cast by the ribs of the mounting grid. Photographs such as that shown on plate 2, figure 5, clearly demonstrate that the a-layer is now lying free at the surface ; the layer is badly broken up, but sufficient remains to establish positively that the a-layer lies on the outside of the nucleus. Where the a-layer is interrupted, the adjoining parts cast shadows on to the b-layer. From the width of the shadow

and knowing the shadowing angle  $(30^\circ)$  we can make an approximate estimate of the thickness of the <sup>a</sup>layer. It is roughly 300 A thick. The <sup>b</sup>layer is itself lying free and torn in places. Judging from the width of the shadows which it casts on to the supporting formvar film below, its thickness is roughly 150 A.

A further proof of the topographical relationship was obtained by means of replicas of the surfaces of whole ~~fixed~~ nuclei. The nuclei were fixed with trichloroacetic acid and dehydrated with alcohol. One of these was then placed on a clean coverslip. A second coverslip coated with a film of collodion in amyl acetate solution was then brought down carefully on to the nucleus before the collodion film had dried out and before the nucleus had dried fast to the lower coverslip. On lifting the coated coverslip the nucleus adhered to the collodion which was then allowed to dry out. After drying, a drop of 0.1% trypsin solution was placed over the nucleus, the coverslip inverted and sealed over a depression slide with paraffin wax. The preparation was incubated at  $37^\circ\text{C}$  for 12 hours. It was subsequently opened, the debris washed away and the portion of collodion film bearing the replica of the nucleus was mounted on a copper grid and shadowed with palladium. Photographs of such preparations gave further indubitable evidence that the <sup>a</sup>layer is on the outside of the nucleus.

It was thought desirable to confirm the estimate of thickness made from shadow length measurements by an independent method. The optical method of multiple beam interferometry described and applied to many physical problems by Tolansky (1948) was used to measure the total thickness of the membrane, the procedure being as follows. Xenopus nuclei were isolated in saline and transferred to carefully cleaned coverslips. The excess saline was now pipetted off and the nuclei ruptured leaving the lower halves stuck to the coverslips as earlier described. The membranes were now fixed in 0.1% phosphotungstic acid solution, washed and dried. After drying, <sup>half</sup> of each membrane was scraped away by means of a fragment of razor blade so as to leave a uniform straight edge on one side of the membrane remaining on the coverslip. A coverslip bearing a piece of membrane and another clean coverslip were silvered contemporaneously in vacuo, The thickness of the silver film being such as to give a reflection coefficient of about 90

about 90% . Pairs of coverslips were now mounted with their silvered faces in contact in a suitable metal clamp placed on the stage of a light microscope . This arrangement was now illuminated with a parallel beam of light from a mercury arc lamp filtered through a monochromatic green filter and the resulting interference fringes observed with a low-power objective . From the displacement of the fringes where they cross the straight edge of the membrane the thickness of the dried membrane was estimated to be approximately 500 A.

Taking the thickness of the membrane from interference measurements ( which agree surprisingly well with estimates from shadow lengths ) and taking figures for pore size and pore separation distance which are means between the measurements on supported <sup>and unsupported</sup> membranes , we are in a position to construct a sectional diagram of the nuclear membrane . Such a diagram is shown in text-figure 2.

#### 4 DISCUSSION

There can be little doubt , from the evidence presented in the foregoing account , that the nuclear membrane of amphibian oocytes is a dual structure. The results of three different kinds of experiment agree in demonstrating that the two surfaces of the membrane are different in character. The external surface is porous : the evidence that this porous structure is not merely an artifact has been presented on page 8 . The internal surface gives no indication of heterogeneity of texture; it is the surface of a discrete layer which can exist in the absence of the external porous material.

At first sight it might be imagined that the thicker porous a-layer acts as a mechanical support for the thinner structureless b-layer . It seems probable that the b-layer determines the permeability properties of the nuclear membrane; the actual pores through which penetrating molecules pass the membrane must be well outside the limits of resolution of the electron microscope since such molecules as egg and bovine plasma albumin , glycogen and gum acacia are unable to penetrate the membrane by ordinary osmotic means. It is conceivable that the absence of visible structure in the b-layer is itself a technical artifact , though this is very unlikely. It seems improbable that the visible porous membrane can play a major

part in determining the limits to permeability though its existence may markedly reduce the surface area of the b-layer available for the passage of materials from nucleus to cytoplasm, or viceversa. In our manipulations of the nuclear membrane we have not found that the presence of the a-layer markedly strengthens the membrane as a whole. The elastic properties and remarkable strength of the nuclear membrane seem to reside mainly in the b-layer since these properties are almost as evident in nuclei isolated in phosphate as in those isolated in chloride solutions. It is true that membranes isolated in phosphate and stretched on grids collapse more readily on drying out than <sup>do</sup> those isolated in chloride, but this is hardly a fair test of the possible supporting functions of the a-layer in life.

The b-layer, insoluble in distilled water, physiological salines and 10% sodium chloride, is formed of protein. (Callan, unpublished, from birefringence properties, quantitative tests and digestion experiments; see also Baud, 1948, 1949a and b). The a-layer too consists at least in part of protein. We have evidence that it also contains a certain amount of lipoid. If unfixed membranes mounted direct on copper grids are exposed to fat extraction by alcohol and chloroform, the a-layer is quite normal in appearance. If, however, the membranes are mounted on stainless steel grids and then exposed to alcohol and chloroform, the characteristic appearance of the a-layer is lost and in its place we see the b-layer with a uniform covering of material derived from the a-layer, all trace of the porous structure having disappeared. This is quite striking evidence that lipoids are concerned in the molecular architecture of the a-layer, since it is known that copper ions in extremely low concentration ( $10^{-5}M$ ) can inhibit lipoid extraction. (Waugh and Schmitt, 1940): this would explain the difference in behaviour of the membranes on copper as opposed to stainless steel grids.

The presence of lipoid in the nuclear membrane was confirmed by the observation that it stains selectively with Sudan Black. Nuclei isolated in saline were transferred to a dilute solution of Sudan Black in 70% alcohol and subsequently examined in water after rapid passage through 70% and 50% alcohol. The stained regions are the membrane, and, incidentally, the borders of the nucleoli. It is therefore possible that the a-layer repre-

sents the "couche lipidique périnucléaire " of Baud 1949, a. and b. ). Polarised light studies on the amphibian oocyte nuclear membrane provide no evidence for the presence of lipoids orientated with their hydrocarbon chains radial to the surface , nor , so far as we are aware, has this been demonstrated for any other kinds of nuclei. ( see, for example, Chinn , 1938 and Schmidt, 1936). Baud has been able to demonstrate positive birefringence with respect to the radius of the nucleus only in the cases of nuclei stained with chrysoïdin ; it may well be that this treatment breaks down lipo-protein complexes thereby allowing the lipoids to become arranged in a film at the surface of the nucleus, there producing the birefringence which Baud has observed . Even if , in life, the lipoids have a regular orientation with respect to ~~the~~ the structural features of the a-layer , it is quite probable that this distribution would give rise to statistical isotropy of the membrane considered as a whole.

#### 5. ACKNOWLEDGEMENTS

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## LEGENDS TO TEXT-FIGURES

## Figure 1.

The disposition of the two layers of the nuclear membrane in relation to the angle of incidence of palladium particles in two kind of shadowing arrangement.

(a) The membrane lying with the a-layer in contact with formvar, the formvar being supported on a copper grid. Being overlaid with the b-layer, the rims of the pores of the a-layer cast no shadow.

(b) The membrane lying with the a-layer attached to a copper grid, the b-layer being backed by formvar. Where the a-layer is not itself in the shadows of the grid, the rims of its pores cast shadows.

## Figure 2.

Reconstructed ~~and~~ sectional diagram of the nuclear membrane, the section passing through the diameters of the pores. The a-layer is shown black, with indentations at the points of mechanical weakness where breakdown gives rise to "annuli". The b-layer is shown cross-hatched. The shape of the structural units of the a-layer is inferred only. The relative magnitudes of layer thickness, pore diameter and separation distances between pore centres have been directly determined. ( x 500,000) ~~after reduction by 1/2.~~

## LEGENDS TO PLATES land 2.

## Plate 1.

Figure 1. Xenopus laevis. Nuclear membrane isolated in distilled water, stretched over copper grid and fixed for 15 minutes in 2% phosphotungstic acid. x 26,000.

Figure 2. Xenopus laevis. Nuclear membrane isolated in distilled water, mounted on forvar film with outer surface in contact with film, fixed for 2 minutes in 0.1% phosphotungstic acid. x 26,000.

## Plate 2.

Figure 3. Xenopus laevis. Nuclear membrane isolated in 0.8% sodium chloride, stretched over copper grid, washed in 10% sodium chloride, fixed for two minutes in osmium tetroxide vapour. x 26,000. Porous layer broken down to form annuli.

Figure 4. Xenopus laevis. Nuclear membrane isolated in 0.2M potassium hydrogen phosphate at pH 6.8, stretched



on copper grid and fixed for 2 minutes in 0.1% phosphotungstic acid. x 26,000. Porous layer disrupted leaving debris supported by the continuous layer.

Figure 5. Xenopus laevis. Nuclear membrane isolated in distilled water, stretched on copper grid, fixed for 2 minutes in 0.1% phosphotungstic acid, ~~backed~~ backed on inner surface with formvar film. Specimen inverted and shadowed through grid at an angle of 30° with palladium. Printed as a negative in order to show shadows dark. x 26,000. "a" indicates shadow of break in continuous layer on to formvar, "b" indicates shadow of break in porous layer on to continuous layer.

Figure 1.

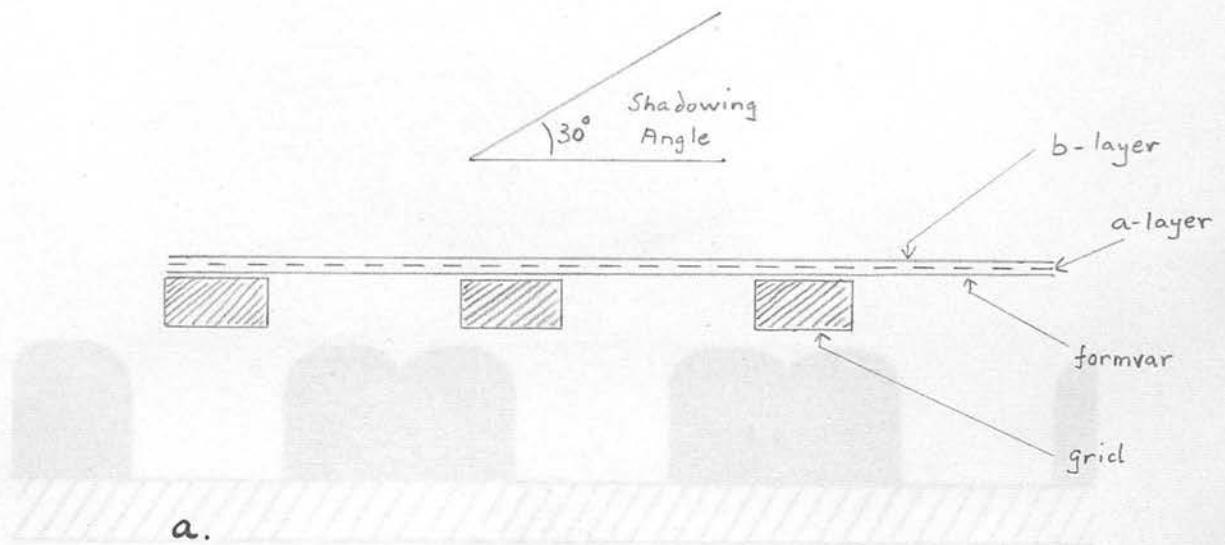
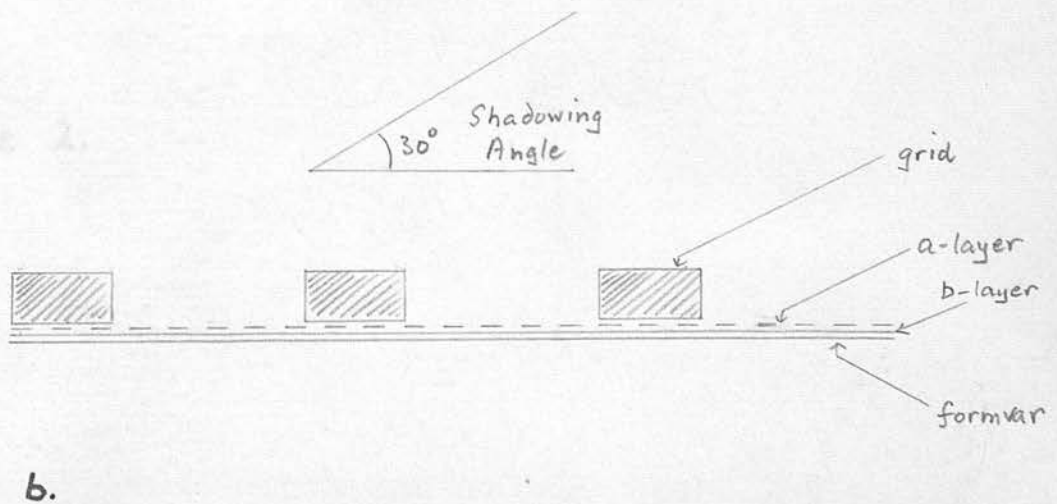


Figure 2.



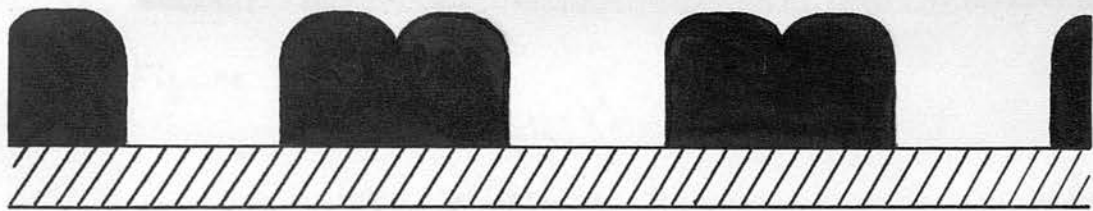


Figure 2.

Plate 1.

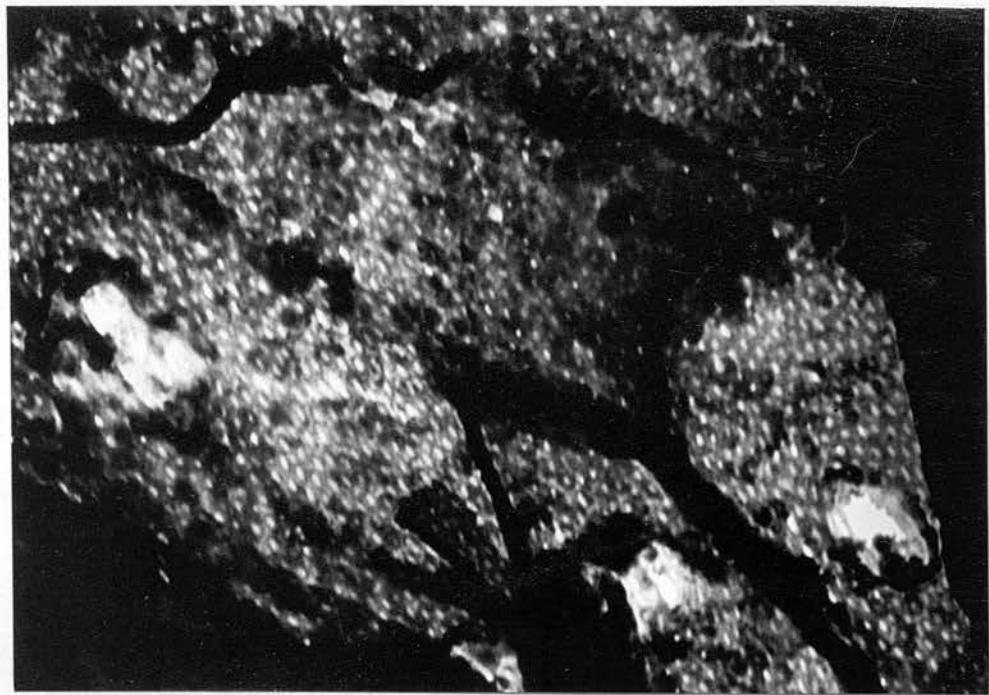


Figure 1.

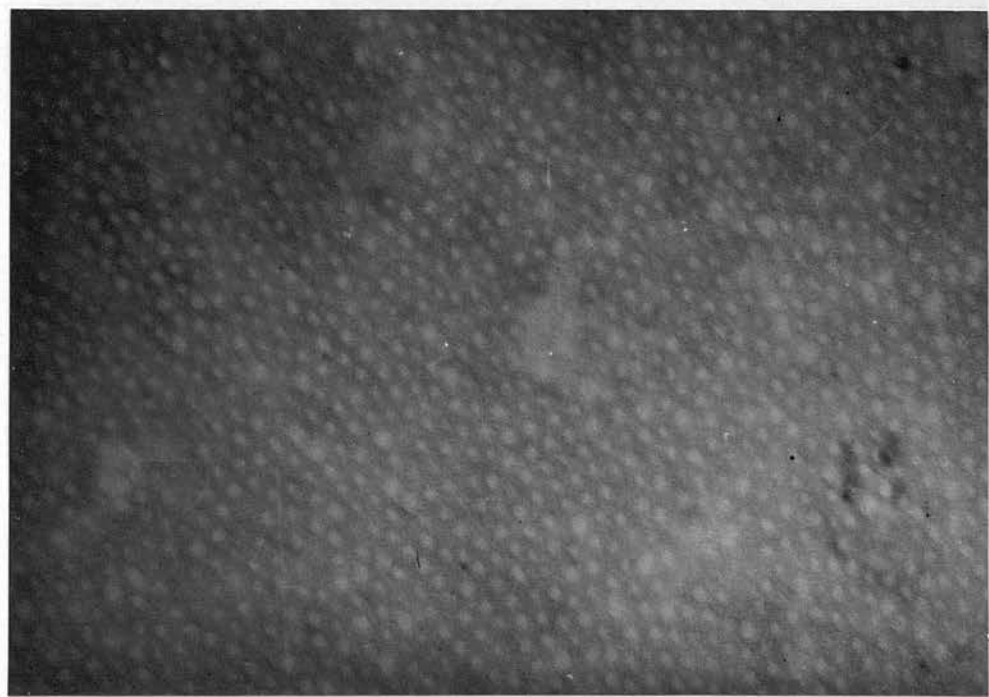


Figure 2.

Plate 2.

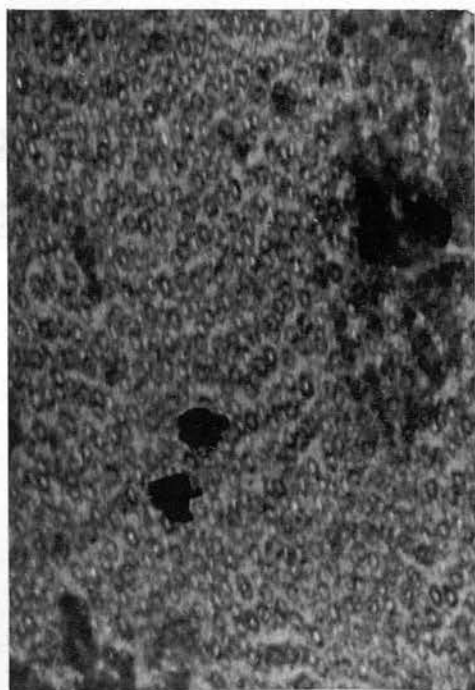


Figure 3.

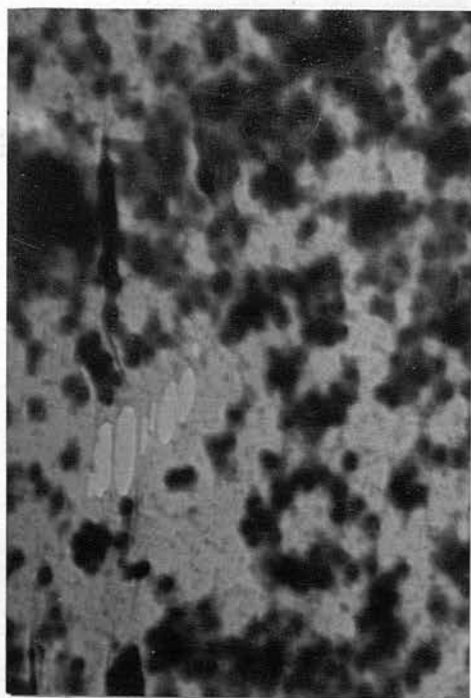


Figure 4.

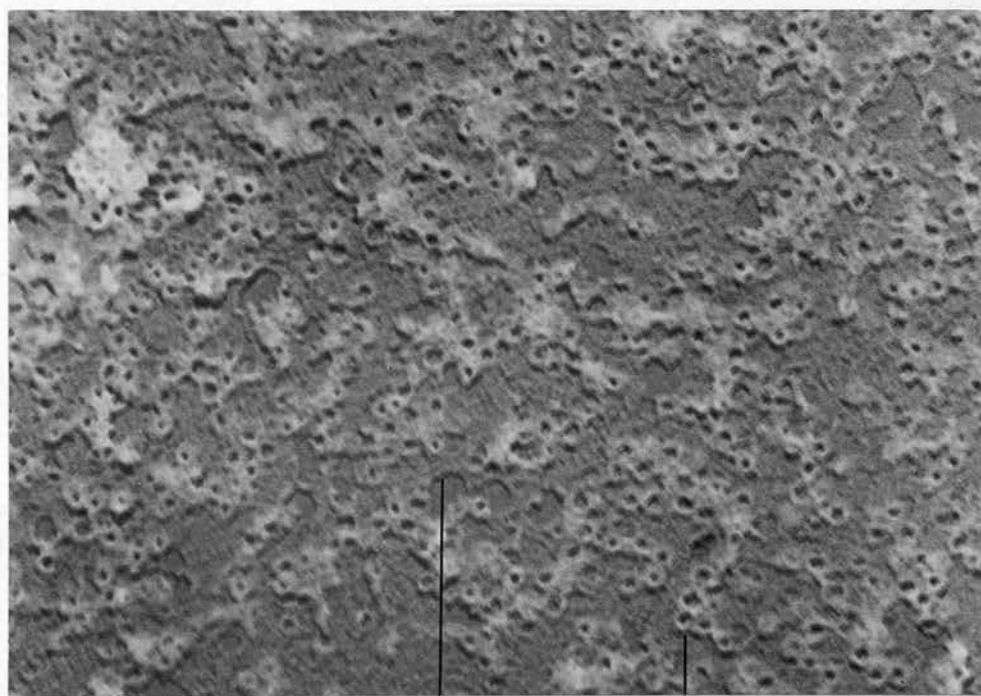


Figure 5.

a

b

EXPERIMENTAL STUDIES ON AMPHIBIAN OOCYTE NUCLEI :

AN INTRODUCTION.

By

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Organisation, Agricultural Research Council,  
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INTRODUCTION

The past twenty years have witnessed a remarkable increase in our knowledge of the biological properties of the cell nucleus. In the one hand, the discovery of nucleosomes, which is the direct study of the inner cell, has revealed the nucleus a field of great complexity and order. On the other hand, the discovery of the nucleosome has revealed the nucleus a field of great complexity and order.

1. The nucleus is a highly organized structure, the study of which has revealed the nucleus a field of great complexity and order.

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The present manuscript is a summarised review of my observations and experiments to date on giant nuclei. Only passing reference is made to work already published or in course of publication since detailed accounts are available among the appended publications. As yet unpublished and fragmentary work is treated somewhat more extensively.

Frequency and distribution, polyloidy, chromosomal inversions, translocations and relations and other genetically interesting systems.

H.G.Callan,

Edinburgh,

9th March, 1950.

However, when one turns from a consideration of the biological properties of the cell nucleus to the more fundamental chemical and physical properties, the gap in our knowledge is seen to be enormous. Microscopical literature abounds in papers attempting to explain the nature of the activity of the nucleus, forces of various kinds are postulated to account for movements of the chromosomes and the like which can be laid down with reasonable exactness at the biological level of analysis but little or no information on chemical and physical information.

It was for this reason that I decided three years ago, after having worked for some time in the field of cytogenetics, to transfer my attention to a direct study of the nucleus.

## INTRODUCTION

The past twenty years have seen a remarkable increase in our knowledge of the biological properties of the cell nucleus. On the one hand the subject of cytogenetics, which is the direct study of the chromosomal basis of heredity, has become a field of great observational precision on which genetical prediction can be based. This has resulted from two major discoveries :

- a. that the cytologically-observable "chiasma" at meiosis is the material counterpart of genetically-detectable "crossing-over"
- b. that the cross bands of the salivary gland chromosomes of the fruit fly, Drosophila, indicate the spatial arrangement of the genes of this organism.

Cytogenetics has thus become a method for the preliminary investigation of genetical processes in ~~organisms~~ organisms not yet studied by breeding methods. In organisms which are already under genetical observation, it is a complementary study which can give immediate information on such questions as number of linkage groups, cross-over frequency and distribution, polyploidy, chromosomal inversions, translocations and deletions and other genetically interesting circumstances.

On the other hand the study of the relationships between nucleus and cytoplasm in development has progressed by the study of hybrid amphibians and echinoderms in a wide variety of experimental situations, also by the "graft-hybrids" of Acetabularia. Great promise in this field of study is offered by the new manipulative techniques whereby nuclei may be transplanted from one cell to another.

However, when one turns from a consideration of the biological properties of the cell nucleus to its more fundamental chemical and physical properties, the gap in our knowledge is seen to be enormous. Cytological literature abounds in theories attempting to explain this or that activity of the nucleus, forces of various kinds are postulated to account for movements of the chromosomes and laws which can be laid down with reasonable exactitude at the biological level of analysis have little or no foundation on chemical and physical information.

It was for this reason that I decided, three years ago, after having worked for some time in the field of cytogenetics, to transfer my attention to a direct exper-



imental study of the more fundamental properties of the cell nucleus.

Most cells are small objects and the nuclei inside them are still smaller. It is evidently the small size of most nuclei which has placed them outside the range of direct experiment by means other than micromanipulation. Techniques of micromanipulation can give useful information but this is often hard to evaluate owing to difficulties in defining the precise experimental conditions. Moreover where nuclear material has to be accumulated for chemical or physical study the larger the nucleus the less laborious is the collection procedure.

For this reason I decided to concentrate my attention on a cell nucleus of exceptional size: the oocyte nucleus of amphibians. The nucleus of an almost mature oocyte of the crested newt, Triturus cristatus, may be slightly exceed 0.5mm. in diameter.

The experiments to be described in this thesis were, with very few exceptions, conducted freehand with the aid of a low power binocular dissecting microscope.

#### DESCRIPTIVE HISTORY OF THE GROWTH OF THE OOCYTE NUCLEUS

The species which I have studied most completely from this standpoint is the Italian crested newt, Triturus cristatus carnifex. The youngest oocytes are to be found in the follicular epithelium of the ovary. Their nuclei are slightly larger than those of ordinary epithelial cells, being about  $20\mu$  in diameter: the chromosomes are visible in fixed and stained preparations; they run throughout the volume of the nucleus and show the usual "chromomeric" structure of earliest meiotic prophase. There are no nucleoli at this stage.

As the oocytes grow in size the chromosomes pass successively through the early prophase stages, being already at diplotene when the nuclei have a diameter of about  $40\mu$  (oocyte diameter about  $70\mu$ ). At a slightly later stage lateral outgrowths from the chromomeres give the chromosomes a "rough" appearance. Many nucleoli now appear adjacent the inner surface of the nuclear membrane and a few are to be seen amongst the chromosomes.

By the time the oocyte has a diameter of  $500\mu$

visible accumulations of yolk are formed in the cytoplasm and shortly thereafter the cytoplasm becomes entirely opaque. Nuclear ~~diameter~~ diameter is now approximately  $200\mu$ . By this time the chromosomes have increased greatly in length and so, too, have their lateral outgrowths. These outgrowths, the "lampbrushes" of other authors, are in the form of loops: they project on an average about  $15\mu$  from the main axis of the chromosome. The chromosomes still run throughout the whole volume of the nucleus; a number of nucleoli lie amongst them and many lie adjacent the nuclear membrane where they form a regular pattern.

The lateral outgrowths from the chromosomes reach their maximum development in oocytes of diameter approximately  $800\mu$ . The chromosomes are themselves maximally extended at this stage. Subsequently the lateral outgrowths regress and the chromosomes start to contract. The chromosomes no longer run through the whole volume of the nucleus: instead they form a central mass separated from the nuclear membrane by a zone of nuclear sap devoid of chromosomes. Nucleoli lie amongst the chromosomes and adjacent the nuclear ~~membrane~~ membrane.

In oocytes of diameter  $1500\mu$  the nuclei are of about  $500\mu$  diameter. The central mass of chromosomes has now shrunk to about  $130\mu$  diameter. Nucleoli in very large number lie adjacent the membrane, some are to be seen amongst the chromosomes and occasional nuclei show radial lines of nucleoli running between the periphery and the central chromosome mass. The lateral outgrowths from the chromosomes are now minute.

The oocytes do not increase much more in size, their diameter at maturity being about  $1600\mu$ . In the interim period the peripheral nucleoli decrease in number and finally disappear: while this happens a dense coat of nucleoli is formed around the central mass of chromosomes, the latter decreasing in length and losing their lateral outgrowths until they take on the normal appearance of chromosomes just prior to the formation of the first meiotic spindle.

Practically the whole of the growth phase of the oocyte, which extends over a period of two to three years, is a much expanded diplotene stage so far as the nucleus is concerned. The breakdown of the nuclear membrane and the completion of the first meiotic division has not been studied in this material.

In the following pages, experimental work on the nuclear membrane, sap, nucleoli and chromosomes will be described.

### THE NUCLEAR MEMBRANE

The structure of the nuclear membrane is discussed in some detail in the appended paper by H.G.Callan and S.G. Tomlin ; "Experimental studies on amphibian oocyte nuclei. 1. Investigation of the structure of the nuclear membrane by means of the electron microscope". (Proc. Roy. Soc. in press)

The membranes were prepared from nuclei of Xenopus laevis and Triturus c. cristatus at the stage when the chromosomes form a small central mass surrounded by nucleoli and when the peripheral nucleoli have started to decrease in size and number,

The membrane is a double structure. It consists of an outer layer of about 300 A thickness bearing pores 400 A in diameter regularly arranged with their centres 1000 A apart, and an inner layer of about 150 A thickness without visible ~~structure~~ fine structure in the electron microscope.

The membrane is insoluble in distilled water, physiological saline and 10% sodium chloride solution. It gives positive protein reactions in the Millon and Xanthoproteic tests; It dissolves in a few minutes in 0.1% trypsin at pH 8.3 at 18°C., similarly in 0.01% pepsin at pH 1.5, though remaining undigested in boiled controls. The evidence is thus that it consists, at least in part, of an elastin-like protein. It stains selectively with the standard elastic fibre stains orcein and resorcin-fuchsin.

The membrane stains selectively with sudan black. It also reacts positively in cytochemical tests for the presence of alkaline phosphatase. The porous outer layer is disrupted, though leaving a residue, in the presence of the phosphate ion at 0.2M concentration over a wide pH range. It is similarly disrupted, leaving a residue, on treatment with alcohol and chloroform, though not in the presence of traces of the copper ion. The evidence is thus that phospholipids and protein are together involved in the molecular architecture of the porous layer. The inner layer, in the other hand, would appear to consist of protein only. The membrane is hydrophobic and readily comes to lie at an air-water interface.

The membrane is elastic up to a symmetrical area increase of  $\times 2.25$ . In the process of stretching it becomes birefringent, presumably as a result of the mechanical deformation of originally isotropic elements in its structure. The birefringence is entirely of the "form" type and disappears on immersion of the membrane in media of the same refractive index as proteins (ca 1.5). Above the elastic limit birefringence does not increase. (see appended paper entitled "Alcune proprietà fisiche della membrana nucleare").

Tests of the permeability of the nuclear membrane to a few substances have been made by studying volume changes when nuclei are isolated into media containing different concentrations of the substances in question together with the requisite electrolytes. The membrane is permeable to : water, simple electrolytes, xylose, glucose, sucrose, raffinose, depolymerised nucleic acids and dextrin. Graphs showing the rate of change of nuclear volume in sugar solutions of different concentrations have been constructed and similar curves may be prepared for other substances : from these it will be possible to estimate the rates of penetration of different substances and also to calculate the colloid osmotic pressure of the nuclear sap in life, though this has not yet been attempted.

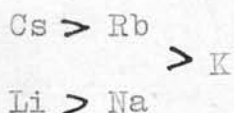
The membrane is impermeable to egg albumin, bovine plasma albumin, synthetic soluble celluloses, gum acacia and glycogen. The permeability limit for globular organic molecules thus lies somewhere between molecular weights 1,500 and 40,000. The existence of this limit to the penetration of protein molecules raises some interesting questions regarding the movement of the products of gene synthesis from nucleus to cytoplasm. Unless gene products are only passed to the cytoplasm during cell division, an unlikely supposition, we must presume that the immediate products of gene synthesis are molecules of relatively low molecular weight which are produced with an orderly distribution in space and time such that specific complexes can be formed outside the membrane. Alternatively the passage through the membrane might be by means other than normal osmosis. Whatever the mechanism involved, the presence of alkaline phosphatase in the membrane may possibly be connected with the energetics of molecular transport across the membrane.

## THE NUCLEAR SAP

### 1. Colloidal properties

If oocyte nuclei are isolated into potassium chloride solutions of different concentrations they hydrate until the back tension developed in the membrane balances the colloidal osmotic pressure of the nuclear sap. If one makes the reasonable assumption that the elastic properties of the membrane are not noticeably affected by potassium chloride over a concentration range 0 - 0,5 M, the degree of swelling of the nuclei is a measure of the colloidal osmotic pressure (i.e. the degree of hydration) of the sap at various electrolyte concentrations. There is a sharp minimum to sap hydration at 0.2 M, which is near to the electrolyte concentration of typical extra-cellular physiological media. (see fig.1.)

The hydration of ~~the~~ colloids in the presence of equimolecular concentrations of various electrolytes throws light on their electrical properties. I have studied the hydration of nuclear sap in the presence of 0.2 M solutions of the chlorides of five monovalent metals. Minimal hydration occurs in the presence of potassium, maximal in the presence of Caesium and lithium. (see fig.2). This can be written as follows :-



Teunissen has shown that this is the order to be expected of a negative colloid, such as a protein at a pH higher than its isoelectric point. In these circumstances the colloidal particles are predominantly anions.

There is no evident relationship between the hydration of the sap and the Hofmeister series of inorganic anions, though potassium salts of di- and tri-basic acids produce greater hydration than do similar salts of monobasic acids at the same molarity. This is probably a result merely of differences in cation concentration.

The effects of pH of the isolating medium bear out the contention that the sap colloids are anionic at physiological pH. In a nucleus freshly isolated into paraffin oil, the sap is translucent and no particles are visible even when examined with dark-ground illumination. The nucleoli are clearly visible with refractile outlines, the

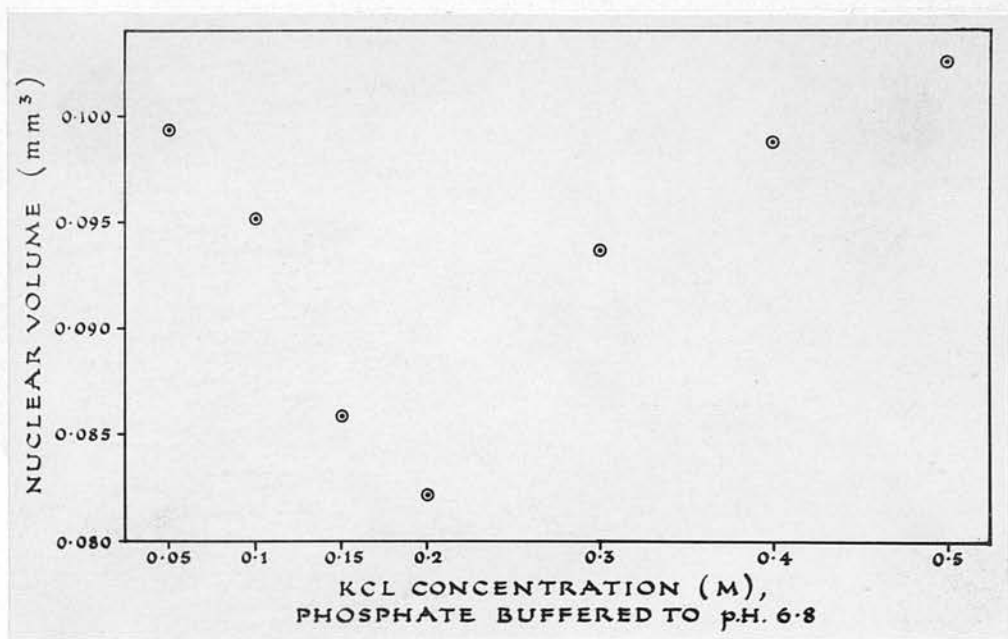


Fig.1. The hydration of nuclear sap in the presence of diverse KCl concentrations. Nuclei isolated from oocytes of Triturus c.cristatus, of diameter 1.3mm, and measured after one hour.

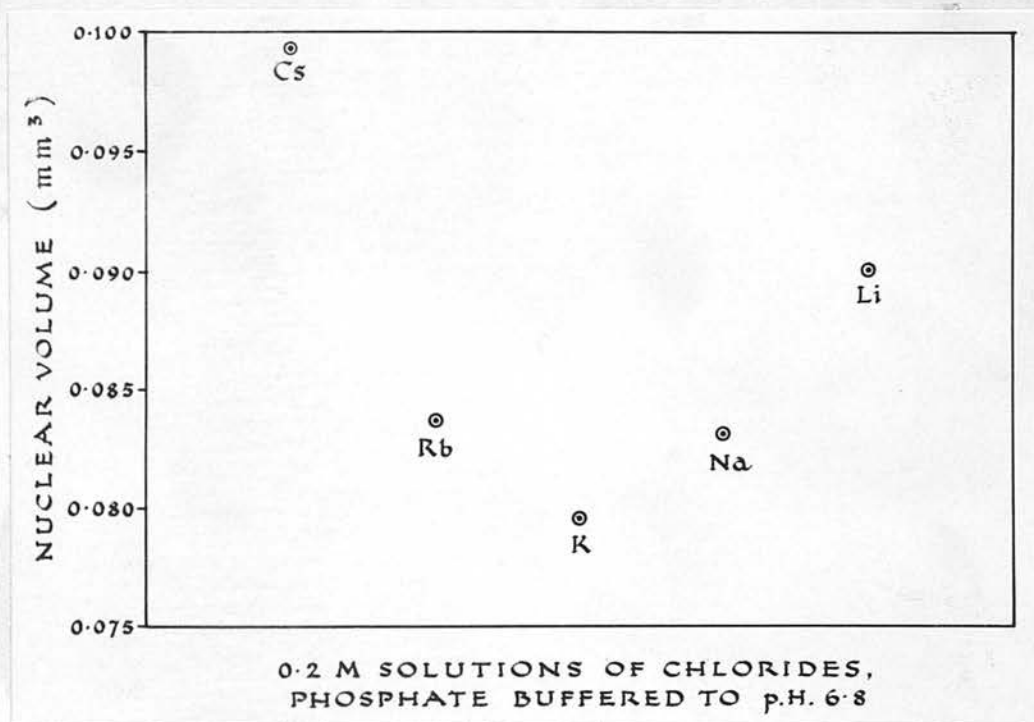


Fig.2. The hydration of nuclear sap in the presence of diverse monovalent cations. Nuclei isolated from oocytes of Triturus c.cristatus, of diameter 1.3mm, and measured after one hour.

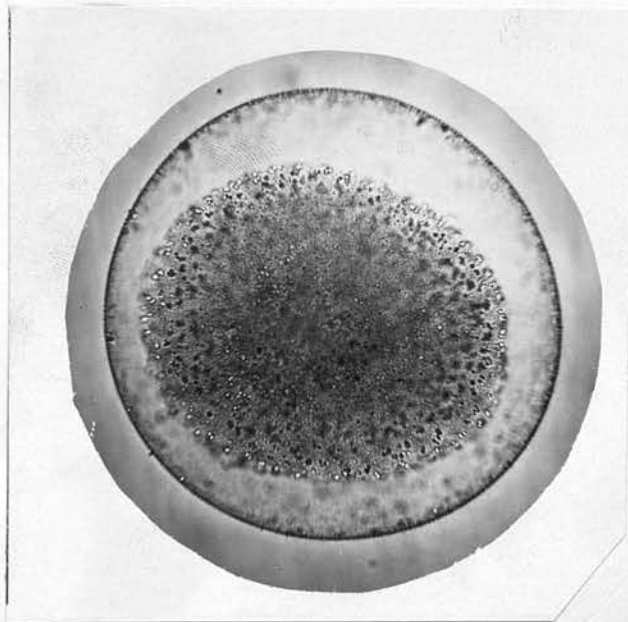


Fig.3. Oocyte nucleus of T.c.cristatus isolated in 0.2 M potassium phosphate, saturated with magnesium phosphate, at pH 6.0. Structural colloid coagulated. (x80)

Fig.4. Oocyte nucleus of Xenopus laevis isolated in 0.2 M potassium phosphate, saturated with magnesium phosphate, at pH 6.4. Structural colloid intact and not hydrated. The nuclear membrane of Xenopus is sacculated. (x80)

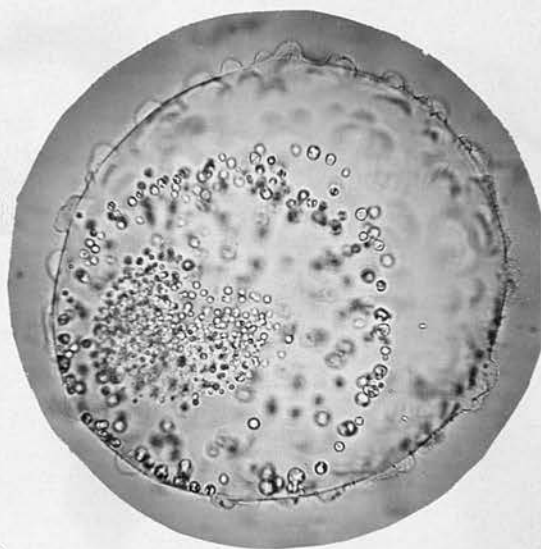
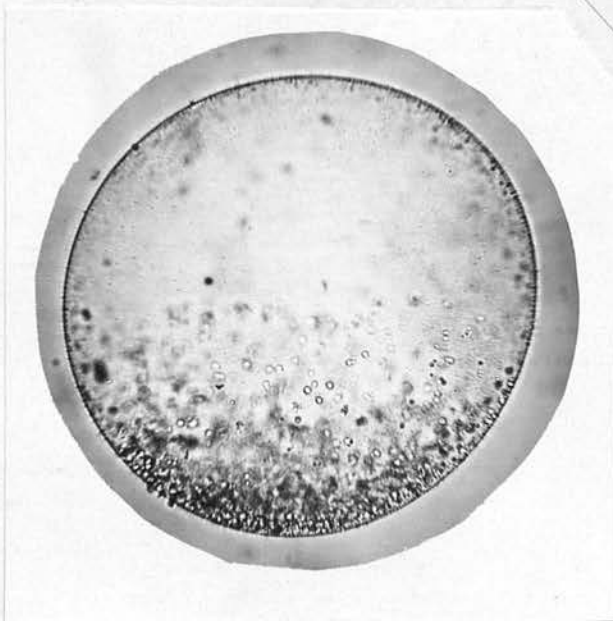


Fig.5. Oocyte nucleus of T.c.cristatus isolated in 0.2 M potassium phosphate, saturated with magnesium phosphate, at pH 7.0. Structural colloid dispersed and nucleoli fallen under action of gravity. (x80)



central chromosome group visible as such though individual chromosomes cannot be resolved. Nuclei were isolated in solutions of 2.3%  $\text{KH}_2\text{PO}_4$  and 1.5%  $\text{K}_2\text{HPO}_4$  mixed in various proportions to give different pH values. At pH 5.8 and below the sap coagulates on isolation but the membrane nevertheless distends (see fig.3). From pH 6.3 - 6.8 there is no immediate coagulation and the membrane distends. However there is a component of the sap which remains undistended and which maintains the disposition of the nucleoli and chromosomes as in life.(see fig.4). Later this unswollen component does in fact disperse and at lower pH s slight coagulation occurs. At pH 7 and above the membrane distends and the nucleoli and chromosomes sink to the bottom of the nucleus without the intervention of a stage when a component of the sap remains unswollen.(see fig.5)

There are evidently two colloidal phases : a structural colloid with mechanical rigidity, presumably consisting of fibrous molecules forming a meshwork, and a disperse colloid which is fluid and which is responsible for the colloid osmotic pressure developed by the nucleus. ~~The latter presumably consists of globular molecules~~ The disperse colloid presumably consists of globular molecules. Physiological pH would appear to lie somewhere between 6.3 and 6.8 and this is borne out by some preliminary experiments with J.F.Danielli involving the micro-injection of colloidal indicator substances having negligible protein errors.

The effects of chlorides of the alkali earth metals have also been studied. Calcium and strontium chlorides have a powerful disruptive action on the structural phase of the nuclear sap and this action also occurs in mixtures with potassium chloride. On the other hand magnesium and to a lesser extent barium chlorides protect the structural phase from hydration on isolation of the nuclei. The best preservation of life-like conditions of the nuclear sap for nuclei isolated into colloid-free media occurs when potassium and magnesium chlorides at 0.2 M concentration are mixed in the proportions 19 : 1-2. It seems probable that these two cations are in physiological balance in life though the proportions may well be different since the nucleus of the intact oocyte has colloidal materials on both sides of the membrane.

It is logical that the structural colloid should be more sensitive to abnormal pH and cation species and



concentration in the environment than the disperse colloid, if the structural colloid in fact consists of fibrous molecules with exposed side chains. The chromosomes, probably for the same reason, are also highly sensitive to environmental ionic and pH conditions. The disperse colloid coagulates only at pH s below the range obtainable with phosphate buffer solutions.

## 2. Chemical properties

Since the discovery by the Stedmans of proteins other than histones and protamines in nuclei of various kinds, there has been considerable argument amongst biologists and biochemists as to the precise localisation of the substances known from bulk analyses to be present in nuclei. (see the appended letters to NATURE on "The distribution of nucleic acid in the cell"). I hold the view that desoxyribose nucleic acid is confined to the chromosomes, at least in those stages when the chromosomes are visible in the light microscope. Preliminary study of the nuclear sap from amphibian oocytes by ultra-violet absorption and paper chromatography of the acid hydrolysate definitely shows that the sap is a complex protein or proteins and that it does not contain nucleic acid. (see the appended paper by G.L. Brown, H.G. Callan and G. Leaf entitled "The chemical nature of nuclear sap"). The presence of small quantities of basic proteins is not as yet ruled out: this question is being studied at the present moment. An electrophoretic study of the sap to decide ~~whether~~ whether it is chemically homo- or hetero-geneous is also under way.

## THE NUCLEOLI

I have not yet studied the nucleoli of amphibian oocyte nuclei in other than a cursory fashion. The nucleoli are formed at certain characteristic regions of the chromosomes: these regions are very probably the low-temperature sensitive regions which I have described both at mitosis and meiosis in the appended paper entitled "Heterochromatin in Triton".

The nucleoli leave the chromosomes after they have grown to a certain size; there are stages, particularly just before the final accumulation of nucleoli around the central chromosome mass in almost mature oocytes, when sufficient

nucleoli are attached to the chromosomes as to render chromosome "mapping" a feasible proposition. The disposition and size of the nucleoli are similar, perhaps identical, in homologous chromosomes.

It seems certain that the nucleoli migrate across the nuclear sap towards the nuclear membrane in well-defined channels, this accounting for their transient radial distribution from the central mass observable at certain stages.

When the nucleoli are formed amongst the chromosomes, their outlines are round and much less refractile than after they have left the chromosomes. The size range of nucleoli as they leave the chromosomes in oocytes prior to yolk accumulation is 0.8 - 3.5 $\mu$ . They pass through an "amoeboid" stage while lying at the surface of the membrane, increase greatly in size (to 10 - 15 $\mu$ ) and droplets appear inside them from the resolving limits to a maximum diameter of 2.5 $\mu$ . The nucleoli later have round outlines once more. They become reduced in size and number as the oocytes reach maturity.

The appearance of the nucleoli is a good index of the suitability of their environment under experimental conditions since they are extremely sensitive to electrolyte type and concentration. In potassium chloride solutions in the concentration range 0 - 1.5 M they swell, at 0.2 M they are well preserved and from 0.3 M upwards they dissolve. Among the chlorides of monovalent metals at 0.2 M concentration they are well preserved only in the presence of potassium. They dissolve in 0.2 M solutions of potassium bromide, iodide, nitrate, thiocyanate, thiosulphate, oxalate and almost completely in potassium sulphate. They swell in phosphate solutions over a wide pH range and are only well preserved in potassium chloride and bicarbonate.

The nucleoli have a strong absorption in ultra-violet light at 2600 A, indicating the presence of nucleic acid. They give positive Millon's reaction for protein. They contain alkaline and acid phosphatases. A very striking feature is a lipid layer at the surface of the nucleoli which is detectable as such in polarised light and which stains selectively with sudan black.

## THE CHROMOSOMES

The "lamp-brush" chromosomes of amphibian oocytes have been remarkably little studied considering their many interesting features. I have not yet studied them in detail though I have started an investigation, in collaboration with S.G. Tomlin, on lamp-brush chromosomes in the electron microscope.

In the past ten years a number of scientists have attempted to study chromosomes in the electron microscope, None of these attempts have been strikingly successful since either a. the chromosomes studied were too thick, or b. thread-like material was recovered from masses of heterogeneous tissue, after violent extraction procedures, which might or might not have been chromosomes and which in any case gave no information concerning fine structure. The only successful investigation to date is that of S.L. Palay and A. Claude. These workers studied collodion replicas of the surfaces of salivary gland chromosomes of Drosophila and confirmed earlier interpretations of the structure of these chromosomes based on observations in the light microscope.

It has proved possible to prepare single lamp-brush chromosomes and to photograph them in the electron microscope. The only stage examined so far come from oocytes of Triturus c. carnifex of 1.6mm diameter. The chromosomes at this stage have lateral outgrowths which have already passed their maximum development.

A nucleus is isolated in 0.2 M potassium chloride and washed free from yolk. The membrane is now broken and removed by means of tungsten needles and the jellylike sap chopped away from the central chromosome mass. A drop of potassium chloride solution is now placed on a coverslip coated with a lightly metallised formvar film and the chromosome group transferred to it. Using a binocular microscope with magnification x 87 the sap material is now agitated away from the chromosomes by the freehand use of a tungsten needle. The visibility of the chromosomes rapidly improves as the sap disperses. Finally a single chromosome arm is picked up by the needle and tacked down on to the formvar film. By a rotary movement of the needle the rest of the chromosome is freed from sap and attached to the film. Chiasmata may be suitably laid out on the film by attaching the four free chromosome arms in turn with

slight tension applied to each. After renewed washing in potassium chloride solution the preparation is fixed for two minutes in 0.1 % phosphotungstic acid, washed in distilled water and dried. The film is later stripped from the coverslip, mounted on a copper grid and subsequently shadowed with palladium. Composite photographs have been built up which are too cumbersome to include with the present text, but they can be examined in the laboratory.

The chief information which has been obtained so far is that :

- a. the classic idea of chromosomes as consisting of larger genic units strung together on a fine thread is confirmed. The thread is astonishingly thin : it has a uniform diameter of the order 100 - 200 A.
- b. contrary to previous statements, homologous chromosomes can be held together by chiasmata despite the fact that the axial threads are still single.

Point b. requires some qualification and explanation. It has always been thought that the lampbrush chromosomes are at diplotene of the first meiotic division. Homologous chromosomes are certainly joined together at points whose number and position resemble those of chiasmata at diplotene of spermatogenetic meiosis. I suggest that the chromosomes of amphibian oocyte nuclei throughout the oocytes' growth phase in the ovary are at a stage intermediate between pachytene and diplotene, corresponding to the so-called "diffuse" stage which is often found in spermatogenetic meiosis. The homologues have separated from one another except, for an~~d~~ as yet unexplained reason, at points where typical diplotene chiasmata will later appear. The coexistence of single chromosome threads with such " pro-chiasmata" would suggest that the " torsion theory" of chiasma formation propounded by Darlington is no longer tenable. Further work on this problem is in progress.

There is also a distinct possibility that we may learn something of gene structure by means of the electron microscope when the technique of chromosome preparation has been more thoroughly explored.