

# THE AMPHIBIAN OOCYTE NUCLEUS

Herbert Cecil Macgregor

A Thesis Submitted for the Degree of PhD  
at the  
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**- THE AMPHIBIAN OOCYTE NUCLEUS -**

A Thesis presented for the degree

of

Doctor of Philosophy

to

The University of St. Andrews

by

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MS 2187.

**- DECLARATION -**

I hereby declare that this Thesis is based upon the results of my own work and that, apart from Appendix I, it has been written by me without assistance. Professor H.G. Callan and I are jointly responsible for Appendix I.

My thesis has not been submitted for any other degree.

**- CERTIFICATE -**

---

I certify that Mr. Herbert Cecil Macgregor has spent nine terms at research work on The Amphibian Oocyte Nucleus, that he has fulfilled the conditions of Ordinance No.16 (St.Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

- UNIVERSITY CAREER AND RESEARCH EXPERIENCE -

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I entered the University of St. Andrews in October 1954 and was awarded a first class Honours B.Sc. in Zoology in June of 1958. During my last year as an undergraduate student I began my work on the "lampbrush" chromosomes of newts, and at the end of that year I wrote a thesis entitled Chemical Studies of Lampbrush Chromosomes. Since September 1958 I have been occupied with various problems of morphology and physiology in the oocyte nuclei of newts. The up-to-date results of my researches are presented here as a thesis for the degree of Doctor of Philosophy.

- ACKNOWLEDGEMENTS -

---

It is a great pleasure to express my warmest thanks to all those people with whom I have discussed many aspects of my researches. I am indebted to Professor J.M.Dedd (Director of the Gatty Marine Laboratory until September 1960) for his technical assistance and for advice on matters of vertebrate endocrinology, to Dr. G.R.Tristram and Dr. G.A.J. Goodlad (both of the Department of Biochemistry, St. Andrews) with whom I have discussed the actions of proteolytic enzymes and the biochemistry of nucleic acids, and to Dr. J.G.Gall (University of Minnesota) with whom I had the good fortune to spend many hours talking about lampbrush chromosomes and "nucleoli".

Above all, I wish to thank Professor H.G. Callan for introducing me to a fascinating and rewarding line of research, for keen interest in my work and for generous guidance, and for setting an example which has been a constant source of inspiration.

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## INTRODUCTION

### CHAPTER I

The object of this thesis is to add to existing knowledge of the chemistry and physiology of the cell nucleus:

It has been known for more than half a century that cytoplasm deprived of a nucleus cannot survive. The interchange of materials between nucleus and cytoplasm is now an accepted general phenomenon and much current research is concerned with analysing the nature of the substances exchanged, with the mechanisms of control of cytoplasmic processes by the nucleus, and vice-versa. The picture emerging shows a dynamic relationship between nucleus and cytoplasm, a complex interplay of feedback systems, in the midst of which we can recognise genetic material as almost the sole unvarying component.

Recent developments in cell biology have made it possible to identify genetic material with a particular substance, deoxyribonucleic acid (DNA), and the fact that this substance is localised in the cell nucleus and in the chromosomes of higher organisms is of the utmost significance. Nuclear genes, segregating according to the laws of Mendelian inheritance and in some cells visible as concentrated packets of DNA along the chromosomes, exercise control over patterns and schemes of development. Studies of the potentialities for enzyme production in micro-organisms have shown alterations

which are genetically controlled, and frequently the specificity of an enzyme can be referred to a single gene locus.

Furthermore, the fact that nuclear genes can exercise direct control over the sequence of amino acids in the molecule of a single cytoplasmic protein seems well attested in the case of haemoglobins. Besides the more specific effects deriving from "primary genetic material", there are also relatively non-specific ways in which this material can control and modify cell metabolism. DNA controls the energy available for nuclear synthetic processes. DNA participates in the synthesis of ribonucleic acid (RNA) which in turn is thought to transmit information necessary for the manufacture of cytoplasmic proteins. DNA even possesses the capacity to modify its own behaviour.

Constancy in the amount of DNA per nucleus, constancy in chromosome number, and constancy in the banding pattern of salivary gland chromosomes of Diptera strongly suggests that in any organism each cell carries a full complement of genetic factors. Yet if we accept the current dogma of "one gene one enzyme" then we may reasonably suspect that this genetic omnipotence is seldom totally expressed within a single cell. Nuclei differentiate, in the course of development, in response to changes in the cytoplasm in which they lie, and in keeping with the function of the tissue of which they are a part. Where chromosomes are visible during interphase, as are the

polytene chromosomes of Diptera, they too have shapes and patterns of synthetic activity which are not only tissue specific but also characteristic of particular stages in the development of the organism. These phenomena have justly been interpreted as expressions of differential gene activity.

The principles outlined above are the outcome of the application of a wide variety of techniques to an even wider variety of biological materials. It is however, a feature of cell biology that the nature of the problem dictates the type of material which shall be used in its analysis, and in turn, the material places limitations upon the choice of technique. Where the need is to examine the fine structure of nuclear components or to record morphological changes within single cells then large cells with large nuclei are favourable; nuclear components must be evident and simple techniques as well as more elaborate modern methods should be applicable.

An amphibian oocyte is generally speaking a large cell, and although when fully grown it owes much of its characteristic size to an accumulation of yolk, it also contains all the other potentialities which will be required during the early development of an embryo. The "germinal vesicle" or oocyte nucleus is immense in comparison with its counterpart in a somatic cell. The largest germinal vesicle which I have recorded in newts had a volume of more than  $10^8$  cubic micra. A comparable figure for a typical mammalian germinal vesicle

is about  $3 \times 10^4$  cubic micra. The chromosomes of yolky oocytes are generally large, and they offer exceptional opportunities for the study of chromosome form and function. They are without doubt physiologically active and they represent the full genetic complement of the parent individual. Oocyte nuclei always contain at least one conspicuous extrachromosomal body, which may or may not be considered a nucleolus; but a feature of oocytes from many sources, vertebrate and invertebrate, is that their nuclei contain an astonishing variety of objects, all of which until recently were collectively referred to as nucleoli. The inaptitude of this term will be apparent later.

Let us now consider the specific virtues of newts as a source of experimental material. Many species of newts are easy to keep in captivity and can be maintained throughout the year in breeding condition. Newt ovaries contain several thousand oocytes, some of which are more than 1 mm in diameter. Newt oocyte nuclei are correspondingly large, and they contain "lampbrush" chromosomes whose morphology has been the theme of much research. The studies of Duryee (1937, 1941, 1950), Dodson (1948), Gall (1954, 1956a), Callan (1955, 1957) and Callan & Lloyd (1960) are especially noteworthy.

I too have studied the morphology of newt oocyte nuclei and lampbrush chromosomes; but I have also tried to relate changes in morphology to changes in physiological activity, as Beerman (1952, 1956, 1959) and Pavan & Breuer (1955a, b,) have done

so cleverly for the giant polytene chromosomes of Chironomus and Rhynchosciara, respectively. Bridges (1935), Beerman (1950, 1952), and Pavan & Breuer (1952) characterised the salivary gland chromosomes of Drosophila, Chironomus and Rhynchosciara, and in so doing they provided a necessary basis for subsequent physiological studies on this kind of material. Likewise Callan (1952, 1955) and Callan & Lloyd (1960) have characterised the germinal vesicles and lampbrush chromosomes of T.cristatus. My own work is based upon their findings and without the information contained in their reports I could not have proceeded far. In this thesis I hold to the terminology and conventions established by Callan & Lloyd (1960) when defining sites on the lampbrush chromosomes of T.cristatus.

My own work has been along three main lines. First, I reinvestigated the chemistry of newt lampbrush chromosomes and tried to explain certain features of these chromosomes which seemed incompatible with accepted cytological data. This work has been prepared for publication and the text and illustrations of the paper form Appendix I to this thesis.

My second line of work represents an attempt to unravel the reasons for variability in certain features of newt oocyte nuclei which is superimposed upon the stable species and individual characteristics. Previous authors, in describing oocyte nuclei and lampbrush chromosomes of newts, have naturally and deliberately disregarded this variability. They have confined their attention to problems of general morphology,

in order to map those characteristics of lampbrush chromosomes which are species-specific. I on the contrary chose the most variable features of oocyte nuclei and studied them to see with which factors their variability was correlated.

Throughout my studies I paid particular attention to the "nucleoli" of newt oocytes, in view of their doubtful nature and origin. I soon learned enough about "nucleoli" to see the need for their thorough reinvestigation. My observations on "nucleoli" are set out in Chapter III.

## CHAPTER II

### PHYSIOLOGICAL CHANGES IN OOCYTE NUCLEI

#### 1. INTRODUCTION

This chapter deals with a study of morphological variability in the oocyte nuclei of the newt Triturus cristatus carnifex.

To make the situation clear I shall begin by defining three types of variability which we may expect to find in newt oocyte nuclei. "Genetic" variability comprises individual-specific differences; it most frequently involves the presence or absence of a single loop pair on a particular lampbrush chromosome at a particular site. "Developmental" variability

includes those changes which regularly accompany the growth of an oocyte. It is encountered when we compare oocytes of different sizes taken from the same animal at the same time. All variability which cannot be classed as developmental and which is not known to be genetic will henceforth be referred to as "physiological".

No two oocyte nuclei are exactly alike whatever their origin, and it is therefore essential to set a standard of difference beyond which we may attach significance to inter-nuclear variation. The standard which I have adopted is the level of variation which exists between like sized oocytes taken from the same animal at the same time.

Many of the irreversible changes which are expressions of developmental variability in oocytes have already been described, (Ruckert, 1892, Duryee 1950, Callan 1952, Guyenot & Danon 1953, Gall 1954 1955); a useful and detailed account of cytoplasmic developmental variations in frog oocytes is given by Kemp (1956a, 1956b).

Of prime importance in this work are those changes in the morphology of lampbrush chromosomes which we can classify as developmental. The only conclusive data which exists on this topic is an outcome of the work of Callan & Lloyd (1960), who studied the lampbrush chromosomes of Triturus cristatus using oocytes of 0.6 to 1.7 mm. diameter. As my own work

deals exclusively with the sub-species carnifex, I shall mention the findings of Callan & Lloyd only in so far as they relate to this particular sub-species. Fig.1 is a working map (prepared by Callan & Lloyd, 1960) of the lampbrush chromosomes of carnifex. It shows the relative lengths of the chromosomes, positions of centromeres, and loci at which one may expect to find landmark structures. Those loops which may be loosely referred to as "normal", and of which there are several thousand, have been omitted from the diagram. The conventions introduced by Callan & Lloyd for defining particular sites on the chromosomes will be found on pages 13 and 14 of the Appendix I in this thesis. It should be emphasised at once that although interhomologue differences with respect to the presence or absence of "landmarks" are constant from oocyte to oocyte from a single individual, they are not necessarily constant between individuals.

Technical difficulties have so far prevented an accurate characterisation of the early transitional stages during which a chromosome assumes the lampbrush form. In carnifex we know that lampbrush chromosomes are present in oocytes of 0.4 mm. in diameter, but we cannot vouch for the presence of lateral loops on the chromosomes of smaller oocytes. Observations made in this laboratory suggest that by the time an oocyte is 0.6 mm. in diameter its chromosomes have reached their maximum length. There is no further appreciable change

in length until the oocyte is nearing maturity, when a sudden contraction sets in. The lateral loops of the chromosomes are longest in oocytes of between 0.6 and 0.8 mm. in diameter. As an oocyte nears maturity the loops regress and finally they are resorbed into the body of the contracting chromosome. In spite of developmental changes in absolute chromosome length, relative chromosome length within a single complement remains constant.

Some landmark structures show striking developmental changes in their size and general appearance.

The axial granules of the left arm of chromosome I (see Figs. 32 and 33) are small and inconspicuous in small oocytes, whereas they are large and distinct in older oocytes.

The middle region of chromosome II (from 59 to 79 units) carries a series of "lumpy" objects (see Fig. 17, Appendix I) which in small oocytes (less than 0.7 mm. diameter) are by far the most striking landmarks of the entire chromosome complement. In larger oocytes they are consistently smaller and some of them may be difficult to locate.

In oocytes within the size ranges 0.6 to 0.8 mm. and 1.1 to 1.6 mm. (diameter) there are sub-terminal objects on the left arms of chromosomes III, IV, and XI (see Fig. 31a). These objects, which have been called "currant buns" (Callan & Lloyd 1960), occur singly, in pairs, or in clusters of three and four.

In oocytes of 0.8 to 1.1 mm. diameter current buds are inconspicuous or absent and their places are occupied by normal lateral loops.

On the left arm of chromosome V at 2 units and 11 units are solitary spherical objects of up to 15 $\mu$  in diameter (see Fig.28). These are known as "spheres". There is a similar object at 3.5 units on the left arm of chromosome VIII. Spheres tend to be smaller in smaller oocytes. In larger oocytes objects closely resembling the spheres of chromosomes V and VIII are found free in the nuclear sap. These objects are without doubt free spheres, and as Callan & Lloyd have pointed out, their presence suggests that beyond a certain stage in oogenesis there is a regular cycle of growth and detachment at the sphere loci. The situation is complicated however by the fact that after free spheres make their appearance in a nucleus their number seems unrelated to oocyte size. I shall return to this question later.

Chromosome X bears "giant loops" on its left arm at 18.5 units. They have well defined but irregular outlines. They are formed of a stiff material which is highly refractile and which frequently contains vacuoles. The degree to which they maintain the form of loops varies from oocyte to oocyte. Giant loops may or may not be present on both homologues of chromosome X. Their presence or absence is known to be a fixed individual-specific character. They are generally larger in larger oocytes although any increase in their size may be small

or negligible until the oocyte reaches about 1.5 mm. diameter. In oocytes which are nearing maturity and in which chromosome contraction is taking place, the giant loops are often truly enormous. At 24 units on the left arm of chromosome XI there is another pair of "giant loops" which resemble those of chromosome X both in texture and in behaviour (see Figs. 26 and 27). At 22.5 units on the left arm of chromosome XII there is a giant loop which differs from those of chromosomes X and XI only in that it is a multiple structure.

In every account of the morphology of the amphibian oocyte nucleus we find some statement concerning free granules present in the nucleoplasm. They were first described by Ruckert (1892). Weismann (1892) attached significance to them and proposed that "minute, specific, vital particles, and not merely nutritive substances, are produced by the chromosomes during the growth of the egg, and are then emitted through the nuclear membrane into the cell body". They were described by Duryee (1950) in T. pyrrogaster as "a cloud of hyaline and refractile bodies surrounding and emerging from the central chromosome area". Gall (1954) refers to them in T. viridescens as "smaller nucleoli ranging in size from 1 to 4 $\mu$  in diameter"; he points out that morphologically identical structures are found at many loci on the chromosomes. The suggestion follows that these objects fall off the chromosomes in the later stages of oogenesis. In 1955 Gall again draws attention to "clouds

of large granules surrounding the loops of the "lampbrush" chromosomes, as well as hundreds of thousands of minute granules ranging from barely visible to 4 or 5 $\mu$  in diameter, and dispersed throughout the nuclear sap". Callan (1955) draws attention to a variety of objects which lie free in the nuclear sap and which range in size down to the limits of resolution; and again in 1960 Callan & Lloyd refer to tiny granules 2 $\mu$  or less in size which vary greatly in number from oocyte to oocyte. From all accounts it seems that free granules of this description are present in the germinal vesicles of all Amphibia. However, remarkably few authors have proffered any statement regarding developmental variability in the numbers or size of these granules. In T. cristatus they are certainly a feature of germinal vesicles from oocytes of 0.8 to 1.4 mm. diameter, but they are absent from the germinal vesicles of larger oocytes.

The foregoing descriptions of developmental changes in lampbrush chromosomes and oocyte nuclei contribute to the classic representation of the growth of an oocyte as an orderly sequence of nicely timed events. According to this picture we ought to be able to predict the biochemical and morphological conditions which we shall find if we examine an oocyte of a given developmental stage. To some extent this is possible, but frequently our predictions prove wrong. This leads us to seek the answers to three basic questions. First, which of the features which can be recognised and described in fresh material

show significant physiological variability when we compare like-sized oocytes from different animals? Secondly, how can we know that this variability is not genetic? Finally, having recognised the extremes of a variety of morphological conditions and having shown that they result from differences in physiological activity, how can we correlate a given condition with a certain rate of metabolism?

What follows is an attempt to answer these questions. In order to simplify matters I have concentrated upon oocytes ranging from 0.9 to 1.0 mm. in diameter. Oocytes of this order of size will henceforth be referred to as "Standard-size"; they are about half grown and although the lateral loops of the lampbrush chromosomes are not at their longest, landmarks are well developed and chromosomes or parts of chromosomes can readily be identified. Where larger or smaller oocytes are used, sizes will be specified.

The following features show a variability which cannot be classed as developmental and which is not known to be genetic; we will assume for the moment therefore, that it is physiological.

#### 1. Viscosity of the nuclear sap.

In all standard-size oocytes from one animal the nuclear sap may be a stiff gel which takes up to 20 minutes to disperse after removal of the nuclear membrane in 0.07 N saline. Stiff

sap cushions the chromosomes during isolation procedures and facilitates the production of intact lampbrush chromosome preparations. In standard-size oocytes from another animal the nuclear sap may be more fluid; it takes less time to disperse and the isolation of intact lampbrush chromosomes is difficult. A range of intermediate conditions has been recognised.

## 2. Free granules in the nuclear sap

Some nuclei contain vast numbers of small free granules (0.5 to 2 $\mu$  in diameter), others may have no free granules, while some may contain a few large granules (2 to 4 $\mu$  diameter) or countless tiny particles (less than 0.5 $\mu$  diameter). All standard-size-oocyte nuclei taken from the same animal at the same time are similar with respect to the granularity of their sap.

3. The giant loops of chromosomes X, XI, and XII, the currant buns of chromosomes III, IV, and XI, and the sphaeres of chromosomes V and VIII, show a variability in size and general morphology which is superimposed upon and often obliterates their developmental variability.

## 4. Chromosome length

Callan & Lloyd (1960) recorded an extreme variability

in absolute chromosome length within oocyte size classes. They were unable to correlate this variability with any known factor.

Presented with this assortment of physiological variables I set out to test how much variability was in fact due to differences in oocyte metabolism. My experimental approach was simple in principle. The task of the amphibian ovary is to manufacture oocytes. The rate at which it does so will depend upon the "genetic capacity" of the individual cells, and upon the nature of the environment in which these cells grow. If the physiological variables which I have listed do reflect differences in rates of oocyte metabolism, then modification of the cell's environment ought to affect their variability; by standardising the environment it might even be possible to eliminate physiological variability.

## 2. MATERIALS AND METHODS (GENERAL)

The newt which has been used in these experiments is Triturus cristatus carnifex (Laurenti), collected near Naples, Italy, and supplied by Professor P. Dohrn of the Stazione Zoologica.

The laboratory stocks from which experimental animals were selected were kept in stagnant water and were fed twice weekly on earthworms or Tubifex. The tank room was permanently lit and maintained at a temperature of about 16°C. The conditions under which experimental animals were kept will be specified later. Healthy, well fed animals whose ovaries contained adequate numbers of standard-size oocytes were selected for experimental purposes.

To obtain oocytes, newts were lightly anaesthetised by immersing them for 15 minutes in a 0.2% solution of tricaine methane sulphonate (MS222, Sandoz). In some early experiments ether was used for anaesthesia: however, all experiments involved two laparotomies in the space of 14 days and during anaesthesia for the second laparotomy the violent physical reactions of the newt to ether vapour often ruptured the unhealed wound of the first operation. A short ventrolateral incision was made in the skin and musculature of the body wall, and a piece of ovary was excised. The piece of ovary was placed in the well of a clean dry solid watchglass. A glass square was then sealed over the top of the watchglass which was then transferred to a dish containing melting ice.

When a newt was scheduled to undergo two operations the first was always performed on the animal's left side, and the second on its right side. On each occasion the amount of

ovarian tissue removed never exceeded one third of the total bulk of the ovary on that side. Operation wounds were sewn up with two stitches of plain 3/0 catgut suture. Newts were placed in running tap water until they recovered from anaesthesia. Selected experimental animals were labelled with small coloured plastic rings which were attached to a loop of fine nylon thread passing through the base of the animal's tail. Ovariectomies were always performed before 10 a.m. so that oocytes could be examined on the same day.

The technique for isolating oocyte nuclei and removing nuclear membranes was the same as that employed by Gall (1954). Full details of this technique have been given by Callan and Lloyd (1960), where Gall's observation chamber for use with an inverted microscope system is also described. In brief, Gall arranged that chromosomes should spread out over a coverslip which is sealed across the bottom of a small hole ( $\frac{1}{4}$ " ) bored through an ordinary microscope slide.

The oocyte nucleus is first isolated in a 5:1 mixture of 0.1 N potassium and sodium chlorides and is then transferred to Callan and Lloyd's "medium C" in an observation chamber for removal of its membrane. When about 50 mm<sup>3</sup> of medium C is placed in an observation chamber it forms a meniscus convex upwards. Once the nuclear membrane has been removed from a nucleus lying in the chamber a  $\frac{3}{8}$ " square coverslip is dropped

in place over the meniscus. After a few minutes (the actual time depends upon the viscosity of the nuclear sap) the nuclear sap has dispersed and the chromosomes have spread out over the lower coverslip. Oocytes were measured and dissected, and nuclear membranes were removed, with the aid of a Beck binocular dissecting microscope which was fitted with a micrometer eyepiece.

The media which I used for isolating nuclei and removing nuclear membranes were designed by Callan to correspond as nearly as possible to the ionic conditions within the living nucleus, and to preserve the chromosomes in a life-like state, (for justification of this see pages 23 and 24 of Appendix I). Medium C consists of 7 parts of the 5:1 0.1 M KCl/NaCl mixture, together with 3 parts of 0.001 M  $\text{KH}_2\text{PO}_4$ . After sterilisation by boiling, it has a pH of 6.2. Throughout this work the ionic concentration and pH of medium C has been scrupulously controlled. In making up fresh lots, analar reagents were weighed to within half a milligram, dissolved in the appropriate quantity of freshly distilled water, and the pH was checked to within two decimal places at 20°C. Measurements of pH were taken using a Cambridge Bench type meter wired to glass electrodes.

Preparations of lampbrush chromosomes in their observation chambers were observed with an inverted phase contrast microscope (IPCM) (Cooke, Troughton & Simms Ltd., York) giving dark contrast (alternatively known as bright field).

To record morphological changes in lampbrush chromosomes and oocyte nuclei photographs of the primary image formed by the objective lens of the microscope were taken with a single exposure camera loaded with Ilford Micro-neg Pan film. A conventional light source with a Compur shutter of 35 mm. aperture on a vibration-free mount interposed between the light source and the microscope condenser was used. Exposure times varied from 1/100th sec, using the x 10 objective to 1/10th sec. using the x 95 objective. All other methods will be described later.

### 5. EXPERIMENTAL METHODS

The experiments which I shall describe in this section were designed to depress or stimulate the metabolic activity of the newt oocyte.

#### A. Hypophysectomy

It is well known that the condition of the gonads in female Amphibia is controlled by the anterior lobe of the pituitary. The facts are presented and discussed in reviews by Moussay (1949), Smith (1955) and Dodd (1960). In Triturus the effects of hypophysectomy upon the ovaries of mature females have been described by Burns (1932) and Tuchmann-

Duplessis (1945). The latter author reports that fourteen days after hypophysectomy ovarian weight had decreased by 25% as compared with that of the control animals. After one month the figure had risen to over 70%. Smaller oocytes remained unaffected for a considerable time after hypophysectomy: they continued to grow but degenerated upon reaching a diameter of 250 to 300 $\mu$ . Tuchmann-Duplessis interpreted his results as indicating that only the later stages of oogenesis are dependent upon stimulation by pituitary hormones.

I set out to learn about the effects of hypophysectomy upon the nuclei of growing oocytes. Basically my plan was as follows. Newts of comparable sizes were set up in pairs. A sample of ovary was taken from both members of each pair and certain features of oocytes, oocyte nuclei and lampbrush chromosomes were recorded. One member of each pair was then hypophysectomised while the other was control operated. Controls and experimentals were kept under similar conditions for a certain length of time, the "experimental period", at the end of which another sample of ovary was taken from each animal, and the same features of oocytes, their nuclei and their chromosomes, which had been recorded at the start of the experimental period, were re-examined to see if they had changed. Changes which were common to both experimental and control animals were disregarded. Changes which were peculiar to experimental animals were scored as reflecting an alteration

in oocyte metabolism resulting from hypophysectomy.

During the winter months, carnifex ovaries contain large numbers of standard-size oocytes. For this reason, all experiments in the present series were carried out between October and the following March. Each experiment involved 6 animals, 3 of which were hypophysectomised while 3 were used as controls. The animals were set up in pairs, one experimental to one control, on the basis of similarities in body weight, general ovarian condition, and characteristics of their oocyte nuclei and lampbrush chromosomes. The following features were recorded for each experimental animal and for each control:-

(Features 2 to <sup>7</sup> were recorded for five lampbrush preparations from each animal)

1. The number of atretic follicles in the ovary (estimated from an ovarian sample). Also, the sizes of the early corpora atretica.
2. Stiffness of the nuclear sap.
3. Granularity of the nuclear sap. Size of the largest free granules (excluding "nucleoli" and spheres).
4. The number of spheres per nucleus (including free and attached).
5. The average size of the spheres (including free and attached).
6. The size and general characteristics of the giant loops of chromosomes X, XI, and XII.
7. The presence or absence of the currant buns of chromosomes II, IV, and XI.

These same features were examined and recorded at the end of the experimental period. Other features were recorded from time to time. These will be mentioned and their significance discussed later. Only one pair of newts could be examined in one day. Thus the first observations of each experiment occupied 3 days.

To begin with, hypophysectomies were performed immediately after the first laparotomy, but it soon became apparent that removal of the pituitary interfered with healing of the abdominal wound. In subsequent experiments therefore, newts were allowed to convalesce for one week following the first laparotomy. They were then hypophysectomised. The method for removal of the pituitary was as follows \*. The animal was anaesthetised by immersion for 15 minutes in a 0.2% solution of MS222. Ventral side uppermost, it was lightly strapped to a specially designed operating board, and its lower jaw fully retracted. In this way the mucous membrane of the roof of the mouth was exposed. The pituitary could be seen through the mucous membrane and the parasphenoid as a small white patch situated in a median position in line with the angle of the lower jaw. A small hole was bored through the mucous membrane and the parasphenoid at this point, using a dental burr. Care was taken to avoid damage to the brain. The pituitary was then lifted out with a pair of fine forceps, and excised. The entire gland was removed. No attempt was made

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\* All hypophysectomies were performed by Dr. J.M.Dodd.

to close the wound in the roof of the mouth. It healed completely in a matter of a few days. The animal was placed in running tap water to recover from anaesthesia. Control animals received the same treatment, but the pituitary was left intact and was not displaced. The only factor which could not be reproduced in controls was the rupture of the third ventricle of the brain caused by excision of the pituitary.

Successful hypophysectomy was followed in a few hours by a paling of the animal. Those experimentals which did not pale, and those controls which did pale, were rejected and destroyed.

The ultimate goal of these experiments was a description of nuclei from standard-size oocytes of healthy hypophysectomised newts; but to begin with it was necessary to establish a set of conditions under which hypophysectomised newts would survive. Furthermore, it was necessary to fix a suitable experimental period at the end of which standard-size oocytes would be maximally affected by hypophysectomy, but would still contain nuclei and lampbrush chromosomes which could be isolated and examined.

Throughout these experiments, each animal was kept in a separate Kilner jar containing 500 ml. tap water at  $16 \pm \frac{1}{2}^{\circ}$  C. in a constantly lit room. The water was changed every two days. The animals were fed twice weekly with small earthworms. Hypophysectomised newts were handled as little as possible.

In the first two experiments, six animals were hypophysectomised and six others were control operated. One animal stopped feeding, became inactive and died less than 14 days after hypophysectomy. Another (8) was sacrificed and re-examined 14 days after hypophysectomy. The remaining four experimental animals continued to feed and remained active for more than one month; but they did not moult. Controls moulted regularly and looked clean and healthy. Hypophysectomised animals became dark and grimy from accumulation of unshed cornified skin. Two animals (10) and (9) were killed and re-examined after 35 and 42 days respectively. Neither had moulted since hypophysectomy and both were in deplorable condition. Two others died before they could be re-examined. Gross post-mortem dissection of newts which had survived for more than one month following hypophysectomy revealed many of the symptoms of thyroid dysfunction which have been described by Taylor (1936) in Triturus torosus.

It is known that hypophysectomy or thyroidectomy in Urodeles inhibits the sloughing of cornified skin. Conversely, sloughing can be induced in hypophysectomised animals by treatment with thyroxine or thyrotrophic hormone, (Adams 1933, Adams and Gray 1936, Osborn 1936, Jorgensen and Larsen 1960). With these facts in mind I introduced a substitution therapy into subsequent experiments using 3-iodo-thyronine (

or thyroxine (l-thyroxine, sodium salt, obtained from British Drug Houses Ltd.) As hypophysectomised newts are adversely affected by handling or physical injury the therapeutic agent was dissolved in the water in which the newts were kept. 3-iodo-thyronine was found to be most effective when used at a concentration of 1 part in 2 million. After our stock of 3-iodo-thyronine was exhausted, thyroxine was used at an equivalent concentration of 1 part in  $4 \times 10^5$ . All experimental animals responded to treatment with either agent by moulting normally.

Of the first fifteen experimental animals, five became heavily infected with the fungus Saprolegnia. Control animals remained free from such fungus. All attempts to rid hypophysectomised animals of the fungus were unsuccessful. Therefore a method was sought whereby infection could be prevented. The method which I finally adopted was devised by Mr. P. Evenett (Department of Zoology, Leeds University) for the prophylactic treatment of fungus infection in lampreys. It involved bathing the animals in a 0.002% solution of mercurochrome for three days prior to hypophysectomy and for one hour per day following hypophysectomy. Hypophysectomised newts, treated in this way remained completely free from fungus.

With regard to the 3-iodo-thyronine, thyroxine, and mercurochrome, each control animal received the same treatment as its experimental partner.

Under the conditions which I have outlined above,

hypophysectomised carnifex females could be expected to survive for at least three weeks. Survival for more than six weeks was rare.

Let us now turn to the question of the length of the experimental period. An upper limit was set by the viability of the experimental animals. A re-examination of the oocytes of female (8), which was killed 14 days after hypophysectomy, was unrewarding. A re-examination of the oocytes of female (9), which was killed 42 days after hypophysectomy, showed all large oocytes and the majority of standard-size oocytes in the early stages of atresia. Thirty-five days after hypophysectomy the ovaries of female 10 contained many standard-size oocytes with workable nuclei and lampbrush chromosomes. In all subsequent experiments therefore, I aimed at an experimental period of 30 to 35 days. However, those animals which survived for more than fourteen days were kept under close observation, and as soon as they stopped feeding or became inactive they were killed and their oocytes were re-examined. If they remained healthy, then they were killed on the 35th day. Out of a total of twenty seven newts which were hypophysectomised, ten remained healthy for more than one month following hypophysectomy, six were killed and re-examined between the 14th and 30th days, eight died before they could be re-examined, and three were killed after 14 days.

B. Gonadotrophin treatment

Most Amphibia ovulate in response to anterior pituitary material from a wide variety of donors, and to mammalian gonadotrophic hormones of extra-hypophysial origin. T.cristatus is no exception in this respect. The effects of injected or implanted pituitary material or of chorionic and serum gonadotrophins upon growing oocytes are less easily defined, but it seems certain that an acceleration of the growth of immature oocytes accompanies or follows ovulation. Tuchmann-Duplessis (1945) treated hypophysectomised Triton with mammalian serum gonadotrophin and found that it alleviated the regressive changes in the ovary. Wolf (1929) showed that by implanting homoplastic pituitary material ovulation could be induced as early as September in Rana pipiens. The latter normally breeds in late spring. This suggests that the gonadotrophins of the anterior pituitary have a stimulatory effect upon the gonads, causing a telescoping of the normal cycle of growth. March (1937) noted that mammalian anterior pituitary material stimulated the ovaries of summer frogs and induced a "preocious transition from the juvenile to the adult condition, or from a state of static regression to one of accelerated functional activity".

There is nothing to be gained by delving more deeply into past literature on this particular topic. Rather let it be said at once that where one wishes to stimulate the metabolic

activity of growing amphibian oocytes, anterior pituitary material, chorionic gonadotrophin, or serum gonadotrophin are the materials most likely to produce the desired effect.

The present work started with the following assumptions:-

1. The metabolic activity of standard-size oocytes would increase following the injection of homoplastic pituitary material, chorionic gonadotrophin (CG), or serum gonadotrophin (PMS), into carnifex females.
2. Changes in oocyte nuclei or lampbrush chromosomes would be expressions of this increase.

Later I was able to justify these assumptions.

The first hormone which I used was a chorionic gonadotrophin prepared by Ciba Ges., Basel, and supplied by Dr. R. Meier. It was supplied in powder form at a strength of 400 I.U. per milligram. Henceforth I shall refer to this hormone as "CG (Swiss)". The greater part of my work however, involved the use of CG (Pregnyl) and PMS (Gestyl) supplied by Organon Laboratories Ltd., London. CG was supplied in ampoules containing 1,500 I.U., and PMS in ampoules containing 1,000 I.U.

While undergoing treatment newts were kept separately in 7 lb. Kilner jars containing 500 ml. tap water at  $16 \pm \frac{1}{2}^{\circ}\text{C}$ . in a constantly lit room.

All experiments were run according to a common plan.

Animals were generally set up in pairs, one experimental to one control, but in some cases controls were deemed unnecessary and were omitted. Samples of ovary were taken from experimentals and controls. Five lamprush preparations were made from standard-size oocytes of each animal and certain features of nuclei and chromosomes were recorded. Before each experimental animal had recovered from the anaesthesia of the first laparotomy it received an injection consisting of 200 I.U. of hormone dissolved in 1/10 ml. sterile injection water, while each control received the same volume of sterile injection water, but no hormone. Seven days later (168 hours) each animal received a second injection which was the same in quantity as the first. The second injection was administered without anaesthesia. Forty eight hours after the second injection each animal was opened up, a sample of ovary was removed, and those features of the nuclei and chromosomes of standard-size oocytes which had been recorded before the treatment were re-examined to see if they had changed.

Injections were carried out with hypodermic syringes graduated in one hundredths of a millilitre, and fitted with 20 gauge needles. Syringes were sterilised and cleaned immediately before and after use by immersion in boiling distilled water. One syringe was reserved for use on control animals. The injection procedure was as follows. The needle was inserted tangentially through the skin of the newt in the

ventrolateral region of the abdomen about 1 cm. in front of the hind limb. It was passed forwards immediately under the skin for about 1½ cms. and at this point the fluid was injected into the space between the skin and the body wall musculature. The injection fluid formed a large blister which disappeared after about three hours.

Altogether, five series of experiments were performed. These differed from one another with respect to the nature of the substance injected into the experimental animals. Table 1 lists for each series the numbers of the experimental animals, the hormones injected and the total amount of hormone given to each experimental animal.

TABLE 1

Series Number	Numbers of experimental animals	Amount of hormone injected	Hormone
1	<u>1</u> to <u>4</u>	400 I.U.	CG (Swiss)
2	<u>32</u> to <u>41</u>	400 I.U.	CG (Swiss)
3	<u>55</u> to <u>57</u>	400 I.U.	CG (Organon)
4	<u>53</u> and <u>54</u>	400 I.U.	PMS (Organon)
5*	<u>48</u> to <u>52</u> and <u>58</u> to <u>60</u>	200 I.U. of each	CG + PMS (Organon)

\* In series 5 I studied the effects of injecting a synergistic mixture of CG + PMS. Each injection consisted of 1/10th ml. sterile injection water containing 100 I.U. of CG and 100 I.U. of PMS.

C. Homoplastic Pituitary Treatment

Newt pituitary material became available as a result of my own work on hypophysectomised caranfex females and the work of a colleague on hypophysectomised males.

As soon as a gland had been excised it was placed in a small glass bottle which was then tightly stoppered and stored in a deep freeze cabinet. Glands were never stored for more than 12 hours.

Pituitary material was administered by injection. For each injection a prescribed number of glands was transferred on the point of a stainless steel needle to the bottom of a tissue macerator (glass tube and plastic pestle), 1/10th ml. of sterile injection water was added, and the tissue thoroughly macerated. All of the resulting suspension was drawn up into a hypodermic syringe and injected into an experimental animal.

Two series of experiments were run. The first involved four animals of which two were experimental and two were controls. The second involved eight animals, four experimentals and four controls. A sample of ovary was taken from each animal and certain features of the nuclei and chromosomes of standard-size oocytes were recorded. Experimentals and controls then underwent a course of treatment consisting of three similar injections. The first injection was given before the animal had recovered from the anaesthesia of the first

laparotomy. The second and third injections followed at 72 hour intervals. Forty eight hours after the third injection, each animal was again opened up, a sample of its ovary was removed, and those features of the nuclei and chromosomes of standard-size oocytes which had been recorded before treatment were re-examined to see if they had changed. Injections given to control animals contained no pituitary material, but in all other respects experimentals and controls were treated alike.

In the first series of experiments each experimental animal received one gland per injection; but this did not induce ovulation, nor did it bring about any significant changes in the oocyte nuclei. Therefore, in the second experimental series, the dose was increased to three glands per injection.

Throughout my experiments I have used hormones for the one purpose of changing the physiological states of growing oocytes. I have not deliberately investigated any of the endocrinological problems otherwise connected with this type of work. I have adhered to a standard technique which, to the endocrinologist will bespeak a lack of imagination and enterprise; but this technique has proved both adequate and convenient.

#### 4. RESULTS

To curtail what would otherwise be a lengthy and

complex report I shall start by emphasising the fact that homoplastic pituitary material, chorionic gonadotrophin and serum gonadotrophin, acting individually or synergistically all have the same general and widespread effect upon the ovaries of carnifex. I have found, however, that nine homoplastic pituitaries are less potent than 400 I.U. of chorionic or serum gonadotrophin, which in turn is less potent than 400 I.U. of a synergistic mixture of CG + PMS. The most consistent results were obtained with the latter treatment.

A. Chromosome length

I began this work with a search for the origin of variability in absolute chromosome length within oocyte size classes. When first presented with this problem it was known that when an oocyte was between 0.6 and 0.8 mm. in diameter, its chromosomes and their lateral loops were maximally extended, and it seemed that thereafter the chromosomes became shorter as the oocyte approached maturity. Contraction of a chromosome is accompanied by regression of its lateral loops and I visualised regression as reflecting a gradual cessation of the activity of "redundant" genes. If inactivity and contraction of the chromosome follow one another in this way, then activity and absolute chromosome length ought to be related.

Stimulation of the metabolic activity of growing oocytes with gonadotrophic hormones might shorten the duration of the active phase of the lampbrush loops, and chromosome contraction would therefore set in at an earlier stage. Conversely, stimulation might increase both the intensity and duration of chromosome activity so that the chromosomes would contract at a later stage. In either event, I expected stimulation to bring the metabolic activity of like-sized oocytes up to a common peak and so to eliminate variability in absolute chromosome length.

CG(Swiss) was used for treatment of the four experimental animals. There were no control animals. In all the experimental animals chromosome contraction was obviously well under way in oocytes having diameters greater than 1.4 mm. All preparations from which chromosome lengths were recorded were made from oocytes having diameters between 1.2 and 1.3 mm., since in this particular size range any tendency for the chromosomes to contract at an earlier stage as a result of stimulation would be apparent.

Five preparations were made from each animal before and after treatment. Drawings of the axes of one or more chromosomes per preparation were made with a camera lucida attached to an inclined monocular tube magnifying x 1.5. Objective magnification was x 40, eyepiece magnification x 6. In each preparation more than 30 minutes was allowed to pass

after removal of the nuclear membrane so that the chromosomes might settle in one focal plane before they were drawn. Only those bivalents which were unbroken and unstretched were drawn. For each bivalent drawn the lengths of both homologues were measured to the nearest millimeter with an opisometer (map measurer), and they were then averaged. Absolute chromosome lengths in micra were not calculated. Measurements were taken from chromosomes I to IX. Because of their inherent shortness, chromosomes X, XI, and XII are unsuitable for studies of changes in chromosome length. From measurements of drawn chromosomes equivalent values for chromosome V were calculated on the basis of known relative lengths of other chromosomes to chromosome V (see Callan & Lloyd, 1960).

Table 2a lists the values obtained from each animal at the start of the experiment. In table 2b are the values obtained from each animal after treatment.

There is a decrease in chromosome length ranging from 3% in newt 2 to 17% in newt 1; but the values for any one animal, or the overall means for different animals, are more scattered after treatment than they were before. I treated these results as indicating that variability in chromosome length within oocyte size classes is not necessarily due to differences in the physiological states of oocytes, and that the stage at which chromosome contraction sets in cannot be shifted by increasing the rate of growth of an oocyte.

TABLE 2a

Estimated lengths of chromosomes V calculated from lengths  
(in millimeters) of drawn chromosomes

Before gonadotrophin treatment

NEWT	PREPARATION		VALUES	MEANS	OVERALL MEAN
	No.				
<u>1</u>	1		287, 352, 327	315	326
	2		364, 369	366	
	3		300, 325	312	
	4		361	361	
	5		299, 258	278	
<u>2</u>	1		381	381	345
	2		336, 344, 348	343	
	3		330, 364	347	
	4		304	304	
	5		322, 377	349	
<u>3</u>	1		294, 256	275	290
	3		325, 342	333	
	6		260	260	
	7		280, 295, 302	292	
	8		301	301	
	9		275, 277, 285	279	
<u>4</u>	1		351, 304, 360, 287	325	326
	2		279, 285, 317	294	
	3		361	361	
	4		332, 367	349	
	5		284, 316	300	

TABLE 2b

Estimated lengths of chromosomes V calculated from lengths  
(in millimeters) of drawn chromosomes

After gonadotrophin treatment

NEWT	PREPARATION No.	VALUES	MEANS.	OVERALL MEAN
<u>1</u>	1	276	276	270
	4	287	287	
	6	244	244	
	7	255	255	
	8	254	254	
	11	259	259	
	12	310, 329	319	
	13	241, 296, 256, 273	267	
<u>12</u>	1	360, 368, 413	380	334
	2	286	286	
	3	334, 257	295	
	4	324, 374	349	
	5	363, 402	382	
<u>13</u>	1	228, 212	220	261
	2	249, 253, 242, 262	252	
	3	299, 260	279	
	4	275, 308	292	
	6	293	293	
	8	228, 228	228	
<u>4</u>	1	326, 249, 240 254, 260, 257	261	290
	2	331, 297, 286	308	
	3	356, 348	352	
	4	230, 249, 289	256	
	5	290, 280, 251	274	

Since I carried out this experiment Callan & Lloyd (1960) have published the results of a systematic survey of chromosome length in T.c.carnifex, from which it seems that chromosome contraction is not the gradual process which I had anticipated, but that it occurs suddenly towards the end of yolk accumulation. Their survey includes measurements of the absolute lengths of 64 bivalents from oocytes ranging in size from 0.6 to 1.68 mm. (diameter), taken from 13 animals.

Before treatment, the nuclear sap of newts 1 and 3 was fluid; after treatment the sap was stiff. The nuclear sap of newt 3 was at first completely free from resolvable granules but after treatment each nucleus contained large numbers of small (less than 1 $\mu$  diameter) free granules. Free and attached spheres (chromosomes V and VIII) were small and inconspicuous in newt 1 before treatment, but after treatment they were larger and were covered with nodules having the same optical properties as the spheres themselves. In newt 4 the giant loops of chromosome X were at first large sausage-shaped structures projecting more than 30 $\mu$  from the chromosome axis. After treatment these loops invariably took the form of compact spherical structures, 5 to 10 $\mu$  in diameter.

Even from these casual observations it was clear that the following features of oocyte nuclei could rightly be classed as physiological variables:-

1. Viscosity of the nuclear sap.

2. Granularity of the nuclear sap
3. Size and general characteristics of the spheres and giant loops.

I therefore concentrated upon a study of the nature and directions of the changes which could be induced in each of these and other features. The results which I shall now describe are classified by the features studied. In a later section I shall deal with that part of my work whereby I was able to measure the effect of gonadotrophins or hypophysectomy upon the metabolic activity of the oocytes of experimental animals.

#### B. Nuclear sap viscosity

My method of measuring the viscosity of nuclear sap was crude but informative. A nucleus was isolated and rapidly cleaned in 5:1 0.1 M K:NaCl. It was transferred without delay to C medium in an observation chamber and its membrane removed. A stop watch was started at the moment of removal of the nuclear membrane, a coverslip was placed over the observation chamber and dispersal of the nuclear sap was watched using the IPCM and x 20 objective. The watch was stopped when all the chromosomes and free bodies lying amongst the chromosomes had settled in one focal plane. Sap dispersal was then judged to be complete. The time to the nearest minute on the stop watch was noted. Nuclear sap which is extremely watery takes between one to two

minutes to disperse. The stiffest of nuclear saps which I have encountered took up to 20 minutes to disperse. To simplify matters I have awarded to each animal a "SAP FACTOR" which is a number between 1 and 10 representing the average of the dispersal times in minutes for the saps of 5 nuclei taken from that animal. Sap factors given as 10+ indicate that dispersal took more than 10 minutes. Tables 3 and 4 summarise the data which I have collected on this topic.

Even if we allow a wide margin for the personal factor, it is clear that sap viscosity increases following treatment with gonadotrophin, and decreases following hypophysectomy.

Table 3

<u>Hypophysectomised newts</u>				<u>Control newts</u>		
<u>NEWT</u>	<u>Experimental period Days</u>	<u>Sap factor</u>		<u>NEWT</u>	<u>Sap factor</u>	
		<u>Before</u>	<u>After</u>		<u>Before</u>	<u>After</u>
<u>9</u>	42	6	4	<u>9C</u>	9	9
<u>10</u>	35	9	6	<u>10C</u>	10	8
<u>11</u>	35	5	1	<u>11C</u>	4	4
<u>14</u>	35	8	3	<u>14C</u>	8	8
<u>22</u>	35	9	3	<u>22C</u>	6	10
<u>23</u>	30	10	5			
<u>24</u>	30	9	2			
<u>26</u>	37	8	2			
<u>27</u>	46	8	2			

Table 4

<u>Gonadotrophin-treated newts</u>			<u>Control newts</u>		
<u>NEWT</u>	<u>Sap Factor</u>		<u>Newt</u>	<u>Sap factor</u>	
	<u>Before</u>	<u>After</u>		<u>Before</u>	<u>After</u>
<u>33</u>	4	10+			
<u>36</u>	2	5			
<u>58</u>	6	10+			
<u>59</u>	6	10+			
<u>60</u>	2	10+			
<u>55</u>	8	10+	<u>55C</u>	4	5
<u>54</u>	7	10+	<u>54C</u>	8	7
<u>51</u>	8	10+	<u>51C</u>	8	8
<u>48</u>	6	10+	<u>48C</u>	7	5
<u>49</u>	7	10+	<u>49C</u>	10	10

C. Granularity of the nuclear sap

The free granules with which this section deals are not classed as and do not include oocyte "nucleoli". "Nucleoli" are generally vacuolated, often irregular in shape, and of more than 5 $\mu$  in diameter. Granules are never vacuolated; so far as one can tell, they are always spherical, and they rarely have diameters greater than 3 $\mu$ . Figs.4b, 5a, and 12a are low power dark field photographs of lampbrush preparations made from nuclei in which there were only a few scattered free granules. Figs.8a and 8b show lampbrush chromosomes and free granules together with one or two scattered "nucleoli". In Fig:10b there are large numbers of granules and "nucleoli" overlying and blotting out the chromosomes.

All the work outlined in this section was carried out upon standard-size oocytes. As a rule, all standard-size oocyte nuclei taken from a given animal at a given time have the same degree of granularity. Animals which were exceptional in this respect were not used in my experiments.

The method of recording the granularity of a nucleus was as follows. A lampbrush preparation was made in the conventional way. A stop watch was started at the moment of removal of the nuclear membrane. A coverslip was placed over the observation chamber which was then transferred to the stage of the inverted microscope. The entire preparation was observed using the x 10 objective lens together with the x 40 phase annulus: this gave

an effective dark ground illumination in which even the smallest particles in the nuclear sap show up brightly. The dark ground image was photographed about 5 minutes after removal of the nuclear membrane. An exposure of  $\frac{1}{2}$  sec. was used. Photographic processing was standardised throughout.

An average lampbrush preparation, fully spread out, almost fills the field of the x 10 objective lens, and although a few "nucleoli" and granules may become scattered over a wider area, the majority fall amongst the chromosomes. Figs. 2 to 16 are of lampbrush preparations photographed by the method outlined above. Differences between photographs with respect to visibility of the chromosomes are due to differences in the amounts of undispersed nuclear sap overlying the chromosomes.

Early in my work I hypophysectomised an animal (10) whose oocyte nuclei contained large numbers of free granules of about 1.5 $\mu$  diameter. Thirty-five days after hypophysectomy all oocyte nuclei taken from this animal were lacking in free granules. Other factors taken into account, this suggested that a high degree of granularity was an expression of intense activity. A little later however, I came across two animals whose oocyte nuclei contained great numbers of granules ranging in size from the limits of resolution to 3 or 4 $\mu$  in diameter (Figs. 2a and 9a). One of these animals (35) received treatment with CG(Swiss), the other (35C) was used as a control. After treatment, newt 35 ovulated, and showed the characteristics

which I had come to associate with stimulation - but its oocyte nuclei were almost completely lacking in free granules (Fig.2b). The oocyte nuclei of the control (350) were slightly less granular after treatment than they were before (Fig.9b).

Subsequent experiments using CG (Swiss) and PMS + CG gave conflicting results. It became clear, however, that the direction of a change in nuclear granularity induced by treatment with gonadotrophin depends upon the degree of granularity of the nuclei before treatment. If an animal starts with very granular oocyte nuclei, stimulation will lead to disappearance of the granules - examples of this type of change are illustrated in Figs.2, 3, and 4. Animals whose oocyte nuclei are at first non-granular may show, after stimulation, nuclei which are still non-granular, or nuclei which contain small (less than 1 $\mu$  diameter) free granules distributed amongst or immediately around the chromosomes, (Figs.5, 6, and 7). The latter is the condition most commonly found in stimulated animals. On two occasions, however, stimulation has induced a marked increase in nuclear granularity; when this happens the granules are small, the largest rarely exceeding 1 $\mu$  in diameter.

Control animals showed comparatively small changes in oocyte nuclear granularity during the experimental period, (Figs.8 and 9); however the oocyte nuclei of one control (480) changed during the nine day experimental period from the moderately

granular condition illustrated in Fig.10a to a condition of extreme granularity illustrated in Fig.10b.

I have already mentioned the change in oocyte nuclear granularity which followed hypophysectomy in 10 (see page 42, paragraph 3). I observed a similar change in (9), (13), and (30), but a change in the opposite direction in (26)! I shall return to consider (26) later. The most common condition found in the oocyte nuclei of hypophysectomised animals is illustrated in Figs.11, 12, and 13; recordings from corresponding control animals are given in Figs.14, 15, and 16. Although I have observed a variety of changes in nuclear granularity following upon hypophysectomy, I have been able to distinguish two universal characteristics of nuclei from animals which survived hypophysectomy for more than one month; such nuclei are either lacking in free granules, or they contain free granules greater than 1.5 $\mu$  in diameter. Fig.17 shows the sizes and general characteristics of the granules found in the nuclei of one hypophysectomised animal; I have never seen objects like these in the nuclei of a stimulated animal. I have explored the possibility of tracing a sequence of changes in nuclear granularity which may take place during the first two weeks of hypophysectomy, but for the present purposes the task proved more exacting than rewarding.

D. Chromosome changes

(i) Giant loops of chromosomes X, XI, and XII.

There is one existing record of non-homologous fusion between the giant loops of chromosomes XI and XII in carnifex and there are two similar records for T.c.karelinii. The fact that these loops can fuse with one another suggests that their "matrices"\* have certain properties in common. Asserted circumstantial evidence (see Callan & Lloyd 1960) points to the fact that the giant loops of chromosome X in carnifex are texturally similar to those of chromosomes XI and XII. Thus we have in the giant loops of carnifex three conspicuous loci whose products are alike.

Because they are large and easily recognised, giant loops offer excellent opportunities for the study of physiological variability at specific "gene loci". I have devoted some attention to changes in the sizes of giant loops following hypophysectomy and gonadotrophin treatment. Loop size before and after treatment was recorded photographically and/or by means of camera lucida drawings. Photographs of complete chromosomes bearing giant loops were taken with the IPCM and a x 40 objective lens; for drawings the system used was the same as that described on page 33 of this text, but for the present purposes the microscope was fitted with a x 95 oil immersion objective and a x 10 eyepiece.

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\*for a definition of the term "matrix" see page 13 of Appendix I

Records of giant loop size were taken from twelve animals - four were hypophysectomised, four treated with CG (Swiss) and four treated with CG + PMS. As a rule the giant loops were recorded from three freshly-made preparations for each animal before and after treatment, but more extensive records were made for those animals whose giant loops were at first unusually large.

Following the treatment of an animal with gonadotrophin its giant loops may remain unchanged or may decrease in size according to whether they are small or large before treatment. Unusually large giant loops, such as those found in 36, 58, and 59 became smaller after treatment with gonadotrophin, (see Figs. 18 to 21). Records from controls 36C and 59C are given in Figs. 22 and 23. On the other hand I have never known gonadotrophin treatment to be followed by a marked increase in the size of the giant loops. It is noteworthy that in 59, which possessed giant loops on both homologues of bivalent XII, there was at first a marked and consistent size difference between the giant loops of one homologue and those of the other (Fig. 20a); the difference was still apparent after CG + PMS treatment, in spite of a general decrease in giant loop size (Fig. 20b). In 32, 58, and 59 opposite members of giant loop pairs were at first consistently fused on one homologue of chromosome XI but separate on the other homologue, (Figs. 19a, 21a, 26a, and 27a). After treatment this state of fusion was unchanged, (Figs. 19b, 21b, 26b, and 27b).

The effects of hypophysectomy upon the giant loops were less obvious. In the majority of animals the size of the giant loops at the end of the experimental period did not differ significantly from their size before hypophysectomy. I have recorded a significant increase in giant loop size in only one animal (13). Drawings of the giant loops of chromosomes X and XI in 13 before and 22 days after hypophysectomy are given in Figs. 24a and 24b. I have not explored the possibility that marked changes in giant loop size occur during the first two weeks following hypophysectomy, nor have I had the opportunity of hypophysectomising an animal whose standard-size oocytes possessed unusually large or small giant loops; such animals are comparatively rare.

ii) The spheres on chromosomes V and VIII.

The information which I shall offer concerning physiological variability at the sphere loci is mainly a synthesis of isolated observations. Certain well defined characteristics of spheres were repeatedly found in animals which had received a particular treatment; each characteristic can be associated with physiological activity or inactivity according to the nature of the treatment. In the early stages of my work, however, it seemed that hypophysectomised animals had smaller spheres and fewer free spheres per nucleus

than animals which had been treated with gonadotrophins. I tried to assess this difference by measuring the total volume of sphere material per oocyte nucleus in each animal before and after treatment. For a given experimental animal I recorded the total number of spheres, both free and attached, present in five lampbrush preparations made from standard-size oocytes. The number so obtained I divided by 5 to give the values listed in columns (ii) and (iii) of Table 5. Furthermore, using the inverted microscope, camera lucida, x 95 phase objective and x 10 eyepieces, I measured to the nearest  $\frac{1}{2}\mu$ , the diameters of all the spheres present in these five preparations. From diameter measurements, individual sphere volumes in  $\mu^3$  were calculated and a value which I have called the AVERAGE SPHERE VOLUME (columns iv and v, Table 5) was obtained for each animal. Data of this sort were not obtained for control animals.

I have already mentioned the nodules which formed on the spheres of newt 1 after that animal had been treated with CG (Swiss). For purely intuitive reasons I attributed the formation of nodules to hyperactivity of the sphere loci. Following treatment with CG + PMS, I have seen in the nuclei of two animals (59 and 48), spheres which were either in the process of fusing with one another or of dividing (Fig. 29); the latter explanation seems the more reasonable. In 32

before treatment the sphere at 11 units on one homologue of chromosome V was consistently missing. After treatment with CG (Swiss) there were spheres at 2 and 11 units on both homologues of chromosome V, but the sphere at 11 units on one homologue was always small. In 59 one sphere at 2 units on one homologue of chromosome V was missing in all preparations both before and after treatment.

As described by Callan & Lloyd (1960), small spheres are homogeneous; larger examples frequently contain vacuoles, whilst in extremely large spheres there may be vacuoles and a single refractile body within each vacuole. All attached spheres and many of the free spheres in 15 out of 25 experimental animals contained vacuoles and internal refractile bodies (IRBs) after these animals had received treatment with gonadotrophins; but the spheres of hypophysectomised animals never contained vacuoles or IRBs.

With regard to sphere volume and the rate of production of material at the sphere loci, the results listed in table 5, if treated as a single body of data suggest that gonadotrophin stimulation or hypophysectomy have no effect. However, I attached some significance to the values recorded for 32 and 13. The marked increase in the amount of sphere material per oocyte nucleus following the treatment of 32 with CG (Swiss) must result from an increase in the rate of production at the sphere

loci; the converse applies to 13. It is noteworthy that whilst at first 32 had unusually small spheres, the spheres of 13 were relatively large. The increase in the number of spheres per nucleus in 48 and 49 is also highly suggestive. The change in appearance of the attached spheres of 13 is demonstrated in Figs.30a and 30b.

Table 5

NEWT	AVERAGE NUMBER OF SPHERES PER PREPARATION		AVERAGE SPHERE VOLUME $\mu^3$		TREATMENT
	Before	After	Before	After	
32	5	9.2	31	133	
33	6.8	6.4	137	130	
34	7	7.8	182	205	CG (Swiss)
36	7.4	5.8	205	195	
37	8	7.2	146	109	
49	11.6	21.6	139	172	
48	9.2	22.8	199	188	CG + PMS
58	7.6	10	162	185	
59	11.4	16.6	52	35	
10 (35)*	8	8	69	39	
11 (35)	7	4	73	65	
13 (22)	8.4	5	150	57	
17 (20)	5.5	7.2	92	73	Hypophysectomy
19 (20)	10	8.5	61	61	
22 (35)	6	8	107	69	
23 (30)	11	8.5	69	54	
27 (46)	9	9	65	58	

\* Numbers in brackets indicate length in days of experimental period

(iii) "Currant Buns" of chromosomes III, IV, and XI.

I have not at any time made special records of the size or any other characteristics of currant buns, but from casual observations it has become obvious that these structures exhibit a marked physiological variability. They are rarely present in standard-size oocytes from untreated animals, and I have never seen currant buns in standard-size oocytes from animals which have been treated with gonadotrophins. However, if currant buns are present and conspicuous in standard-size oocytes of an untreated animal, then they invariably disappear after treatment of that animal with gonadotrophin (Figs. 31a and 31b). On the other hand, hypophysectomy seems to favour the accumulation of material at the currant bun loci. I have noted that in five experimental animals which at first showed no currant buns there were conspicuous currant buns on chromosomes III, IV, and XI at the end of the experimental period. 13 was exceptional in that it started with unusually large dense currant buns, but 22 days after hypophysectomy all its currant buns were so small and diffuse as to be scarcely recognisable.

(iv) The heteromorphic arm of chromosome I

There is a marked physiological variability at some of the axial granule loci of the left arm (referred to by Callan & Lloyd, 1960, as the "heteromorphic" arm) of chromosome I, but because of the inherent genetic variability of carnifex

chromosome I, a systematic study of physiological variability at each axial granule locus is impracticable. Nevertheless I have recorded some odd conditions which appeared from time to time at the axial granule loci. Records were obtained photographically using the x 95 oil immersion objective, and were standardised by concentrating on a region extending 60 $\mu$  (half the width of the x 95 field) on either side of the mid-point of the heteromorphic arm which bore the greater number of large contorted loops; the approximate mid-point was found by measurement of a camera lucida drawing of the selected chromosome arm.

I have observed one particularly striking post-treatment condition on the heteromorphic arm of chromosome I in 9 out of 13 newts which were treated with Organon gonadotrophins. This condition, which I have called 'bunching' is illustrated in Fig.32. At each axial granule locus, instead of there being a solitary granule, there were two, three or four highly refractile spherical bodies, most of which, by virtue of their refractility, differed from any other class of object found in oocyte nuclei. Bunching at the axial granule loci is often, though not always, accompanied by the presence in the nuclear sap of free granules of the same order of size as those at the axial granule loci.

As a rule, hypophysectomy had no obvious effect upon the heteromorphic arm of chromosome I; but newt 13 was exceptional. Before hypophysectomy each of its axial granule loci carried a single conspicuous granule (Fig.53a). Twenty-two days after

hypophysectomy axial granules could scarcely be distinguished from nearby chromomeres (Fig.33b). The abundance of free granules in Fig.33a and their absence in Fig.33b is worth noting.

(v) The Double Axis Ends of Chromosome XII

Hypophysectomy of 10 was followed by a clearance of the large granules which, at the start of the experiment, were clustered around the double axis regions of chromosome XII and other chromosome regions (Figs.34a and 34b). The free granules in the nuclear sap also disappeared. It was at first difficult to resist the implications presented by the story of newt 10, but a little later I hypophysectomised another animal, 26, whose chromosomes and nuclear sap were completely free from resolvable granules. Thirty-five days after hypophysectomy I recorded a change which was the opposite to that induced in 10.

E. Gross physiological variability in newt ovaries.

By studying gross physiological variability in the carnifex ovary I have been able to contribute something to existing knowledge of the roles of pituitary and extrapituitary gonadotrophins in ovarian development and maintenance; but the changes which I shall describe now were of prime importance as criteria for assessing the effectiveness of each particular experimental treatment.

(a) The effects of gonadotrophins.

All animals which were treated with gonadotrophins or homoplastic pituitary material (9 pituitaries) deposited eggs approximately three days after receiving their first injection. Oviposition continued for the duration of the experimental period but ceased shortly after the second laparotomy. Control animals did not ovulate.

The general condition of the ovaries seemed to improve with treatment and the rate of growth of yolky oocytes seemed greatly accelerated. Oocytes ranging from 0.7 to 1.2 mm (diameter) had a richer blood supply after treatment (Figs. 35a and 35b), and fluid filled spaces often formed between the vitelline membrane and the investing follicular epithelium. The nuclei of oocytes taken from a gonadotrophin-treated animal seemed to be separated from the surrounding yolk by a mass of clear fluid cytoplasm. Upon puncturing such an oocyte the outpouring of yolky cytoplasm is interrupted while nucleus and surrounding fluid pass out into the dissecting medium.

(b) The effects of hypophysectomy.

Atretic follicles are a feature of the ovaries of hypophysectomised newts. Oocytes in the early stages of atresia are flaccid and often hyperaemic. Their cytoplasm is stiff and glutinous. If their nuclei have not already broken down then

they no longer contain lampbrush chromosomes. In several hypophysectomised animals I have found standard-size oocytes which were outwardly normal in appearance but whose nuclei contained only a mass of granules and chromosome fragments suspended in watery nuclear sap (Fig. 36). The presence in such nuclei of large sausage-shaped objects which are certainly entire detached giant loops from chromosomes X, XI, and XII is worth noting. Oocytes with fragmented chromosomes are probably in the very early stages of atresia. Later corpora atretica are bright yellow in colour and mis-shapen. Corpora atretica derived from the larger yolky oocytes are finally reduced to knots of golden brown tissue 100 to 200 $\mu$  across.

In recording the incidence of atresia in hypophysectomised animals I scored all corpora atretica which were outwardly recognisable as such. A small random sample of ovary was taken from each experimental animal at the beginning and end of the experimental period. Without damaging individual oocytes the sample was teased out and all those oocytes which contained yolk were counted - there were usually between 100 and 150. All corpora atretica were then counted and the number expressed as a percentage of the number of yolky oocytes in the sample. The percentage incidence of atresia recorded before hypophysectomy was subtracted from that recorded at the end of the experimental period to give the "percentage increase in atresia". Values so obtained are listed in Table 6.

Table 6

NEWT	Experimental Period Days	% Increase in Atresia	Sizes of Early Corpora Atretica (mm. diameter)
8	14	8	over 1.3
9	42	25	over 0.9
10	35	15	over 1.0
11	35	20	0.9 to 1.2
12	28	18	0.9 to 1.3
13	22	18	over 1.0
14	35	21	over 0.9
17	20	6	1.2 to 1.8
18	20	17	1.0 to 1.3
19	20	12	1.2 to 1.8
22	35	17	0.8 to 1.7
23	30	15	0.9 to 1.8
24	30	21	1.1 to 1.2
26	57	26	over 0.7
27	46	36	over 0.7
28*	135	91	-
29	14	4	over 1.5
30	21	9	over 1.4

\* This animal was, for some unknown reason, quite exceptional in that it remained healthy for 135 days after hypophysectomy. It died on the 136th day. Its ovaries were examined and found to consist entirely of yolkless oocytes of less than 0.4 mm. in diameter.

E. Ribonucleic acid (RNA)

It has often been assumed, though never actually stated, that the RNA-rich granules of oocyte nuclear sap comprise material which is destined to pass through the nuclear membrane into the cytoplasm, there to participate in the synthesis of cytoplasmic proteins.

Treatment of 35 with CG (Swiss) led to a dramatic clearance of the free granules originally present in the oocyte nuclei of that animal. Judging from the results of previous experiments with CG (Swiss), I anticipated such a clearance, and I reasoned that if it betrayed a transfer of RNA from nucleus to cytoplasm, then cytoplasmic RNA concentration might show a temporary increase after treatment of the animal with CG. I set out to test this theory on newt 35 and its control partner, 35C.

At the start of the experiment a piece of ovary from each animal was fixed in Smith's formol dichromate\* for 24 hours, washed in running tap water for 12 hours, and then

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\* Smith's Formol Dichromate was chosen for a fixative because it does not harden the yolk of larger oocytes, and therefore it facilitates the production of good unbroken sections. It is made up as follows:-

Potassium dichromate ...	5 grms.
Glacial acetic acid ....	2.5 ml.
Formalin .....	10 ml.
Water .....	87.5 ml.

The dichromate and distilled water are made into a stock solution; the formalin and acetic acid are added immediately before use.

placed in 10% formalin for 12 hours. It was then passed through 30% and 50% alcohol, and stored in 70% alcohol. After each animal had received the appropriate treatment, a second piece of ovary was fixed in the same way, passed through 30% and 50% alcohol and stored for 24 hours in 70% alcohol. Thereafter all pieces of ovary were treated alike. They were dehydrated and embedded as follows:-

95% alcohol .....	12 hours
1% solution of celloidin in methyl benzoate ....	4 hours
	2 changes
Benzene .....	1 hour
	2 changes
Paraffin wax, (m.p. 54°C.) .....	2 hours
	2 changes

Pieces of ovary taken from the same animal were embedded in the same block so that they could be sectioned simultaneously. 8 $\mu$  sections were cut with an M.S.E. rotary microtome, and mounted on albuminised slides. One slide from each animal was incubated for 4 hours in a solution containing 0.5 mg. of crystalline ribonuclease (L. Light & Co. Ltd., Colnbrook England) per ml. of 0.01 molar  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer, pH 6.2. These and two other undigested slides from each animal were then placed for 30 minutes in a 0.25% solution of pyronine (Geigy) in 0.1 molar acetate buffer, pH 4.7. After staining, slides were washed for 5 minutes in acetate buffer (pH 4.7), and passed through 95% alcohol, absolute alcohol, and xylene. The sections on each slide were then mounted in DePeX (G. T. Gurr Ltd.)

Stained sections were photographed in ordinary light using a conventional microscope system fitted with a x 10 (16 mm.) objective lens. Photographic processing was standardised throughout.

Figs.37a and 38a show pyronine stained sections of oocytes taken from 35 before it had received CG treatment. Figs.37b and 38b show pyronine stained sections of oocytes taken from the same animal after treatment. Stained sections of oocytes taken from the control animal 35C at the beginning and end of the experimental period are shown in Figs.39a and 39b. Ribonuclease incubated sections did not stain with pyronine.

The test showed that the concentration of cytoplasmic RNA in the smaller (less than 0.7 mm. diameter) oocytes of newt 35 increased after that animal had been treated with gonadotrophin. Similar tests were carried out on four other animals but were unrewarding in that pyroninophilia of the smaller oocytes either decreased or remained unchanged following CG treatment.

I abandoned this line of study until I saw how great an effect gonadotrophin treatment has upon the uptake of  $^{32}\text{P}$  into the cytoplasm of standard-size oocytes, (experiments described in a later section, page 63), and until I found another newt with granular oocyte nuclei. Newt 51 suited my purposes well, and with certain technical modifications, I repeated on this animal and its control partner the test

which I had applied to 35.

Because it is a more rapid fixative and yet does not harden tissue excessively, I used Bouin in place of Smith's formol dichromate\*.

Pieces of ovary were fixed in Bouin for 6 hours, and washed in 70% alcohol for 6 hours. They were then impregnated with colloidin and embedded in wax as described above. Nucleic acids are said to lose some of their affinity for basic dyes after prolonged contact with alcohol, (Brachet 1953). Pieces of ovary removed from animals before CG treatment were therefore embedded in wax for the duration of the experimental period, instead of being stored in alcohol. Later they were re-embedded alongside the piece which had been taken from the corresponding animal after treatment.

Fig.40a shows Bouin fixed, pyronine stained sections of oocytes taken from 51 before treatment; Fig.40b is of comparable sections of oocytes taken from the same animal after it had received 400 I.U. of CG + PMS. Corresponding sections from the control animal are shown in Fig.41a and 41b. Once again, the increase in basophilia was noticeable only in oocytes of less than 0.7 mm. diameter.

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\* I abandoned formol dichromate as a fixative partly because Chayen, Gahan and La Cour (1959) claim to have shown that ribonucleo-protein is removed from calf thymus tissue during fixation in formaldehyde calcium.

If my technique is free from sources of artifact then it is more than coincidental that at first, the oocyte nuclei of 35 and 51 were abnormally granular, (see Figs. 2a, 3a, and 4a) and cytoplasmic basophilia in all oocytes was relatively weak, whereas after these animals had been treated with gonadotrophins their oocyte nuclei were free from granules (see Figs. 2b, 3b, and 4b) and the cytoplasm of their smaller oocytes was intensely basophilic. It should be remembered that, due to the accumulation of yolk and a consequent dilution of other materials in oocyte cytoplasm, the histochemical detection of RNA becomes more difficult as the oocyte grows larger, (Brachet 1942, 1960, Wittek 1952, Osawa & Hayashi 1953). Thus it is reasonable to expect that the larger the oocyte, the harder it will be to detect by conventional cytochemical methods, changes in the RNA content.

A more detailed and systematic attack on this problem using dyes other than pyronine, is planned for the future.

#### G. <sup>32</sup>P Incorporation

In the foregoing I have described the physiological variability of certain features in newt oocyte nuclei, and I have listed conditions which are characteristic of the oocytes of gonadotrophin-treated newts and hypophysectomised newts. Consequently it is now possible to construct provisional

definitions of physiologically stimulated and unstimulated standard-size oocyte nuclei.

Stimulated nuclei will have stiff sap. Extra-chromosomal particles embedded in the nuclear sap can be grouped in two classes - small granules (less than  $1\mu$  diameter), and free spheres. Disregarding landmark structures, there will be no clustering of large droplets or granules of material at any point on the lampbrush chromosomes, but often there is more than one granule per axial granule locus on chromosome I. The giant loops of chromosomes X, XI, and XII will not extend more than  $20\mu$  from the chromosome axis to which they are attached. There will be no currant buns on chromosomes II, IV, or XI. The spheres of chromosomes V and VIII will be conspicuous and may contain vacuoles and internal refractile bodies.

An unstimulated nucleus will have fluid sap, either devoid of extra-chromosomal particles or with granules of 1 to  $4\mu$  diameter. There may be clustering of granules at various points on the lampbrush chromosomes, notably on the double axis ends of chromosome XII, but there will only be one granule per axial granule locus on chromosome I. The giant loops of chromosomes X, XI, and XII will be of indeterminate size. Currant buns will be readily visible on chromosomes III, IV, and XI. The spheres of chromosomes V and VIII will be small and will not be vacuolated.

These two definitions are based on the assumption that gonadotrophins, whether extraneous or secreted by the animals own pituitary, govern the metabolic activity of yolky oocytes. While studying the morphological aspects of physiological variability I sensed the need for a method of calibrating in definite terms the effectiveness of injected gonadotrophins and of hypophysectomy, and for this purpose I injected  $^{32}\text{P}$  Phosphorus ( $^{32}\text{P}$ ) into experimental and control animals and made a comparative study of the amount of isotope incorporated in a given time into whole oocytes and oocyte nuclei.

30 $\mu\text{c}$  of  $^{32}\text{P}$  in 1/10th ml of sterile orthophosphate in isotonic saline at pH 7 (supplied by the Radiochemical Centre, Amersham, England) was injected into each newt 24 or 48 hours before the final sample of its ovary was removed for examination. For those animals in the 48 hour group which were under treatment with gonadotrophin, the isotope solution was used as a solvent for the second dose of hormone, so that both isotope and hormone were injected simultaneously.

At first I confined my attention to measuring  $^{32}\text{P}$  uptake in standard-size oocytes and their nuclei, but later I extended my studies to oocytes ranging from 0.4 to 1.9 mm diameter. Each oocyte was mounted in a small drop of 0.1 M 5:1 K/NaCl on a  $\frac{5}{8}$ " circular coverslip which was numbered with black Indian ink. The oocyte was measured and then stripped of its follicle tissue.

The latter process was simple with larger oocytes; by careful manipulation with two pairs of fine forceps the oocyte can be "shelled" out of its follicle tissue without damaging the vitelline membrane. With smaller oocytes it was necessary to puncture the oocyte and squeeze out its contents. Follicle tissue was discarded. Using a fine glass needle the contents of each oocyte were spread out to form a thin disc of material approximately  $\frac{1}{2}$ " diameter, and concentric with the rim of the coverslip. Each preparation was then dried in a dessicator.

The number of ionising particles (high energy beta particles in the case of  $^{32}\text{P}$ ) liberated from each specimen in unit time were counted using an end-window Geiger-Müller tube mounted in a lead castle, and connected to an automatic scaler and register (Ekco, type NS30F). The  $\frac{3}{4}$ " coverslips upon which oocytes were mounted fitted nicely into the aluminium planchets which were used in conjunction with the counting apparatus.

For each preparation I recorded the time taken to collect 1,000 counts. I then calculated the number of counts collected per minute, from which I deducted the 'background' in counts per minute. The resulting value was divided by the volume of the oocyte in  $\text{mm}^3$  to give the number of counts collected per minute per  $\text{mm}^3$  of specimen material, or the "specific radioactivity" (SR) of the specimen.

Nuclei were prepared for counting as follows :-

Only the nuclei of standard-size oocytes were used. Each nucleus was isolated in 0.2 molar 5:1 K/NaCl (in which there is minimum hydration of the nuclear sap; Callan 1952), rapidly cleaned, measured and transferred to a drop of saline on a  $\frac{1}{4}$ " circular coverslip. Because nuclei swell rapidly after isolation my estimates of nuclear volumes are probably too great by up to 20%. However, the time lapse between puncturing an oocyte and measuring its nucleus was standardised to within a few seconds, and the degree of swelling may reasonably be taken as constant. Between the isolation of one nucleus and the next, the bunch of oocytes was thoroughly washed and transferred to clean saline so as to reduce the contamination of the isolation medium with radioactive material. Five nuclei from each animal were mounted on each coverslip. After isolation, each nucleus was pricked and its sap was allowed to disperse. Preparations were then dried and counted. The count/minute-less-background (calculated from the time taken to collect 1,000 counts) was divided by the total volume of nuclear material in the preparation - i.e. the sum of the volumes of each of the five measured nuclei.

The SRs of whole standard-size oocytes and standard-size oocyte nuclei, taken from gonadotrophin-treated newts, hypophysectomised newts and controls, are set out in Table 7. Each experimental treatment produced the desired effect. The mean SR for oocytes of all these newts which received treatment with gonadotrophin or homoplastic pituitary material was 2,310;

Table 7

I Newt number	II Treatment	EXPERIMENTALS			CONTROLS				
		III Oocyte count	IV Nuclear count	V Newt number	VI Oocyte count	VII Nuclear count	VIII T Hours		
38		1337	-	38C	1000	-	48		
39		1498	-	39C	875	-	48		
40	CG (Swiss)	1995	-	40C	1279	-	48		
41		1777	-	41C	427	-	48		
55		2876	368	55C	147	143	24		
56	CG (Organon)	359	NAB	56C	705	159	24		
57		3460	707	57C	151	126	24		
53	PMS (Organon)	2672	314	53C	298	NAB	24		
54		3040	485	54C	515	NAB	24		
48		3927	818	48C	123	NAB	48		
49	CG + PMS	5275	577	49C	438	53	48		
50	(Organon)	3348	815	50C	1051	202	24		
51		2020	501	51C	560	-	24		
52		1909	511	-	-	-	24		
42	5	1581	343	42C	1684	483	24		
43	5	1626	558	43C	1164	359	24		
44	9 Homoplastic	1936	325	44C	925	189	24		
45	9 Pituitaries	3051	549	45C	911	169	24		
46	9	2144	587	46C	267	146	24		
47	9	382	810	47C	210	94	24		
17 (20)		109	34	17C	333	127	48		
18 (20)		100	NAB	18C	105	NAB	48		
19 (20)	Hypophy-	151	51	19C	273	141	48		
22 (35)	sectomy	47	NAB	22C	242	61	48		
29 (14)		563	102	29C	1314	396	48		
31 (14)		328	99	31C	503	146	48		

Key to Table 7

T (column VIII) = time lapse between the injection of  $^{32}\text{P}$  and the removal of a sample of ovary for counting.

"NAB" indicates that the number of counts collected per minute from each specimen was not significantly greater than the expected number of counts per minute which result from background gamma radiation.

The figures in brackets after the numbers of hypophysectomized newts indicate the length in days of the experimental periods.

Each value in columns (iii) and (vi) is the mean of the SRs of three or more separately counted, whole, standard-size oocytes.

Each value in columns (iv) and (vii) is the SR of one preparation consisting of five standard-size-oocyte nuclei.

for control newts it was 612, and for hypophysectomised newts 249. Corresponding values for nuclei were 517, 149, and 47 respectively. Counts obtained from hypophysectomised newts substantiated the results of my morphological studies. Newts 29 and 31 showed, fourteen days after hypophysectomy, a rate of uptake of  $^{32}\text{P}$  which was comparable to that of the corresponding control animals (29C and 31C). In newts 17, 18, and 19 (20 day experimental period) uptake was definitely slower than that of the control animals; whilst in newt 22 (35 day experimental period) uptake had practically reached a standstill.

The situation in newt 47 is worth noting. So far as I was able to tell at the time, the SR of a nucleus from a standard-size oocyte equalled or was greater than that of a whole oocyte of the same size. This could be explained if we assume (a) that an increase in the metabolic activity of the oocyte as a whole is necessarily preceded by hyperactivity of the nucleus, and, (b) that in 47 standard-size oocytes responded more slowly than usual to the relatively mild experimental treatment.

The abnormally low counts from newt 56 were almost certainly due to water leaking into the abdominal cavity of this newt through the badly healed wound of the first laparotomy. The oocytes of this animal looked to be in unusually bad condition for a gonadotrophin-treated newt.

At an early stage in my studies of  $^{32}\text{P}$  uptake I injected  $^{32}\text{P}$  into an intact newt and removed a sample of its ovary 48 hours later. I then made, and collected counts from, thirty preparations of whole oocytes ranging in size from 0.4 to 1.5 mm. (diameter). The data so obtained and each step in my calculations is given in Table 8. A developmental variability in the rate of  $^{32}\text{P}$  uptake was evident and to clarify the pattern of this variability, I constructed Fig. 42. Oocytes were grouped according to their volumes; Group a comprises oocytes of 0.03 to 0.25 mm.<sup>3</sup>; Group b, 0.26 to 0.60 mm.<sup>3</sup>; Group c, 0.61 to 1.0 mm.<sup>3</sup>; Group d, 1.01 mm.<sup>3</sup> and upwards. The mean of all SRs in each group was calculated and was used to determine the height of the corresponding column in Fig. 42. The width of each column was set by the number of oocytes in each group. Thus column c is the highest because the mean SR for Group c was the greatest, and column a is the widest because a relatively large number of the thirty oocytes in my sample fell into Group a.

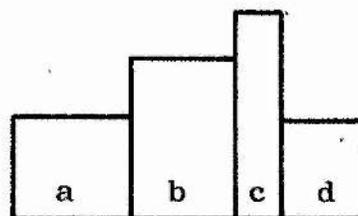


Figure 42

Table 8

	i	ii	iii	iv
	OOCYTE VOLUME in mm. <sup>3</sup>	COUNTS/MINUTE	LESS BACKGROUND	SR
<u>d</u>	1.628	1670	1658	1018
	1.503	1235	1223	814
	1.336	1084	1072	802
	1.336	1148	1136	850
	1.189	1689	1677	1410
	1.052	1707	1695	1611
	1.052	1526	1514	1439
<u>c</u>	0.952	2192	2180	2289
	0.813	1633	1621	1993
	0.704	1655	1643	2334
	0.613	1207	1195	1949
<u>b</u>	0.570	1037	1025	1798
	0.523	1076	1064	2034
	0.509	865	853	1675
	0.411	581	569	1384
	0.392	748	736	1878
	0.342	643	631	1845
	0.283	468	456	1611
	0.256	486	474	1851
	0.256	318	306	1195
	<u>a</u>	0.232	295	283
0.209		269	257	1229
0.209		238	226	1081
0.149		255	243	1197
0.131		156	144	1099
0.131		149	137	1045
0.102		147	135	1323
0.102		74	62	608
0.055		59	47	854
0.039		59	47	1205

Mean SR for Group a = 1086  
 Do. do. b = 1697  
 Do. do. c = 2141  
 Do. do. d = 1135

No. of oocytes in Group a = 10  
 Do. do. b = 9  
 Do. do. c = 4  
 Do. do. d = 7

The foregoing "activity/size" survey prompted me to look at the effects of gonadotrophins and hypophysectomy upon the rate of uptake of  $^{32}\text{P}$  into oocytes of different sizes. My results are presented in Figs. 43 to 47, all of which were constructed as described for Fig. 42. Figs. 42 to 46 and Fig. 47 are strictly comparable with respect to vertical and horizontal scales; but for practical purposes the values which determined the heights of the columns in Fig. 46 were multiplied by ten.

Perhaps the most striking feature of these results is the effect of gonadotrophins upon the rate of uptake of  $^{32}\text{P}$  into large (Groups c and d) oocytes, and its relatively slight effect upon uptake into smaller (Group a) oocytes. If it is reasonable to assume that most of the  $^{32}\text{P}$  incorporated into oocyte cytoplasm is utilised in the manufacture of yolk phosphoprotein, then it seems that the main target for gonadotrophin is yolk synthesis. That other processes beside yolk synthesis are affected, is shown by the fact that many of the Group a oocytes which I counted were previtellogenic, yet their SR was higher in gonadotrophin-treated newts than in the corresponding controls.

The rate of incorporation of RNA precursors into oocyte nuclei is known to be relatively high, (Picq 1955, Gall 1958), and I would suggest that much of the  $^{32}\text{P}$  incorporated into oocyte nuclei in my experiments was used in RNA synthesis.

## II. Total protein content of nuclear sap.

In spite of their great size and other practical attributes amphibian oocyte nuclei and lampbrush chromosomes do not readily lend themselves to techniques for quantitative analysis. Nevertheless, a worthy sequel to my study of morphological changes in oocyte nuclei would be a description of these changes in terms of absolute amounts of chemical substances.

As a starting point I set out to examine the basis for changes in nuclear sap viscosity. My aim was to discredit or substantiate an idea that an increase in nuclear sap viscosity reflected an increase in total nuclear protein content. So far I have met with little success but an account of my efforts may illustrate some of the problems which are characteristic of the material.

My first line of approach was to compare the U.V. absorption curves obtained from solutions of nuclear sap in saline made up from standard-size oocyte nuclei which were taken from the same animal before and after gonadotrophin treatment. I used three animals whose nuclear saps were watery at the start of the experiment. Before and after treatment with 400 I.U. of CG+PMS a preparation of 100 standard-size oocyte nuclei was made from each animal as described below.

First I selected, cleaned, marked and weighed (all weighings were made to the nearest  $\frac{1}{10}$ mg) two small similar glass

bottles each of about 1.5ml capacity. Into one bottle (A) I put a weighed amount of a stock solution of water adjusted to pH 9 by addition of NaOH. Barring the nuclear membrane, all components of an oocyte nucleus go into solution in alkaline water (see Appendix I, pages 25 and 26). Into the second bottle (B) I put an equal amount by weight of the same stock solution. I then isolated, cleaned and transferred to bottle (A) one hundred standard-size oocyte nuclei. Each nucleus was isolated and cleaned in 0.1 M 5:1 K/NaCl. The total volume of nuclear material represented by 100 nuclei I estimated as being about 0.008ml. Inevitably a small quantity of contaminant K/NaCl was pipetted into (A) with each nucleus, so that the final volume of fluid in (A) was about 0.85ml. To compensate for contamination, I added K/NaCl to (B) until the total weight of fluid in (B) equalled the weight of fluid + nuclear material in (A). Nuclear membranes were then broken by stirring the contents of (A) with a glass needle. The fluid in (A) was then transferred to a 2mm\*path silica cell (0.9ml capacity) constructed by Unicam Instruments Ltd. for use with a Unicam SP 500 spectrophotometer). The fluid in (B) was transferred to another identical cell. Both cells were mounted

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\* I began by using 20mm path microcells and a modified standard cell housing; this combination proved unsatisfactory, the nature of my problem did not justify the purchase of a Universal Cell housing, and so I resorted to shorter path cells of standard dimensions.

in the appropriate positions in the spectrophotometer, the solution lacking nuclear sap being used as a control. Readings were taken of optical density at wavelengths ranging from 230 to 300m $\mu$ . One of the curves which I obtained from nuclei taken from a gonadotrophin-treated newt is shown in Figure 48. Others had smaller absorption peaks in the 260m $\mu$  region and showed only weak absorption around 270 to 285 m $\mu$ .

I abandoned this method at an early stage because:-

- (a) A well defined peak for the cyclic amino-acids (275 to 285m $\mu$  at alkaline pH) could not be obtained from 100 newt standard-size-oocyte nuclei, no matter how stiff their sap.
- (b) It is frequently impossible to obtain 200 standard-size oocyte nuclei from one newt within 9 days , and it is virtually impossible to obtain more than 200. Moreover, one must take into account the fact that to obtain 100 standard-size oocyte nuclei from one newt it is often necessary to remove the entire ovary from one side of the animal.
- (c) Whilst all standard-size oocyte nuclei taken from a given animal at a given time have approximately the same volume, treatment of an animal with gonadotrophin may cause oocyte nuclear volume to increase or decrease (see below). Thus even if the U.V. absorption measurements showed a rise in the amount of protein per nucleus it would be hard to dissociate this from an

increase or decrease in the average size of standard-size oocyte nuclei, and no really valid conclusions could be reached concerning the concentration of nuclear protein.

i. Variations in nuclear volume within oocyte size classes

Throughout my spectrophotometry work I was conscious of the possibility that standard-size oocyte nuclei might be larger or smaller after gonadotrophin treatment than they were before.

I investigated the question of nuclear size changes by measuring the oocyte nuclei of three animals before and after gonadotrophin treatment. I chose oocytes of five different sizes - 0.5, 0.9, 1.2, 1.4, and 1.65 mm. (diameter). From a given animal I measured to the nearest 1/100th of a millimeter in 0.2 molar K/NaCl, the diameters of the nuclei of five oocytes of each size. If a nucleus was not spherical, then its diameter was taken to be the mean of the lengths of its longest and shortest axes. No measurement was considered valid unless it was made within 30 seconds of isolation of the nucleus. I then calculated the mean nuclear volume and standard deviation for each size of oocyte. The values so obtained were used to construct Figs.49, 50, and 51.

As far as one can judge from so rough a test, there

is no marked trend towards larger or smaller nuclei following gonadotrophin treatment; but there is evidence to confirm my suspicion that nuclear volume in standard-size and larger oocytes undergoes slight day to day changes.

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My second attempt to assess nuclear protein concentration involved the use of a Baker Interference microscope with which I had hoped to be able to determine at least the dry mass of oocyte nuclei of known dimensions. The method has shown promise but it needs refining before it can be applied to a comparative study. However, it has transpired that the interference microscope is a good tool with which to study certain aspects of the behaviour of isolated nuclei. The work which I have carried out along these lines is reported in Appendix 2 of this thesis.

CHAPTER III

OOCYTE "NUCLEOLI"

1. Introduction

The amphibian oocyte "nucleoli" to which this chapter is devoted do not include any of the objects which I have described in Chapter II. I am now exclusively concerned with those structures which are analogous to the "nucléoles périphériques" described in the oocyte nuclei of Triturus cristatus and Rana temporaria by Guyénot & Danon (1953).

The oocyte "nucleoli" of Triturus cristatus are characteristically large (5 to 50 $\mu$  in diameter) bodies, irregularly rounded, often vacuolated, and appearing bright in phase contrast. In a medium sized newt oocyte there may be as many as 1,500 "nucleoli" not attached to the chromosomes, but lying near or closely appressed to the inner surface of the nuclear membrane. Whilst something is known of the chemistry of amphibian oocyte "nucleoli" from the studies of Gersch 1940, Guyénot & Danon 1953, Gall 1954, and Brown & Ris 1959, (see also Vincent 1955, and Brachet 1960), remarkably little can be said with surety concerning their origin, fate, or function.

Until 1954, theories as to the origin of the amphibian oocyte "nucleolus" were based upon (1) the preconceived notion that any object which had hitherto merited the name NUCLEOLUS must originate at some point on a chromosome, and (2) numerous

reports in which it was stated that there were present amongst the chromosomes of oocyte nuclei objects which looked like the peripheral "nucleoli". Ideas varied according to observers, their methods and the source of their material, but basically it was thought that all discrete bodies which were present in amphibian oocyte nuclei were produced on the chromosomes and shed by the chromosomes. Those bodies which were destined to become peripheral "nucleoli", once shed from a hypothetical "nucleolar" organiser locus, migrated through the nuclear sap to occupy their characteristic position near to the nuclear membrane.

The most painstaking studies of amphibian oocyte "nucleoli" were those of Gersch (1940) and Guyénot & Danon (1953). Guyénot & Danon discuss the origin and role of "macronucleoli" in generating the "filaments nucléoplasmiques", and they refer to "micronucleoli" lying amongst and around the chromosomes of larger oocytes. Their "macronucleoli" were clearly analogous to what we now describe as giant lampbrush loops; whilst their "micronucleoli" were almost certainly the free granules which I described in Chapter II. Although Guyénot & Danon in several respects created confusion by their account of the development of the chromosomes of amphibian oocytes, their description of the mode of origin and growth of peripheral "nucleoli" is worth noting. They state that in the primordial germ cell nuclei of female Triturus larvae there

are one to four nucleoli which are the true "plasmosome nucleoli" and are thought to give rise to the "macronucleoli" of larger oocytes. In oocytes which were at a stage corresponding to zygotene, they recognised a few "peripheral nucleoli" - but they do not venture to suggest how these first arose. The subsequent development of peripheral "nucleoli" is described by Guyénot & Danon as follows:-

"Pendant toute la période de croissance, leur nombre s'accroît à peu près comme la surface de la sphère, mais ce rapport n'est que très approximatif car les nucléoles varient aussi de dimensions.

"L'augmentation numériques des nucléoles résulte d'un phénomène de multiplication, tenant de la division et du bourgeonnement. On voit les nucléoles primitivement sphériques s'allonger, s'étaler contre la membrane nucléaire en prenant des formes irrégulièrement bosselées. Par étirement les bosses s'écartent les unes des autres, encore reliées par les ponts étroits, des filaments, puis deviennent indépendantes. D'autres nucléoles acquièrent, vus de face dans des calottes de noyaux, des formes très irrégulières, en raquette, polygonales, ramifiées; ils émettent, à la façon de bourgeons, des nucléoles plus petits, rattachés temporairement à la masse principale par des ponts filamenteux, puis libres.

....."Cette multiplication se répète assez rapidement pour engendrer des groupes de 4, 8, 12 nucléoles, de plus en plus petits et de taille variable, et peut aboutir parfois à la genèse de véritables micronucléoles. Les groupes ne tardent pas à se dissocier; les nouveaux nucléoles se disposent assez régulièrement à la face interne de la membrane nucléaire et présentent, dès lors, un accroissement de leur masse".

The same authors state that at the end of oogenesis all "nucleoli" migrate through the nuclear sap and congregate around the central chromosome group. Their description of "nucleolar" behaviour in Rana is impossibly complicated by

numerous references to "filaments nucléoplasmiques", and to a wide variety of ill defined "nucleolar" types. Latterly however, they betray their views concerning the origin of "peripheral nucleoli" when they describe

"des nucléoles périphériques arrondis, accompagnés chacun d'un grain rouge sombre, après coloration au Feulgen .....extérieur au nucléole et généralement situé du côté de la membrane nucléaire".

They conclude that this Feulgen positive granule

"correspond sans doute a l'organisateur décrit par Painter & Taylor (1942)."

In a special study of the oocyte "nucleoli" of Rana esculenta and Rana temporaria, Gersch (1940) treats all discernible extra-chromosomal bodies as "nucleoli". According to him, one "nucleolus" is present in young oocytes (he gives no indication of the actual sizes of the oocytes which he studied), while

"Mit einsetzendem Oocytenwachstum entsteht vor allem durch Teilung und Zerschndrung zunächst eine Vielzahl von grosseren und kleineren Nucleolen, die sich unregelmässig im ganzen Kernraum verteilt finden".

Prior to 1954 Duryee championed not only the chromosomal origin of oocyte "nucleoli", but also the eversion of "nucleoli" through the germinal vesicle membrane. He states in 1941 that in T.pyrrhogaster

"nucleoli are produced at definite loci on three or four pairs of chromosomes .... consequently an accumulation of nucleoli is evidence of chromosome function".

As knowledge has accumulated concerning the nature and properties of the nuclear membrane authors have refrained from expressing their views on the extrusion of visible particles from the germinal vesicle. The significance of Duryee's observations has been questioned (Gall 1954, Callan 1952, Tandler 1957), and in a recent publication Callan & Lloyd (1960) suggest

"that objects visible with the light microscope detach from lampbrush chromosomes only if they are being synthesised more rapidly than they are transforming into nuclear sap."

In so saying Callan & Lloyd infer that material leaving the oocyte nucleus does not do so in the form of visible particles.

Concerning the ultimate fate of oocyte "nucleoli", one thing is certain: in a mature oocyte all "nucleoli" are grouped together in the centre of the nucleus and after the breakdown of the nuclear membrane at the onset of first meiotic metaphase, the "nucleolar" material is exposed to the cytoplasm. What happens to oocyte "nucleoli" thereafter is a question which has been investigated by only one 20th century author - Yamamoto (1956). He showed that in the mature oocytes of the flounder Liopsetta obscura the "nucleoli" assume various bizarre shapes after breakdown of the nucleolar membrane, and their complete disappearance coincides with the re-appearance of "minute rod shaped chromosomes".

To return to the question of the origin of amphibian oocyte "nucleoli" Gall (1954) published convincing evidence that

in Amblystoma tigrinum a single lampbrush chromosome locus carries one or more refractile bodies which are similar in appearance to some of the peripheral "nucleoli" and that this locus is homologous with the somatic nucleolar organiser locus established by Dearing in 1934. Gall also reported that in Triturus viridescens there is a positional homology between a region of "attached oocyte nucleoli" on the lampbrush chromosomes and one of the five secondary constrictions of the somatic set. Since 1954 the theory that amphibian oocyte "nucleoli" originate at specific loci on the lampbrush chromosomes has never been seriously challenged.

The currently accepted dogma is briefly as follows:-

1. Oocyte "nucleoli" originate at certain specific lampbrush chromosome loci.
2. "Nucleolar" material accumulates at these loci and is periodically shed into the nuclear sap.
3. Each new "nucleolus" migrates through the nuclear sap to the periphery of the nucleus.
4. The total number of "nucleoli" in an oocyte nucleus increases steadily throughout the period of growth of the oocyte.
5. The increase in "nucleolar" numbers results from constant production of "nucleoli" by the nucleolar organiser loci, or by auto-duplication of pre-existing "nucleoli" or by both processes occurring simultaneously.
6. "Nucleolar" material passes into the cytoplasm either by diffusion through the nuclear membrane or by extrusion of whole "nucleoli".
7. Those "nucleoli" whose material does not pass into the cytoplasm before the end of oogenesis migrate inwards to the centre of the nucleus and disappear after the breakdown of the nuclear membrane.

Accordingly, in the growing oocytes of a given species of newt one may expect to find a few specific lampbrush chromosome loci carrying objects which resemble oocyte "nucleoli" in size, shape, and optical properties. One may also expect to find evidence that these loci are actively producing and periodically shedding new oocyte "nucleoli". Now it is known from the work of Parmenter (1926), Fankhauser & Humphrey (1943) and Elsdale, Fischberg & Smith (1958) that the number of nucleoli per somatic nucleus in Amphibia is genetically determined. Thus if the oocyte "nucleolar" organiser loci are indeed homologous with the somatic nucleolar organiser loci then we may expect to find in Triturus as many nucleoli per somatic nucleus as there are lampbrush chromosome loci carrying attached oocyte "nucleoli".

In this laboratory we are familiar with the lampbrush chromosomes of four sub-species of Triturus cristatus, and of one other species - Triturus marmoratus. In each of these newts the lampbrush chromosome loci which should be considered as possible oocyte "nucleolar" organisers can be identified with ease. Of all the attached objects, the giant loops of the three shortest chromosomes are most similar in appearance to the oocyte "nucleoli". Giant loops are characteristically present on chromosomes X (Fig.52), XI (Fig.53), and XII (Fig.54) of T.c.carnifex, on chromosomes X and XII of T.c.karelinii, on chromosomes XII of T.c.cristatus and T.c.danubialis, and on

chromosomes X and XII of T.marmoratus. Callan & Lloyd (1960) have shown that within a given sub-species the presence or absence of giant loops at particular loci is an individual-specific character, and that if giant loops are present at certain loci in one oocyte of an animal then they will be present at the same loci in all oocytes ranging from 0.6 to 1.7 mm. diameter taken from that animal.

Morton (unpublished) tackled the problem of equivalence between the number of recognisable giant loop loci and the number of nucleoli per somatic nucleus in the four sub-species of T.cristatus which I have named above, and in an F1 hybrid from the cross carnifex x karelinii. She was able to show (1) that for a given individual the maximum number of nucleoli per somatic cell (fusion can result in fewer nucleoli) does not correspond with the number of giant loops per oocyte nucleus, (2) that the maximum number of nucleoli per somatic cell varies between sub-species, and (3) that there is a segregation of the numbers of nucleoli per somatic cell in hybrid material.

Following upon Morton's work I became interested in some of the problems presented by the amphibian oocyte "nucleolus". Certain casual observations which I made during my work on the actions of enzymes on lampbrush chromosomes, together with some of the results which I have reported in Chapter II, raised doubts as to the correctness of the current dogma and I therefore set out to test the "facts" upon which the dogma is based. This

chapter tells of my efforts to date.

## 2. Comparative Studies

Most of my work on oocyte "nucleoli" was carried out on material obtained from T.c.carnifex. Where I used other sub-species, I shall say so. However, many of the techniques used for obtaining, processing, and examining material in my study of oocyte "nucleoli" are described elsewhere in this thesis; I shall refer to the appropriate page when necessary. An account of each special technique will be intercalated immediately prior to a report of the results obtained through its application. My studies of isolated "nucleoli" were performed upon the "nucleoli" which spill out of an oocyte nucleus when one tears its membrane. Thus the method for isolating "nucleoli" is the standard technique for making lampbrush chromosome preparations.

To begin with, I must emphasise the fact that the oocyte "nucleoli" of T.cristatus sub-species have a characteristic appearance on account of which they cannot be confused with any other class of free intranuclear object. I must also re-emphasise the fact that of all the structures attached laterally to the lampbrush chromosomes of carnifex, cristatus or karelinii the giant loops of chromosomes X, XI or XII most often look like oocyte "nucleoli". With the exception of the "lumpy loops" of

the middle region of chromosome II, no other attached structure warrants attention. I shall discuss the lumpy loops of chromosome II later.

Oocyte "nucleoli" first attracted my attention when I was examining toluidine blue stained preparations of lampbrush chromosomes (see page 21 Appendix I). "Nucleoli" and giant loops stained brilliantly and orthochromatically with toluidine blue, but in those slides which had been treated with ribonuclease prior to staining, the "nucleoli" stained more intensely than the giant loops (pre-treatment with ribonuclease reduced but did not eliminate toluidine blue staining of lampbrush chromosomes and oocyte "nucleoli" - see page 22 of Appendix I). Nothing stained in preparations which had been immersed for 15 minutes in boiling tri-chloro-acetic acid.

While studying the effects of enzymes upon unfixed lampbrush chromosomes I again became aware of differences between "nucleoli" and giant loops. Pepsin at pH <sup>6</sup>2 caused a slow reduction in the size of giant loops (see page 36 Appendix I), but had no morphological effect upon "nucleoli". Ribonuclease had no effect upon "nucleoli": the nature of its effect upon giant loops is illustrated in Figs. 45 and 46 of Appendix I. At a pH of 6.2 and a concentration of 0.25 mg/ml trypsin quickly reduced giant loops to a fraction of their original size; but even after 2 hours of enzyme action the "nucleoli" were relatively unaffected, (Figs. 55 and 56). Although resistant to proteolytic enzymes at

physiological pH, "nucleoli" were completely dissolved by trypsin or pan-protease at pH 7.8 (Figs. 57 and 58), and by pepsin at low pH.

I have found that when "nucleoli" are isolated in low concentration salines (less than 0.03 molar 5:1 K/NaCl) they often swell, burst and exude their contents - a sequence of events which I have called "decapsulation". Within a few minutes of decapsulation "nucleoli" dissolve completely. The more dilute the saline the more quickly they dissolve. A freshly decapsulated "nucleolus" can be seen to consist of a blob of diffuse material - the "CORE" of the "nucleolus" lying beside an empty "SHELL". For a long time I regarded the phenomenon of decapsulation with suspicion, but quite recently I stumbled upon evidence which seemed to me suggestive to be ignored. In all previous studies of the action of trypsin on chromosomes and "nucleoli" I had used a sample of trypsin which had been kept in our laboratory for more than 2 years. The experiment which I am about to describe was performed using a new sample of trypsin (Armour) which I shall characterise by calling it "T2"\*. o/

The effect of T2 upon isolated unfixed "nucleoli" was quite original. Within five minutes of application of the enzyme solution, all "nucleoli" became less bright, (Figs. 59 and 60).

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\* I used T2 at a concentration of 0.25mg/ml dissolved in C medium at pH 6.2 (see page 30 of Appendix I for details).

"Nucleolar" vacuoles became larger, and where there were two or more in one "nucleolus" they merged to form a single large vacuole. All vacuoles seemed to be full of minute particles which were in violent Brownian motion. About ten minutes after the start of digestion, each "nucleolus" began to swell, its vacuole grew, and the wall of the vacuole - representing all the remaining solid matter of the "nucleolus" - became thinner. After twelve to fifteen minutes of enzyme action, some "nucleoli" burst, and their contents flowed out and dispersed, leaving empty shells (Fig. 61 and 62). The latter were slowly digested, and disappeared after about one hour. The effect of T2 upon "nucleoli" from cristatus, karelinii and danubialis was exactly as I have described for the "nucleoli" of carnifex. I have interpreted decapsulation and the action of T2 as indicating that newt oocyte nucleoli possess a shell, on account of which they differ from giant loops and from any other class of intra-nuclear object.

### 3. The giant loops as "nucleolar" organisers

I have shown that "nucleoli" and giant loops differ in certain important respects; so that if we adhere to the belief that the giant loops are "nucleolar organisers" then it becomes necessary to add that "nucleoli" metamorphose after detaching from the chromosomes.

Let us now examine the questions of "nucleolar" production at giant loop loci, migration of "nucleoli" through the nuclear sap to the nuclear membrane, and increase in "nucleolar" numbers during oogenesis.

As I have described in Chapter II, gonadotrophin treated newts usually have small giant loops, whilst large "nucleolus-like" giant loops are characteristic of untreated or hypophysectomised animals. These facts alone are not incompatible with the idea of "nucleolar" production by the giant loops, and they would be wholly acceptable if there were a marked variability in giant loop size from oocyte to oocyte in a given animal at a given time. Some giant loops would be small because they had just shed a "nucleolus"; others would be larger because they carried "nucleoli" which were almost full grown. However I have found, not variability, but a conspicuous uniformity in giant loop size and appearance within a given animal at a given time (Figs. 18 to 25), irrespective of the treatment which that animal had received. If, for example, in the standard-size oocytes of a particular newt, the giant loops on homologue A of bivalent X are fused to form a single spherical object measuring  $8\mu$  in diameter, then we may expect to find a similar object at the corresponding locus in all other standard-size oocytes of that newt. Nevertheless, uniformity in giant loop size and shape is not so odd as it may seem, nor can it be taken as evidence that "nucleoli" are

not periodically shed from the giant loop loci. Consider the spheres of chromosomes V and VIII. Free spheres are a feature of all carnifex oocyte nuclei above a certain size, and because free and attached spheres resemble one another so closely we have never doubted that the former are detached products of the sphere loci (Callan & Lloyd 1960); but as with the giant loops there is a remarkable uniformity in the size of homologous attached spheres in standard-size oocyte nuclei taken from the same animal at the same time. In newt 32 for instance the sphere at 11 units on one arm of chromosome V was small in every oocyte which I examined, whilst the sphere at 2 units on the same chromosome was consistently large.

#### 4. Migration of "nucleoli"

I have already remarked upon the peripheral distribution of oocyte "nucleoli" but it is also worth mentioning the fact that in an average standard-size-oocyte nucleus the "nucleoli" are remarkably evenly spaced over the surface of the nuclear membrane. A new "nucleolus" migrating from its organiser locus to take up its position near to the nuclear membrane must therefore seek out a vacant space on the surface of the membrane or all other "nucleoli" must readjust their spacing to accommodate the newcomer, an unlikely story whichever way one looks at it!

If peripheral "nucleoli" are produced on the chromosomes

then how frequently are they shed and what is the likelihood of seeing one as it migrates through the sap of a standard-size-oocyte? Because "nucleoli" are relatively large objects and nuclear sap is often a stiff gel, migration if it took place, would be a slow process. The frequency with which it would occur is not difficult to estimate, and part of my work was directed towards such an estimate.

I selected a young carnifex female (No.61) and examined its left ovary through a small ventrolateral abdominal incision. The largest oocytes were approximately 0.3 mm. in diameter and were yolkless. The animal was given one injection of 100 I.U. CG + PMS per month for four months, during which time it was given "V.I.P." treatment with respect to feeding and other environmental factors. At the end of four months I removed its left ovary and found that the largest available oocytes were 1mm. in diameter. Standard-size oocytes showed all the characteristics which I had come to associate with intense metabolic activity. Five lampbrush preparations were made from the largest oocytes, and in each preparation the nuclear membrane was spread out over the bottom of the observation chamber, instead of being discarded. I then counted all the "nucleoli" in each preparation. Counts were obtained using the IPCM x 10 objective, bright field illumination, and a camera lucida. As each "nucleolus" was counted its position was marked by a small black dot on a sheet of paper pinned to the drawing board of the camera lucida. In such a way I was able to count the number of "nucleoli" per nucleus with more than 90% accuracy.

The numbers of "nucleoli" in each of the five preparations which I examined were as follows:-

912; 1,014; 1,161; 982; 991.

The mean of these five counts is 1,012.

From the experiment outlined above we know that the time taken for an oocyte to grow from 0.3 to 1.0 mm. diameter is at least four months. A complete oogenesis may occupy little more than five months. Assuming that an oocyte starts with one, two or three nucleoli and has 1,012 "nucleoli" when it reaches 1 mm. in diameter, we can calculate the rate of production of "nucleoli" during a five month oogenesis as 7 per day. Thus if the giant loops are the only sources of "nucleoli" then no statistical wizardry is necessary to show that if one studies a large number of nuclei then the chances of seeing newly shed migratory "nucleoli" are good.

Hoping that I might spot some migratory "nucleoli" in the oocyte nuclei of newt 61, I made a special study of 50 freshly isolated nuclei from oocytes ranging in size from 0.6 to 1.0 mm. (diameter).

Each nucleus was isolated in 0.2 molar 5:1 K/NaCl, transferred without delay to an observation chamber and examined with the IPCM using the x 20 objective and bright field illumination. The whole of the nuclear interior was scanned for objects resembling "nucleoli". Scanning was completed within two minutes of isolation of the nucleus.

I saw nothing which could positively be identified as a "nucleolus" anywhere but in contact with or near to the nuclear membrane. However, if I allowed two or three minutes to pass between isolating and examining a nucleus then the nuclear sap hydrated and some of the "nucleoli" left the nuclear

membrane and fell through the nuclear sap to the bottom of the nucleus. I believe that many of the reports of "nucleoli" distributed randomly throughout the nuclei of medium sized amphibian oocytes derive from studies of nuclei whose sap was allowed to hydrate before the positions of the "nucleoli" were recorded.

It is worth adding at this point that although I have examined more than 200 stained sections of newt ovaries, I have never seen "nucleoli" anywhere but around the inner edge of oocyte nuclei, except in oocytes which are nearly full grown.

##### 5. "Nucleologensis"

Only a brief glance at Figs.2 to 16 is necessary to see that there are many more "nucleoli" in lampbrush preparations made from the standard-size oocytes of gonadotrophin-treated newts than there are in lampbrush preparations made from the standard-size oocytes of control or hypophysectomised newts. At first I attributed this difference to a greatly accelerated "nucleolar" production following gonadotrophin treatment but later I realised that the lack of "nucleoli" in lampbrush preparations from hypophysectomised newts was due to the fact that in the standard-size oocytes of these animals more than 80% of the "nucleoli" were firmly attached to the nuclear membrane

(see Figs.63 to 65); whereas in the standard-size oocytes of gonadotrophin-treated newts there were no "nucleoli" attached to the nuclear membrane, (see Fig.66). To confirm and extend these observations I selected five carnifex females, examined their oocyte nuclei and noted that in the standard-size oocytes of all five animals the "nucleoli" which were attached to the nuclear membrane outnumbered those which were free. Each newt then received 400 I.U. of CG + PMS (as described on Page 28 of Chapter II). After treatment I re-examined the oocytes of all five newts and could not find a single oocyte measuring more than 0.8 mm. in diameter whose nucleus contained "nucleoli" which were attached to the nuclear membrane.

It is known from the reports of Guyénot & Danon (1953) and Gall (1954) that in T.cristatus and T.viridescens the peripheral "nucleoli" of small oocytes are flattened against and firmly attached to the nuclear membrane. Hitherto I had believed that attachment of "nucleoli" to the nuclear membrane reflected a nucleo-cytoplasmic transfer of "nucleolar" material the overall duration of which was jointly governed by the demand for "nucleolar" material in the cytoplasm and the supply of "nucleoli" by the lampbrush chromosome "nucleolar" organiser loci. So long as the demand persisted "nucleolar" production would keep pace with the loss of "nucleolar" material into the cytoplasm; but when the demand ceased, newly formed "nucleoli" would no longer affix themselves to the nuclear membrane, those "nucleoli"

which were already attached to the nuclear membrane would round off and fall back into the peripheral nuclear sap, and coincident with the regression of normal lateral loops, the "nucleolar" organiser loci would cease to function. Such a theory would be acceptable but for the lack of evidence to show that "nucleoli" are produced on the lampbrush chromosomes, and the fact that in gonadotrophin-treated newts all "nucleoli" are free from the nuclear membrane in relatively young oocytes where one would expect to find intense "nucleolar" production.

In a final search for facts which fitted the dogma I tackled the question of "nucleolar" numbers. The medium and large sized oocytes of gonadotrophin-treated newts seemed the ideal starting point and the first questions to which I sought answers were these : is there an increase in "nucleolar" numbers after the peripheral "nucleoli" cease to affix themselves to the nuclear membrane; and if so, is the increase of an order which could be accounted for by continued production of "nucleoli" at one or more "nucleolar" organiser loci.

I selected one gonadotrophin-treated caruifex female (62) and determined for that animal the maximum sizes of oocytes in which I could be sure of finding all peripheral "nucleoli" flattened against, and securely attached to, the nuclear membrane. Oocytes of 0.55 to 0.6 mm diameter suited my purposes well, and I isolated the nuclei from ten such oocytes and estimated the

total number of "nucleoli" per nucleus.

Each nucleus was placed in an observation chamber containing 0.2 molar 5:1 K/NaCl. The observation chamber was then transferred to the stage of the IPCM and the nucleus was brought into view. The "nucleoli" which were in contact with that part of the nuclear membrane which was farthest from the objective lens were then drawn to show their distribution over an area of approximately  $3,000\mu^2$ . Drawings were made using the x 95 oil immersion objective, x 10 eyepieces, and the camera lucida. Immediately after the completion of a drawing the diameter of the nucleus was measured using the x 10 objective lens and a micrometer eyepiece. The observation chamber was then removed and tilted on its side so as to roll the nucleus into a new position. A second drawing was made and the nuclear diameter was again measured. If perchance some "nucleoli" were not attached to the nuclear membrane then they fell out of the plane of focus as the nuclear sap became hydrated. I rejected all nuclei in which I found unattached "nucleoli". Some of the drawings which I made according to the method described above constitute Figs. 69 to 75.

Given two values for the diameter of a nucleus and two values for the number of "nucleoli" per unit area of its membrane one can estimate with reasonable accuracy the total number of "nucleoli" in that nucleus.

e.g. Number of "nucleoli"/ $2500\mu^2$  of  
nuclear membrane in drawing (1) = 12  
Diameter of nucleus after  
drawing (1) = 265 $\mu$

Number of "nucleoli"/ $2500\mu^2$  of  
nuclear membrane in drawing (2) = 10  
Diameter of nucleus after  
drawing (2) = 272 $\mu$

Surface area of nucleus after  
drawing (1) = 220506.5 $\mu^2$

Therefore, total number of  
"nucleoli" calculated from  
drawing (1) =  $\frac{220506.5}{2500} \times 12 = \underline{1058}$

Surface area of nucleus after  
drawing (2) = 232309.8 $\mu^2$

Therefore, total number of  
"nucleoli" calculated from  
drawing (2) =  $\frac{232309.8}{2500} \times 10 = \underline{929}$

The total number of "nucleoli" in this particular nucleus would then be taken as the mean of the estimates made from each drawing, that is 993.

I then counted the total number of "nucleoli" per nucleus in ten standard-size-oocytes (counts were made using the method described for newt 61 on page 90). Finally by the same method I counted the total number of "nucleoli" per nucleus in five oocytes each of which measured between 1.2 and 1.4 mm. diameter. The counts which I obtained from small and standard-sized oocytes are listed in Tables 9a and 9b. In preparations from large sized oocytes I found that the "nucleoli" were often clumped together in groups of 2 to 5, each member of a group being smaller than any of the solitary "nucleoli" in the same preparation. In some instances members of groups were attached to one another by thin strands (Figs. 67 and 68)\*. I assumed that each group resulted from the division of a single large "nucleolus" and on the basis of

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\* The precise nature of these strands remains obscure. They look like irregular strips of material rather than threads of uniform thickness, and they can only be broken or dissolved by agents which destroy the shells of the "nucleoli" which they join. My own view is that they arise accidentally from the shell of the parent "nucleolus".

this assumption I counted in each preparation, the number of solitary "nucleoli", the number of groups, and the number of small "nucleoli" which were members of groups. The data so obtained is set out in Table 9a.

Table 9a

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Oocyte diameters .... 0.55 to 0.6 mm.

Mean nuclear diameter ... 290 $\mu$

Estimates:-	715	
	866	
	803	
	948	
	907	MEAN = <u>968</u>
	956	
	1000	
	1240	
	1102	
	1142	

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Table 9b

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Oocyte diameters .... 0.89 to 1.0 mm.

Mean nuclear diameter ... 502 $\mu$

Estimates:-	811	
	859	
	979	
	959	
	1040	MEAN = <u>1014</u>
	1020	
	1155	
	1052	
	1109	
	1160	

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Table 9c.

Oocyte diameters ..... 1.2 to 1.4 mm.  
 Mean nuclear diameter .. 570 $\mu$

i Prep.	ii Solitary "nucleoli"	iii Groups of "nucleoli"	iv Total "nucleoli"	v Grand Total "nucleoli"	vi Total of groups of "nucleoli" + solitary "nucleoli"
1	546	512	1056	1602	858
2	687	336	878	1565	1023
3	438	303	960	1396	741
4	676	327	916	1592	1003
5	611	325	978	1580	936

Mean for the last column = 912

The figures listed in tables 9a and 9b show that the total number of "nucleoli" per small-oocyte nucleus (a) is not different from the total number of "nucleoli" per standard-size oocyte nucleus (b). The figures listed in column vi of Table 9c show that if one counts each group of "nucleoli" as a single "nucleolus" then there are as many "nucleoli" in small oocytes (a) as there are in large ones (c).

A curious feature of the "nucleolar" numbers in newt 62 is that the mean of the counts obtained from its standard-sized oocyte nuclei is almost identical to the mean of five counts obtained from the standard-sized-oocyte nuclei of newt 61, (see Page 90). This struck me as being more than mere coincidence

and it prompted me to develop the present line of research.

My first action was to reopen newt 62, remove another sample of its ovary, and isolate the nuclei of three oocytes having diameters of 0.13, 0.26, and 0.42 mm respectively. By the method described on pages 95 and 96 I then estimated the total number of "nucleoli" in each of these nuclei. Details of my estimates are given in table 9d. Figs. 69, 70, and 71 are three of the six drawings from which the estimates were made.

Table 9d

Oocyte diameter mm	Nuclear diameter $\mu$	Estimated No. of "nucleoli"
0.13	89	981
0.26	176	836
0.42	272	1014

The data presented above show quite clearly that in a nucleus of 80 to 90 $\mu$  in diameter, in which we cannot even guarantee the presence of lampbrush chromosomes, there are as many objects scattered over the inner surface of the nuclear membrane as there are free "nucleoli" in the nucleus 570 $\mu$  in diameter, whose lampbrush chromosomes are past the peak of their activity. It would thus seem that "nucleoli" do not increase in number during the lampbrush phase of oogenesis.

When do oocyte "nucleoli" first appear and from whence do they come? I cannot yet answer these questions with certainty

but I can offer some further information which may help to complete the picture. I have counted "nucleoli" in a third carnifex female (63), and in karelinii (64), cristatus (65), and danubialis (66) females. The "nucleolar" numbers in newt 63 (Table 10) corresponded well with those in newts 61 and 62. Karelinii (Table 11) proved to be uninteresting as its "nucleoli" did not differ in appearance or in number from those of carnifex. On the other hand, cristatus and danubialis offered some useful clues.

In cristatus, the number of "nucleoli" per oocyte nucleus varies from about 350 to about 1,000 (Table 12). This variability is not related to oocyte size, but it is related to "nucleolar" size. A nucleus of 283 $\mu$  diameter contained 923 "nucleoli", each of which occupied between 9 and 40 $\mu^2$  of nuclear membrane. Another nucleus of 288 $\mu$  diameter from the same ovary contained 355 "nucleoli" each of which occupied between 30 and 250 $\mu^2$  of nuclear membrane. The "nucleoli" of oocytes 19 and 26 (Table 12) although variable in size, were larger and less numerous than those of 21 or 28. Indeed oocyte No. 19 contained the largest "nucleolus" which I have yet seen; it was nearly round and measured 68 $\mu$  in diameter.

Small oocytes containing only three or four hundred "nucleoli" are common enough in cristatus, but I have looked in vain for a large oocyte containing less than 600 "nucleoli". Likewise I have looked in vain for evidence of "nucleolar" division. The extreme variability of "nucleolar" numbers in

Table 10

Nucleolar counts T.c.carnifex (No.63)

	i	ii	iii
	Cocyste Diameter	Nuclear Diameter	Number of "nucleoli"
	mm	$\mu$	
1.	0.38	260	919
2.	0.42	315	824
3.	0.51	344	1112
4.	0.74	380	1102
5.	0.81	421	874
6.	0.85	404	1215
7.	1.15	511	941
8.	1.20	502	990
9.	1.41	556	1008

Table 11

Nucleolar counts T.c.karelinii (No.64)

	i	ii	iii
	Cocyste Diameter	Nuclear Diameter	Number of "nucleoli"
	mm	$\mu$	
1.	0.43	179	1247
2.	0.46	207	1130
3.	0.50	213	1253
4.	0.56	251	1096
5.	0.59	308	1012
6.	0.80	371	972
7.	1.00	445	967
8.	1.12	480	1141
9.	1.19	472	896
10.	1.36	501	1050

Table 12

Nucleolar counts      T.c.cristatus (No.65)

i Oocyte diameter mm.	ii Nuclear diameter μ	iii Number of "nucleoli"	
1.	0.15	83	355
2.	0.16	95	601
3.	0.20	111	526
4.	0.40	226	346
5.	0.42	221	491
6.	0.44	239	491
7.	0.46	230	629
8.	0.50	240	618
9.	0.53	261	629
10.	0.55	269	994
11.	0.56	283	923
12.	0.57	288	624
13.	0.59	288	355
14.	0.73	346	511
15.	0.65	311	719
16.	0.75	358	772
17.	0.78	364	659
18.	0.81	386	613
19.	0.87	420	443
20.	0.90	425	714
21.	1.03	468	947
22.	1.03	434	756
23.	1.08	450	768
24.	1.12	450	678
25.	1.12	435	757
26.	1.15	502	608
27.	1.16	521	679
28.	1.16	504	919
29.	1.18	516	709
30.	1.50	554	823

Values 1 to 14 in column iii are estimates made from camera lucida drawings.

Table 13

Nucleolar counts      T. c. danubialis (No. 66)

	i	ii	iii
	Oocyte diameter	Nuclear diameter	Number of "nucleoli"
	mm	μ	
1.	0.26	152	975
2.	0.27	147	1110
3.	0.30	177	1455
4.	0.30	163	1034
5.	0.36	198	1561
6.	0.36	192	1759
7.	0.38	198	1176
8.	0.39	288	1586
9.	0.39	272	1394
10.	0.40	275	1300
11.	0.41	288	1324
12.	0.41	246	836
13.	0.41	304	1537
14.	0.41	307	1602
15.	0.68	345	3890
16.	0.78	377	776
17.	0.81	393	672
18.	0.90	411	781
19.	1.06	475	844
20.	1.15	490	804
21.	1.31	524	855
22.	1.36	524	891
23.	1.37	535	976
24.	1.50	655	892

cristatus oocytes of 1 mm or less in diameter may be due to a variable rate of division of pre-existing "nucleoli". It may however be due to something quite different; this I will discuss later.

A glance at Table 13 will show that the situation is still more complex for in danubialis, "nucleoli" are consistently more numerous during the first half of oogenesis. The smallest danubialis nucleus which I isolated was 100 $\mu$  in diameter. There were no "nucleoli" attached to its membrane. Instead the inner surface of the nuclear membrane bore curiously shaped patches of dense material (Figs. 73 and 74). Similar patches were present in nuclei of the same size taken from two other danubialis females. In nuclei of 120 to 300 $\mu$  in diameter I found small peripheral "nucleoli". These were all firmly attached to the nuclear membrane; they were arranged in groups of two to five, and members of groups were often attached to one another by slender threads, (Fig. 75). In all larger nuclei there were typical peripheral "nucleoli" which were more or less uniform in size and evenly distributed over the surface of the nuclear membrane. Values (1) to (14) in column (iii) of Table 13 are estimates of the number of GROUPS in each nucleus; whilst values (15) to (24) in column (iii) are counts of solitary peripheral "nucleoli".

In view of what I have reported in this chapter it seems that current views concerning the origin of amphibian

oocyte "nucleoli" are unsatisfactory. Now, I shall present what I believe to be a more reasonable theory.

The oocyte "nucleoli" of newts form, not one by one upon the chromosomes, but simultaneously upon the inner surface of the nuclear membrane. In carnifex they probably begin to form in oocytes of 0.08 to 0.1 mm in diameter, and in oocytes of about 0.15 mm in diameter they are recognisable as small (1 to 5 $\mu$  across), dense, roughly hemispherical bodies, which I shall call primary "nucleoli". These are firmly attached to, and evenly distributed over, the inner surface of the nuclear membrane (Figs.69 and 76)\*. They are not vacuolated, and they do not produce the bright haloes which are so characteristic of the "nucleoli" of larger oocytes, when viewed in phase contrast. They are often attached to one another by slender threads, and in some cases a thread emerges from a primary "nucleolus" and ends blindly against the nuclear membrane.

Each primary "nucleolus" grows, spreads itself out over the surface of the nuclear membrane and gives rise to a refractile, non-vacuolated, plate of material - a secondary "nucleolus" - which clings to the nuclear membrane by pseudopodia-

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\* Figs.76 to 79 are photographs of the interior surfaces of the nuclear membranes of whole intact nuclei which were isolated in 0.1 molar K/NaCl. To obtain a picture of this sort an observation chamber containing a nucleus is placed upon the stage of the IPCM and the nucleus is brought into view. A photograph is then taken of that part of the nuclear interior which is nearest to the objective lens of the microscope.

like processes, (Figs. 70, 77, and 78). Secondary "nucleoli" are evenly distributed around the periphery of the nucleus. The largest of them may be 10 to 15 $\mu$  wide. Some are connected to their neighbours by threads, whilst the pseudopodia of others end in threads which merge into the substance of the nuclear membrane. Secondary "nucleoli" are characteristic of oocytes ranging from 0.2 to 0.6 mm. in diameter.

When an oocyte reaches about 0.5 mm. in diameter its secondary "nucleoli" start to thicken, draw in their pseudopodia and gradually revert to a spherical or nearly spherical shape (Figs. 71 and 79). As soon as a "nucleolus" has rounded off, it detaches itself from the nuclear membrane and becomes a free "nucleolus". Free "nucleoli" are held in place by gelatinous nuclear sap; they therefore retain their positions relative to one another. All "nucleoli" do not round off and detach at once. A few free "nucleoli" are usually present in oocytes of 0.6 mm. diameter; in oocytes of 0.8 to 0.9 mm. diameter there are usually about as many free "nucleoli" as there are attached secondary "nucleoli", and in a normal healthy animal one rarely finds attached "nucleoli" in oocytes of more than 1.1 mm. diameter.

Each free "nucleolus" grows and when it reaches a certain maximum size it may break up into several small daughter "nucleoli". Coincident with the start of chromosome contraction the "nucleoli" begin to leave the periphery of the nucleus and

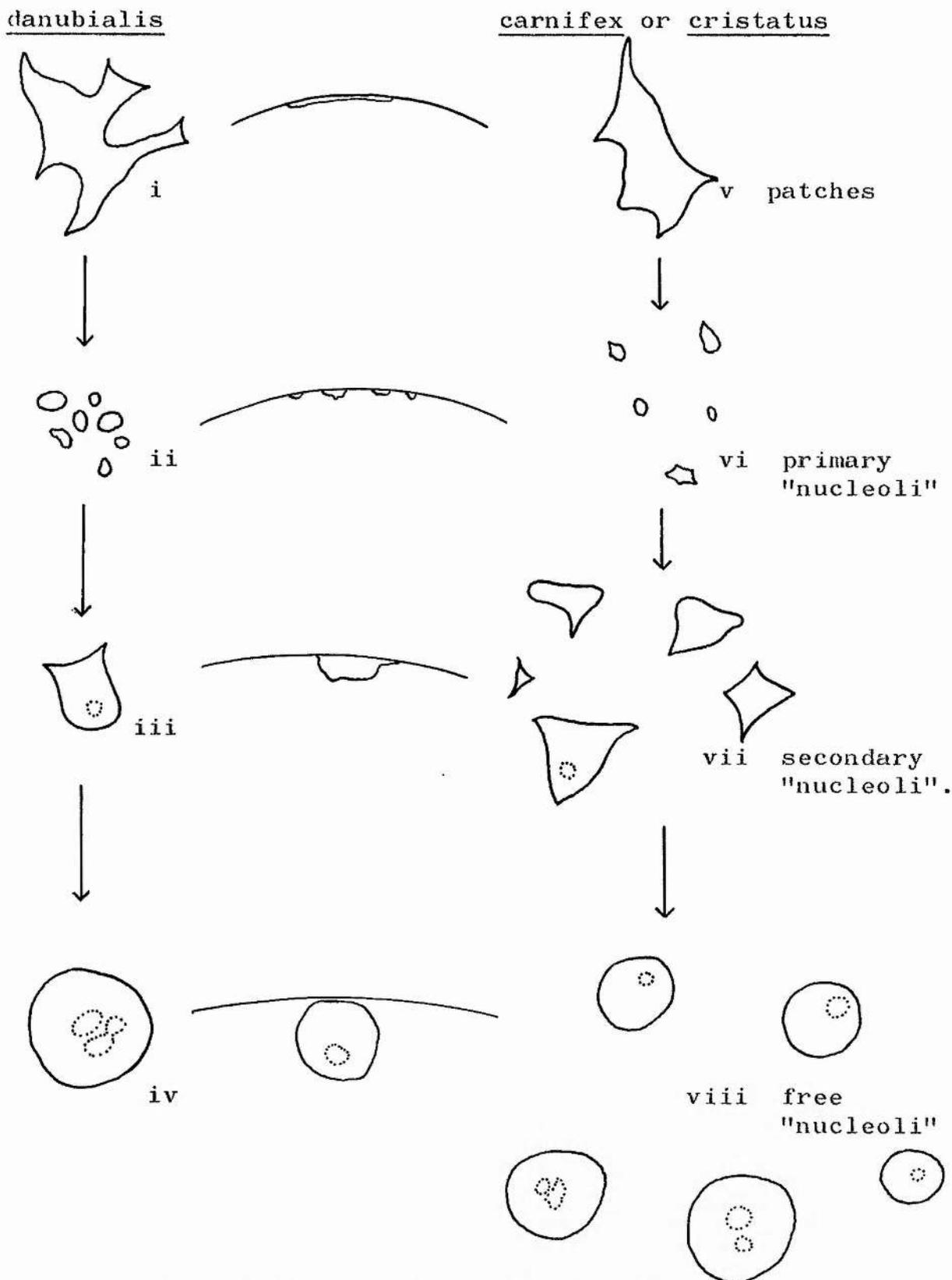
migrate inwards. In an oocyte of about 1.5 mm diameter a few "nucleoli" are clustered around the central chromosome group; others are scattered throughout the nucleus and are often arranged in columns as though they were moving through channels which converged upon the central cluster.

The ball of closely packed "nucleoli" which is so characteristic of mature oocytes measures some 50 to 80 $\mu$  in diameter.

Now let us look at some of the features of "nucleolar" development in danubialis. We have seen that in this species the nuclear membranes of very small oocytes bear curious patches of material. I think these patches are the antecedents of "nucleoli". Moreover I suggest that each patch gives rise to a group of primary "nucleoli" the members of which subsequently rejoin to form a single secondary "nucleolus". I have little doubt that the primary "nucleoli" of carnifex and cristatus arise from patches which are similar to those of danubialis, but I cannot be sure of this until I have succeeded in isolating and examining nuclei of sufficiently small carnifex and cristatus oocytes. Fig. 84 is a diagrammatic representation of "nucleolar" development in danubialis, and in carnifex and cristatus; it may help to clarify my description of the process.

The main points which I would emphasise concerning the development of newt oocyte "nucleoli" are these:-

Figure 84



Diagrammatic representation of "nucleolar" development in *danubialis* (i - iv) and in *carnifex* or *cristatus* (v - viii). In *danubialis* one group of primary "nucleoli" gives rise to one free "nucleolus"; in *carnifex* or *cristatus* each primary "nucleolus" gives rise to a free "nucleolus". A sectional view of each stage in "nucleolar" development is shown in the middle of the page.

(1) In an oocyte of less than 150 $\mu$  diameter a complete generation of "nucleoli" arises on the inner surface of the nuclear membrane; (2) each "nucleolus" continues to grow so long as it remains in contact with or near to the nuclear membrane; (3) at a certain stage in the growth of an oocyte, secondary "nucleoli" begin to round off and detach from the nuclear membrane; (4) the detailed pattern of "nucleolar" development is characteristic for a given sub-species of newt.

Given a scheme for the development of newt oocyte "nucleoli", let us now turn to the question of "nucleolar" physiological variability. Why are all "nucleoli" attached to the nuclear membranes of standard-size oocytes of hypophysectomised newts, but free in the standard-size oocytes of gonadotrophin-treated newts? I suggest that gonadotrophin treatment, by stimulating oocyte metabolic activity, causes "nucleolar" material to accumulate more quickly, and leads to the detachment of "nucleoli" from the nuclear membrane at an unusually early stage. Hypophysectomy produces the reverse situation and indirectly prohibits the detachment of "nucleoli" from the nuclear membrane. But hypophysectomy affects "nucleoli" in other ways. The late secondary "nucleoli" of hypophysectomised newts are by no means normal. They are small half round objects, and they are often grouped together in threes or fours (Figs.63 and 64). Why this should be so I cannot yet say. There are many more aspects of "nucleolar" physiological variability which would be worth

studying. It would, for example, be helpful to know whether "nucleolar" size, or the incidence of "nucleolar" vacuolation and division could be changed by treating newts with gonadotrophin; and indeed I think that information of this sort must be obtained before we can offer any sound ideas concerning the function of the amphibian oocyte "nucleolus".

Let us now look for a moment at some facts which I have hitherto deliberately disregarded and at certain assumptions which I have included in my arguments.

The similarity between oocyte "nucleoli" and giant loops is more striking in cristatus than in any other sub-species of newt which I have examined. Moreover, in preparations of cristatus lampbrush chromosomes the giant loops of chromosome XII are often surrounded by free objects which are undoubtedly the shed products of this locus, but which are optically indistinguishable from nearby "nucleoli" (Fig. 80). I suggest that material which is synthesised at the giant loop locus in cristatus is shed more rapidly than it is transformed into nuclear sap, and that it accumulates in the nucleus in the form of droplets which resemble free "nucleoli". If this were so then it might account for the fact that larger cristatus oocytes seem to have more "nucleoli" than smaller ones.

Earlier on I said that the "nucleoli" of newts are

irregularly round, but this is not always true (see Figs. 81, 82, and 83). Free "nucleoli" sometimes have the most peculiar shapes, and a single nucleus may contain several "nucleolar" types, which differ from one another in size, shape, degree of vacuolation, and optical properties. I have avoided newts with odd "nucleoli", but I do not mean to suggest that variability in "nucleolar" form is without significance.

Lastly, the theory which I have offered for the origin of newt oocyte "nucleoli" discounts the possibility that primary "nucleoli" are the product of a chromosome locus. The lumpy loops of chromosome II in T. cristatus sub-species are known to be at their largest in small oocytes and at one time I was ready to consider these structures as generators of "nucleoli". However, we have seen that where lampbrush chromosome loci are concerned, large size does not necessarily indicate active synthesis, and even if it did, how could one explain the sudden appearance of a "rash" of primary "nucleoli" distributed over the entire surface of the nuclear membrane?

Electron Microscopy.

The work which I shall now describe was done with the Seimens Elmiskop I in the Department of Zoology, University of Edinburgh, and I am indebted to Mr. D. C. Barker and Mr. F. Collier for their invaluable assistance and for introducing me to many of the basic techniques of electron microscopy.

I am also indebted to Dr. J. G. Gall for teaching me the essentials of his technique for embedding and sectioning lampbrush chromosomes and oocyte "nucleoli".

I first used the electron microscope to see the nature of the contact between secondary "nucleoli" and the nuclear membrane. For this purpose I used whole isolated nuclei from oocytes of 0.5 to 0.6 mm. in diameter in which the majority of "nucleoli" were firmly attached to the nuclear membrane. Nuclei were isolated in 0.1 molar 5:1  $\text{KH}_2\text{PO}_4$ / $\text{Na}_2\text{HPO}_4$  buffer, pH 7. After fixation for 15 minutes at 1°C., nuclei were washed in 0.1 molar K/NaCl, rapidly dehydrated in an alcohol series and embedded in methacrylate. Thin sections were cut on a Porter-Blum microtome with glass knives and were picked up on carbon coated grids. More than 100 sections of 5 nuclei taken from the oocytes of one newt were examined.

Figs. 85, 86, and 87 are electron micrographs of sections through the nuclear membrane and secondary "nucleoli". Four

points are worth noting. First, wherever the microtome knife had passed through the region of contact between a "nucleolus" and the nuclear membrane there was a gap in the nuclear membrane. Secondly, "nucleolar" material and nuclear membrane had similar electron absorbing properties. Thirdly, the structure of the "nucleoli" shown in Figs. 85, 86, and 87 might be interpreted as a complex system of folds - between which there is no nuclear sap. Finally, I would stress that the isolated nuclei which I have examined with the electron microscope were badly fixed, and judging from the state of the nuclear membrane (as seen in Fig. 87) my results are certainly not trustworthy.

This work is to be repeated on whole oocytes fixed in  $\text{OsO}_4$  prepared according to Caulfield (1957), embedded in Vestopal W, and stained with uranyl acetate.

In my second electron microscope study I sought to compare the ultrastructure of free "nucleoli" and giant loops in T.c.carnifex. I also hoped to find more evidence of a "nucleolar" shell.

Preparations of lampbrush chromosomes and free "nucleoli" were made according to the method described on pages 8 and 9 of Appendix 1. Each preparation was set aside under a dust cover for half an hour to allow the nuclear sap to disperse and the chromosomes to settle. A low power camera lucida drawing of the chromosomes was made and the positions of the giant loops were

marked. Each preparation was then placed in a chamber saturated with formaldehyde vapour for 15 minutes, (treatment with formaldehyde was needed to stick the chromosomes and "nucleoli" to the glass bottom of the observation chamber). The bottom coverglass of the observation chamber, bearing the chromosomes and "nucleoli" was then prised off the bored slide and allowed to fall into a solid watchglass containing a chilled 1% solution of  $\text{OsO}_4$  in veronal acetate buffer pH 7.2. In many cases the chromosomes and "nucleoli" were lost at this stage in the proceedings through not being attached to the glass. After 15 minutes fixation in  $\text{OsO}_4$  preparations were washed in veronal acetate buffer (pH 7.2), immersed in a 0.01% solution of uranyl acetate for 15 minutes and washed again in distilled water. They were subsequently dehydrated in an acetone series and placed in 25%, 50%, and 75% Vestopal/acetone mixtures for 1, 2, and 4 hours respectively, and in 100% Vestopal for 8 hours. Each coverglass bearing chromosomes and "nucleoli" was then inverted over the mouth of a gelatine capsule which was filled to the brim with Vestopal + initiator and activator. The position of the coverglass was adjusted so that the chromosome group lay in the centre of the mouth of the capsule. The Vestopal was polymerised at  $80^\circ\text{C}$  for 12 hours, after which the coverslips were removed from the gelatine capsules, leaving the chromosomes and "nucleoli" embedded in the surface of a block of Vestopal. Clean separation of the coverslip and Vestopal block was made easy by holding a piece of dry ice against

the back of the coverslip for a few seconds.

With an ordinary microscope fitted with a x 10 objective and under certain critical conditions of illumination I was able to see the embedded chromosomes and "nucleoli". By reference to the original camera lucida drawing I located the larger landmark structures and I trimmed the block to include only that chromosome region which I wished to section. Sections about 600Å thick were cut on a Porter-Blum microtome and were picked up on carbon coated grids.

So far I have seen sections of about 50 "nucleoli" taken from the standard-size oocytes of two carnifex females and every one of these "nucleoli" possessed an electron-dense shell about  $\frac{1}{2}\mu$  thick (Figs. 89, 90, and 92). Some of the "nucleoli" which I sectioned were vacuolated and the vacuoles appeared empty and smooth walled (Figs. 90 and 92). Looked at with higher magnifications the core and shell of a "nucleolus" appear to consist of the same granular material (Fig. 92).

The giant loop on chromosome XII (Figs. 88 and 91) has no shell, its vacuoles are rough walled and it appears to be structureless.

The results of this study serve to emphasise the fact that "nucleoli" and giant loops are in most respects different from one another. However, the question of the "nucleolar" shell is still subject to doubt. It is possible, for example,

that the shell is an artifact brought about by the brief formaldehyde or uranyl acetate treatments, in which case we must assume that one or other of these reagents took as long as 15 minutes to penetrate the dense material of the "nucleolus" to a depth of  $\frac{1}{4}\mu$ . As an alternative I suggest that the proteins of the "nucleolar" shell react with  $\text{OsO}_4$ . "Nucleoli" are known to be rich in -SH groups (Gersch 1940, Brachet 1939, personal observation\*), which according to Bahr (1954), reduce  $\text{OsO}_4$  rapidly. Moreover, if the "nucleolar" skin were characterised by a high concentration of protein -SH then it might not be absurd to suggest a parallel between "nucleoli" and erythrocytes. The integrity of the latter is said to depend upon -SH groups in the proteins of the cell wall (Benesch & Benesch 1954).

I cannot yet explain the extraordinary differences between my pictures of secondary "nucleoli" embedded in methacrylate and those of free "nucleoli" embedded in Vestopal, nor can I yet explain the fact that my findings are so different

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\* I adopted the method of Barnett & Seligman 1952 (the D.D.D. reaction) for the detection of protein bound -SH groups and applied it to isolated lampbrush chromosomes and "nucleoli" which had been fixed in 80% alcohol and T.C.A. "Nucleoli" were a deep red in colour after staining; giant loops (chromosomes X and XI) and lumpy loops (chromosomes II and VII) were less intensely coloured than "nucleoli"; normal loops and chromomeres were faintly pink. Nothing stained in preparations which had been previously kept in 0.1 molar iodoacetate for 24 hours. I am indebted to Dr. A. M. Seligman for supplying me with samples of 2,2-dihydroxy-6,6-dinaphthyl disulphide, and tetra-azotised diorthoanididine.

from those of previous workers (Kemp 1956a and 1956b, Brown & Ris 1959).

This work is entirely preliminary and it has been sufficiently promising to warrant a much more detailed and systematic attack when I find myself within easy reach of an electron microscope.

CHAPTER 4

DISCUSSION

Callan (1955), Beerman (1956), and Gall (1958) have drawn attention to the fact that the loops of lampbrush chromosomes and the puffs, Balbiani rings and nucleoli of salivary gland chromosomes are all expressions of synthetic activity at specific gene loci. Essentially a lampbrush loop is a stretch of chromonema spun out from its "parent" chromomere (Callan 1960), and surrounded by the materials whose synthesis it directs. A puff or Balbiani ring is derived from a single euchromatic band of salivary gland chromosome. At such loci the individual chromonemata have bent out from the chromosome and formed miniature loops, each of which is surrounded by a material whose properties are probably locus specific (Stich 1959). The first point which I wish to make here is that a puff or Balbiani ring is essentially a polytene lampbrush loop.\*

In salivary gland chromosomes there is no sharp distinction between a puff and a bulb. The difference between these structures is said to depend upon the quality of the product synthesised at a locus (Beerman 1956). Likewise, there is no sharp distinction between a normal lampbrush loop and a giant loop.

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\* Beerman (1956, 1959) describes each individual loop of a Balbiani ring as a "lampbrush". In the present discussion one miniature loop of a Balbiani ring is compared to one loop of a typical lampbrush chromosome.

Therefore I propose that the changes which I have been able to induce at the giant loop loci in carnifex are comparable to the changes which Beerman (1956) induced in puffs and Balbiani rings by controlled temperature alteration. When Chironomus larvae were kept at temperatures lower than  $10^{\circ}\text{C}$ , for a few hours and then transferred to water at  $20^{\circ}\text{C}$  for one hour, droplets formed at the sites where puffs are normally present; after 2 to 8 hours at  $20^{\circ}\text{C}$  Beerman found that the droplets disappeared.

Let us consider how similar changes might be manifest at the giant loop loci in a newt oocyte, assuming that the main target for gonadotrophin is protein synthesis in the egg cytoplasm, and that a metabolic balance exists between the various components of the egg. Under normal circumstances there would be a steady turnover of materials at the giant loop loci, the gene product diffusing away from the loops and being used up as rapidly as it was being formed. A sudden failure in the supply of gonadotrophin would cause a decrease in the rate at which protein was being synthesised in the egg cytoplasm: this would temporarily upset the balance between the lampbrush chromosomes and the rest of the cell: material would be synthesised on the giant loops more quickly than it could be used in other parts of the cell: the nuclear sap would become "saturated" with the products of the giant loops, and the latter would become large and bloated. An artificially

supplied stimulus, such as an injection of gonadotrophin, would accelerate cellular metabolism and increase the demands made upon the chromosomes. For a time gene product material would be used up more quickly than it was being formed, and the loops themselves would become smaller.

Physiological variability on chromosomes is common enough if we include under this heading the variability of nucleoli in somatic cells. A true nucleolus is attached to a specific locus on a chromosome (Heitz 1931, McIntock 1934). The nucleolar substance is a gene product in the sense that it is synthesised or collects at a specific locus and has certain peculiar properties. Yet nucleolar size is said to be proportional to the rate at which protein is being synthesised in the cytoplasm, (Caspersson & Schultz 1940, Caspersson & Santesson 1942, Ehrenburg 1946, Edstrom & Eichner 1958, Stich 1959). The lampbrush chromosome loci in Amblystoma tigrinum and T. viridescens which are thought to be homologous with the nucleolar organiser loci of somatic cells (Gall 1954) would be worth studying to see whether they become more or less conspicuous after gonadotrophin stimulation. The results of my own studies on physiological variability of the lampbrush chromosomes of carnifex suggest that where the giant loops are concerned, large size does not necessarily indicate intense metabolic activity, either on the chromosomes or elsewhere in the cell.

The spheres of chromosomes V and VIII (carnifex) deserve to be considered separately. They differ from other objects found on carnifex lampbrush chromosomes in that they contain little or no RNA, they are always spherical, they are found free in the nuclear sap, and their size and number does seem to be proportional to the metabolic activity of the oocyte as a whole. I mention them because they have taught us two important principles. First, the presence of free spheres indicates that there is a regular cycle of growth and detachment at the sphere loci. Presumably similar cycles prevail at other loci. Secondly, since in any one nucleus, free spheres are never larger than those which are attached to the chromosomes, it seems logical to assume that after detachment, spheres slowly transform into nuclear sap. Perhaps the products of other loci behave likewise. These facts and speculations have been discussed in more detail by Callan & Lloyd (1960).

Now let us consider the free granules of oocyte nuclei. To recapitulate; free granules are optically dense; they are always round; they vary in size from the limits of visibility with the light microscope to 3 or 4 $\mu$  in diameter; they contain cytochemically demonstrable RNA; they are dissolved by trypsin and pan-protease at pH 8 and by pepsin at pH 2 but they are not dissolved by ribonuclease. The granules which characterise the giant granular loops of chromosome XII (cristatus) and the "marker" loops at 56 units on chromosome II (carnifex) have

similar properties.

I consider that the unqualified view that all free granules are the shed products of lateral loop synthesis is unjustified. I do not deny that the smallest free granules (up to 1 $\mu$  in diameter) may have been formed upon and shed by the lateral loops, nor do I deny that the substance which makes up both large and small free granules was ultimately derived from the loops. But the larger the granule the less plausible is the suggestion that it originated directly from a locus on a lampbrush chromosome. The facts which bear upon this problem are as follows:-

1. The oocyte nuclei of untreated newts sometimes contain free granules which are too large for them to have originated directly from any lampbrush chromosome locus.
2. Oocyte nuclei from gonadotrophin-treated newts rarely contain free granules of more than 1 $\mu$  in diameter.
3. The largest free granules which I have seen were in the oocyte nuclei of newts which had survived hypophysectomy for more than one month.
4. Wherever lampbrush chromosomes are smothered in attached granules there are many free granules in the nuclear sap, yet the converse does not always hold.
5. In some gonadotrophin-treated newts large refractile granules were bunched at the axial granule loci on chromosome I; free granules of comparable size lay nearby yet the remainder of the nuclear

sap was clear

6. In one newt there were swarms of free granules in the immediate vicinity of the giant loops on chromosome X, but not elsewhere (Figs. 93 and 94).

Most normal lateral loops carry minute granules which are often barely visible with the light microscope. In my opinion, these granules are regularly shed into the nuclear sap where under favourable conditions they dissolve and their material passes into the cytoplasm. Now let us suppose that there is a fall in the rate at which protein is being synthesised in the cytoplasm. As a result the transfer of materials from nucleus to cytoplasm would slow down but, for a time, metabolic activity in the nucleus would remain unchanged. The products of lateral loop synthesis would accumulate in the nucleus. Each loop would continue to shed its granules, but these would not dissolve. Eventually a kind of "recrystallisation" and accretion of gene products would take place as a result of which granules would become larger and fewer in number. According to this scheme, granules are not a permanent feature of oocyte nuclei, and large free granules (more than  $1\mu$  in diameter) or large numbers of free granules are characteristic of nuclei whose anabolism is not synchronised with synthetic processes in other parts of the cell.

On the other hand, when an oocyte as a whole is intensely active, large masses of granular material may be shed from a certain locus so rapidly that a zone of saturation forms in the

neighbourhood of the locus. Newly shed granules would have to escape from this zone before they could dissolve in the nuclear sap. Thus I account for the conditions illustrated in Figs. 32, 93, and 94, and for the large numbers of minute free granules which lie amongst the chromosomes in the oocyte nuclei of gonadotrophin-treated newts. I would stress here that only once have I seen evidence to suggest that granules or lumps of material can detach from the giant loops of carnifex, and indeed the striking physiological variability at these loci points to the fact that in carnifex the giant loops cannot regulate their size by a mechanism of this sort. No doubt the situation is different in cristatus and in T. viridescens (Gall 1954) where it seems that large bodies are regularly shed from the giant loops.

Callan & Lloyd (1960) consider that objects visible with the light microscope detach from lampbrush chromosomes only if they are being synthesised more rapidly than they are transforming into nuclear sap. My own views on this matter are essentially in agreement with those of Callan & Lloyd, but I would suggest two qualifications. First, in T. cristatus large granules or bodies, more than 1 $\mu$  in diameter, may be shed from a few specific loci on the lampbrush chromosomes of oocytes which are intensely active. Secondly, the large free granules commonly found in the oocytes of unstimulated newts are not formed as such upon the chromosomes, although they do consist of gene product material.

While discussing the nature of free granules I have assumed that the RNA which they contain will eventually take part in the synthesis of cytoplasmic proteins. This assumption is largely based upon evidence from autoradiography experiments of Ficq (1955) and Callan and Gall (unpublished), which showed a high rate of turnover of nuclear RNA in amphibian oocytes and a transfer of RNA from nucleus to cytoplasm. However, I have also taken into account the results of some of my own experiments. For example, gonadotrophin treatment causes a disappearance of free granules from an oocyte nucleus, the formation of a cytoplasmic perinuclear zone of clear fluid, an increase in the concentration of cytoplasmic RNA, and, as judged by the rate of incorporation of  $^{32}\text{P}$ , an increase in the rate of protein synthesis in the egg cytoplasm. Brachet (1942) described an accumulation of RNA in the perinuclear cytoplasm of growing oocytes. I have not found evidence of such an accumulation in stained sections of newt oocytes, but I consider that the perinuclear fluid which I have identified in fresh material may be significant in this respect.

Gall (1956b) drew attention to a likeness in the sizes of the RNP particles which are associated with the membranes of the endoplasmic reticulum (Porter 1954, Palade 1955) and those present on the normal lateral loops of lampbrush chromosomes. The particles which he describes are of the order of  $300\text{\AA}$  in diameter. I do not know how these particles are related to the

granules which one can see with the light microscope and I do not propose to discuss this matter here.

Hitherto I have rather presumptuously used the word "dogma" when referring to current views on the origin of oocyte "nucleoli", and in my introduction to Chapter III, I placed much of the responsibility for this standpoint upon Gall (1954, 1958). My own views about the origin of oocyte nucleoli are reactionary, and presently I shall muster more facts to support these views. To begin with however, I must make clear that I think one or other of the more conspicuous objects which one finds attached laterally to the lampbrush chromosomes of carinifex are homologous with the nucleoli of somatic cells. I have not established such a homology because, unlike Gall, I have not tried to do so. I have instead scrutinised the theory that peripheral "nucleoli" are generated at one or more lampbrush chromosome loci. The conclusion which I have reached is that peripheral "nucleoli" are not generated upon the lampbrush chromosomes, but that they arise upon the inner surface of the nuclear membrane. They are therefore unrelated to somatic nucleoli.

Cytological literature offers little support for my conclusions, but I have been encouraged by the fact that since Wagner (1835) first described the "nucleoli" of germinal vesicles more than 200 cytologists have remarked upon the peripheral distribution of these bodies in the oocyte nuclei of various animals; yet scarcely anyone has sought to demonstrate the

significance of this distribution. It is true that many people have presented ideas concerning the origin and role of peripheral "nucleoli", but most of these ideas have been founded upon intuition, not upon evidence. I have gained most encouragement however, from conversations with Dr. A. Ficq and Dr. J. G. Gall, both of whom have studied the incorporation of labelled RNA precursors into the "nucleoli" of amphibian oocytes. These workers mentioned that the "nucleoli" which are attached to the inner surface of the nuclear membrane are often asymmetrically labelled, the "hottest" region being towards the nuclear membrane. Their observations supplement the findings of Pantelouris (1958) who showed, amongst other things, that peripheral "nucleoli" in newt oocytes incorporate labelled amino acids most rapidly during the early stages of oocyte development, and that they do so at the expense of tracer in the cytoplasm.

In 1898 Montgomery compiled a review on the subject of the "nucleolus" and he also recorded the results of his own observations. He assembled information from the studies of numerous previous authors and stated that large numbers of peripheral "nucleoli" are characteristic of the oocytes of Selachians, Teleosts, Amphibians, Reptiles and some Nemertines. Montgomery's conclusions regarding the origin of peripheral "nucleoli" were similar to my own, although they were less conservative. He states that

"the nucleolar substance in many if not all cells has an extra-nuclear origin, and although it may undergo

a chemical change upon entering the nucleus, it can be regarded neither as an excretion or a secretion of the latter".

Until recently, the idea of nucleolar material being derived from the cytoplasm was unpopular. This is scarcely surprising since there is every reason to believe that the nucleoli of somatic interphase nuclei are formed at particular chromosome regions. There is, however, one other type of nucleus in which nucleoli form upon the inner surface of the nuclear membrane.

I refer here to the male pronucleus of the rat. Austin (1951, 1952, 1960) found that in the early development of this pronucleus, small nucleoli form, grow, and coalesce to form one large nucleolus. Thereafter, new nucleoli form but differ from the earlier nucleoli in that they are found in contact with or even embedded in the nuclear membrane, whereas the early nucleoli lay free within the nucleus. The new or "secondary nucleoli" grow and detach from the nuclear membrane about half way through pronuclear development. Whether or not these secondary nucleoli are associated with Feulgen stainable material is not certain. Alfert (1950), and Braden & Austin (1953), claim to have detected small amounts of DNA around the periphery of the mammalian pronucleus, yet pyronine-methyl green staining showed the presence of DNA about the early nucleoli but not the later ones (Ludwig 1953, 1954). If there is no DNA about the secondary nucleoli of pronuclei then we may reasonably assume that their mode of origin is comparable with that of amphibian oocyte "nucleoli".

Whether or not the two types of nucleolus are comparable in other respects I cannot say. Certainly they both form in contact with the nuclear membrane and circumstantial evidence points to the fact that in both cases the nucleolar material is extra-nuclear in origin.

Finally let us consider the roles of the different components of the amphibian oocyte both during oogenesis and after the breakdown of the nuclear membrane at the end of oogenesis. As I have already pointed out, one function of lampbrush chromosomes is assuredly the production of a cytoplasmic RNA fraction. That they perform another function is suggested by the facts that the germinal vesicle enlarges greatly in the course of oogenesis, and that it ceases to enlarge after the lampbrush chromosomes retract their loops and revert to a condensed form (Marechal 1907, on elasmobranchs).

The nuclear sap consists mostly of protein (Brown, Callan & Leaf 1950) and we may reasonably ask whether this protein is of a specific type whose synthesis is directly controlled by the chromosomes themselves. The fact that increased metabolic activity on the lampbrush chromosomes is accompanied by a rise in the viscosity of the nuclear sap may be significant here. At the end of oogenesis, the chromosomes condense, and from this time onward they probably cease to be involved in the physiological economy of the cell. Whether or not they are essential for cleavage is not certain, although the experiments of Pankhauser

(1929, 1934), and Briggs et al. (1951) seem to prove that a full intact complement of chromosomes is not essential.

Without doubt, the non-chromosomal material of the germinal vesicle is indispensable for cleavage. A condition of cytoplasmic maturity must be attained in the egg before the latter can be fertilised and before asters can form, (Hertwig 1887, Morgan 1896, 1899)\*. Moreover, the experiments of Delage (1899, 1907)\*, Wilson (1903)\*, and Yatsu (1904, 1905)\* showed that cytoplasmic maturity is attained only after the breakdown of the germinal vesicle membrane. The way in which the nuclear contents condition the cytoplasm at this stage is not yet known and there is little scope for speculation.

The function of oocyte "nucleoli" is worth special attention, but first let us review the chemistry of these bodies. Nucleoli in general contain high concentrations of proteins (Serra and Lopes 1945, Pollister and Ris 1947, Caspersson 1950, Nurnberger et al 1952, Vincent 1952, Vincent and Muxley 1954) and amphibian oocyte "nucleoli" are not exceptional in this respect (Gersch 1940, Brown and Ris 1959). The latter also contain RNA which is said to account for about 15% of their dry mass (Brown and Ris 1959), and they are rich in protein bound -SH groups (Brachet 1938, Gersch 1940, personal observation).

As I have mentioned earlier in this thesis, each "nucleolus" continues to grow from the time it first appears on

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\*In The Cell in Development and Heredity, E.B.Wilson : 405.  
MacMillan, N.Y., 1925.

the nuclear membrane until the lampbrush chromosomes retract their lateral loops; and at the end of oogenesis all "nucleoli" move toward the centre of the nucleus, diminish in size and number, and become tightly packed around the condensed chromosomes. According to Callan & Gall (personal communication) free "nucleoli" as well as those which are attached to the nuclear membrane incorporate tritiated uridine rapidly; but labelling of "nucleoli" is not necessarily preceded by labelling of the lampbrush chromosomes. However, Callan & Gall have also found evidence of an intracellular "store" of uridine, and probably of other metabolites, from which the precursors of lateral loop synthesis are drawn. If such a store were located in the "nucleoli" then by teleological reasoning one might explain the concurrent increases in "nucleolar" size and lateral loop activity, and the decrease in "nucleolar" size and number after the lateral loops regress. As an alternative theory of "nucleolar" function and fate I suggest that "nucleolar" material enters and is retained in the nucleus in readiness for some event which follows the breakdown of the nuclear membrane.

Three things happen in an immature egg after the contents of the germinal vesicle have dispersed in the cytoplasm. The first meiotic spindle forms, morphogenetic substances are distributed to various parts of the cytoplasm, and changes take place in the egg cortex which render the egg fertilisable (Lillie & Just 1924, p.478). While there is no direct evidence to show

that "nucleolar" material is involved in any of these events, there is circumstantial evidence which ought not to be ignored. Guyénot & Danon (1953) state that in the larger oocytes of Rana

"Les nucléoles sont arrondis, de tailles variées; ils s'accumulent contre un pôle du noyau ..... Ce pôle correspond à la région du cytoplasme où le boyau vitellin a pris le plus grand développement: c'est le futur pôle végétal de l'ovule. Au pôle opposé du noyau, les nucléoles sont rares: ....."

This observation stands by itself and consequently it would be unwise to use it as a basis for speculation. I attach more significance to the fact that "nucleoli" contain all the main ingredients of the "achromatic apparatus".

A single protein which is homogenous upon electrophoresis is said to be the main constituent of the achromatic apparatus (Mazia 1955). "Nucleoli" consist largely of protein (see above). Spindle and asters, like "nucleoli", are rich in RNA (Brachet 1942, Stich 1954, Shimamura & Ota 1956, Mazia 1955). The formation of the achromatic apparatus is said to involve protein-bound -SH groups (Mazia 1955); the latter are present in "nucleoli" although their concentration is unknown. Mazia (1955) excludes the possibility of the germinal vesicle contributing directly to the achromatic apparatus on the grounds that the dry mass of the NUCLEUS of an unfertilised sea urchin egg is less than that of a single mitotic figure. The latter is said to have a dry mass of about  $2 \times 10^{-6}$  mg (Mazia & Dan 1952). The estimated dry mass of a single "nucleolus" isolated from a newt

oocyte is said to be between  $4.8 \times 10^{-9}$  and  $9.1 \times 10^{-9}$  mg. (Brown & Ris 1959). A thousand "nucleoli" would thus give something of the order of  $5 \times 10^{-6}$  mg. of nucleoprotein, which is probably more than enough for the formation of the first meiotic spindle!

Undoubtedly the greatest source of error in cytology is unwarranted generalisation, and in this respect the nucleolus offers enormous scope. Therefore I have not compared the "nucleoli" of amphibian oocytes with those of somatic cells. Had I done so I would have found likenesses, but these alone tell us nothing; they merely confuse the issue, which is that a nucleolus has not yet been identified in an amphibian oocyte.

Lastly, I suggest that the term NUCLEOLUS be reserved for bodies which are homogenous when examined by the light microscope, spheroid, conspicuous in interphase nuclei, few in number, basophilic but Feulgen-negative, and demonstrably attached to a chromosome. Only by adopting a strict definition can we hope to discover the function of the nucleolus and so assess the worth of the doctrine which ascribes to the nucleolus a key role in the synthesis of cytoplasmic protein.

SUMMARY

A study has been made of the effects of hypophysectomy, and of subcutaneous injections of serum and chorionic gonadotrophins and macerated homoplastic pituitary, on whole oocytes, oocyte nuclei and lampbrush chromosomes of the newt Triturus cristatus carnifex.

Hypophysectomy leads to a reduced rate of incorporation of  $^{32}\text{P}$  into whole oocytes and oocyte nuclei of all sizes; large yolky oocytes are more severely affected than are small oocytes lacking yolk. Hypophysectomy also leads to: a decrease in the viscosity of oocyte nuclear sap; changes in the numbers and character of free granules in the nuclear sap; changes in the sizes of objects present at certain loci on the lampbrush chromosomes; and, in particular, decrease in size of the spheres of chromosomes V and VIII. In medium and large oocytes from hypophysectomised newts most peripheral "nucleoli" are firmly attached to the inner surface of the nuclear membrane, whereas in similar oocytes from unoperated newts most peripheral "nucleoli" lie free in the nuclear sap.

Injection of mammalian gonadotrophins or of homoplastic pituitary material leads to a considerable increase in the rate of uptake of  $^{32}\text{P}$  into cytoplasm and nuclei of yolky oocytes; the reaction of oocytes prior to yolk accumulation is similar, but less marked. Gonadotrophin treatment also leads to an

increase in the viscosity of the nuclear sap, a decrease in the numbers and/or sizes of free granules in the nuclear sap, and decrease in the amount of material accumulated at lampbrush chromosome loci which were hitherto thought to be responsible for the production of oocyte "nucleoli". The sphere loci of chromosomes V and VIII show signs of hyperactivity in gonadotrophin treated newts. When gonadotrophin treatment evokes a reduction in the number of free granules in the nuclear sap, there is an accompanying increase in the concentration of RNA in the cytoplasm. After gonadotrophin treatment all "nucleoli" of medium and large oocytes lie free in the nuclear sap.

Each of the features remarked upon above varies from one newt to another. This variability is superimposed upon stable genetic characteristics and upon changes which normally accompany oocyte development. The variability reflects differences in physiological state of the ovaries of different newts. With respect to objects attached laterally to newt lampbrush chromosomes, large size does not necessarily reflect intense metabolic activity. Likewise, large numbers of free granules in oocyte nuclear sap indicate physiological maladjustment rather than physiological hyperactivity.

The theory that peripheral "nucleoli" in newt oocytes are generated at one or more lampbrush chromosome loci has been scrutinised. Objects which are characteristically present at

three loci on the lampbrush chromosome of carnifex, and which were thought to be "attached oocyte nucleoli", often look like peripheral "nucleoli" when viewed in phase contrast, but they differ from peripheral "nucleoli" in certain respects. Free peripheral "nucleoli" possess a shell which is relatively resistant to proteolytic enzymes, and which is recognisable in sectioned "nucleoli" examined with an electron microscope. The objects attached to lampbrush chromosomes have no such shell. Evidence has been sought for the production of "nucleoli" by the hypothetical "nucleolar" organiser loci, but none has been found. "Nucleoli" do not increase in number during the lampbrush phase of oogenesis other than by division of pre-existing "nucleoli". This may occur towards the end of oogenesis. There are usually about 1,000 "nucleoli" per oocyte nucleus of carnifex. These first appear on the inner surface of the nuclear membrane in oocytes of 0.1 to 0.15 mm diameter. When an oocyte is about half grown its "nucleoli" detach from the nuclear membrane. "Nucleoli" grow from the time they first appear until the lampbrush chromosomes retract their lateral loops. In general, sub-species crisatus has fewer and larger "nucleoli" per oocyte nucleus than carnifex, karelinii, or danubialis.

The evidence suggests that in newt oocytes peripheral "nucleoli" are not generated by the lampbrush chromosomes. They are unrelated to the nucleoli of somatic cells.

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-- APPENDIX I --

- THE ACTIONS OF ENZYMES ON LAMPBRUSH CHROMOSOMES -

by

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## 1. INTRODUCTION

The dissection of organic matter by means of enzymes has become a potentially refined operation now that several reasonably pure enzymes are available. The present paper describes the effects produced by the enzymes trypsin, 'pan-protease', pepsin, ribonuclease (RNase) and deoxyribonuclease (DNase) on lampbrush chromosomes isolated from newt oocytes. It also describes the actions of salines of various pH values and ionic strengths on these chromosomes, actions which must be taken into account when we attempt to interpret the effects of enzymes. Furthermore, the inaction of a chelating agent - versene - on isolated lampbrush chromosomes is described.

Because of the key role played by chromosomes in determining schemes and details of biological organisation, interest in their chemical make-up has been for a long time widespread, and many analyses have been attempted. Indeed, if we equate chromosomes with sperm head nuclei, we may date the earliest of these analyses back to the fundamental discoveries made by Miescher in 1871.

Biochemical analyses of sperm head nuclei in bulk have repeatedly shown that the two main constituents are deoxyribonucleic acid (DNA) and arginine-rich protamine, (Felix, 1952). Analyses of interphase nuclei in bulk, and of the so-called "chromosomes" prepared by centrifugation of disrupted interphase nuclei,

have demonstrated that the main constituents of such material are DNA, histone, tryptophane-containing protein and ribonucleic acid (RNA) (Mirsky & Ris 1947, Mirsky 1947). If allowance be made for polyploidy, constancy in the quantity of DNA per interphase nucleus from a given organism, irrespective of the tissue of origin, has been established, and this quantity has been demonstrated as twice that present in sperm head nuclei from the same organism (Vendrely & Vendrely 1948, Swift 1950, Mirsky & Ris 1951, Leuchtenberger 1952, Frazer & Davidson 1953). There is, furthermore, some evidence for the assumption that there is a quantitative parallel between the DNA and histone contents of interphase nuclei (Vendrely & Vendrely 1953, Alfort 1955, Bloch & Godman 1955, Mirsky & Ris 1951).

Inferences concerning the chemical make-up of chromosomes have been drawn from these bulk analyses, and some of these inferences are contradictory, (Mirsky & Ris 1947, Mirsky 1947, Polli 1951). Since interphase nuclei are not just chromosomes, and since there are doubts as to whether Mirsky's interphase "chromosomes" are genuine (Lamb 1950) the contradictions are hardly surprising. If compelling evidence for the chemical make-up of chromosomes is sought, then clearly the observations must be made on chromosomes as such.

No one has yet succeeded in collecting mitotic or meiotic chromosomes, uncontaminated by other cellular components, for bulk analysis; neither has this been achieved for the giant

polytene chromosomes of larval dipteran salivary glands. On the other hand there have been several qualitative analyses of giant chromosomes, both from dipteran salivary glands and from amphibian oocyte nuclei, and some of these studies have included observations of the results of enzymatic digestion. We can at least be sure that such studies relate to genuine chromosomes, but nevertheless they too have given conflicting results.

The particular qualities of lampbrush chromosomes, which enable their enzymatic degradation to be critically observed, were emphasized by Gall (1954) in his paper on Triturus viridescens. Whereas D'Angelo (1946) and other workers have tried but have failed to separate unfixed and undamaged polytene chromosomes from the cytoplasm and nuclear sap of dipteran salivary gland cells, the isolation of lampbrush chromosomes in life-like condition in saline (for justification see page 23) is a simple procedure. The isolated chromosomes may then be observed with an inverted phase contrast microscope, the system first advocated and used by Gall (1954), and provided they lie in a suitable saline their morphology remains unchanged for several hours. Enzymes or other agents can now be applied to such preparations, and any morphological changes which ensue can be observed directly. Because one can dispense with fixatives and stains when studying lampbrush material, artifacts introduced by these aids to observation can be eliminated. Such artifacts have been largely responsible for the conflicting

results obtained by previous workers in this field.

Newts have larger lampbrush chromosomes than other organisms, and hence were chosen for the present study. Lampbrush chromosomes are morphologically heterogeneous, and we wished to take account of this heterogeneity when observing the effects of enzymes. Callan & Lloyd (1960b) have described the morphologies of the lampbrush chromosomes of four geographical races of Triturus cristatus (Laurenti) in some detail, and in the present study we have concentrated attention on selected chromosome regions from newts belonging to three of these races. To avoid a repetition of much 'background' information, the reader is referred to Callan & Lloyd's (1960b) paper. We now follow the terminology of that paper, and hold to the conventions there established when defining particular sites on the chromosomes.

As well as the lampbrush chromosomes, there are other bodies in newt oocyte nuclei which can be seen with a light microscope and which in life, like the chromosomes, are embedded in nuclear sap. These bodies are unavoidable contaminants of preparations of lampbrush chromosomes. They fall into two classes: detached chromosome products: and "nucleoli". Observations on the "nucleoli" are deliberately omitted from the present paper; the "nucleoli" of amphibian oocytes are almost certainly not homologous to the nucleoli of somatic cells, nor in all probability are they detached

chromosome products. Their peculiarities will be discussed elsewhere.

Nuclear sap diluted in saline is also a contaminant of preparations of lampbrush chromosomes. Brown, Callan & Leaf (1950) have demonstrated that amphibian oocyte nuclear sap consists in the main of protein or proteins which, on hydrolysis, yield an extensive range of amino-acids. These findings have been confirmed by Gall (1952). The sap protein is soluble in certain salines which do not dissolve components of the lampbrush chromosomes, and hence contamination with nuclear sap is of little consequence when structural components of the chromosomes are being qualitatively analysed.

## II MATERIALS AND METHODS

The three subspecies of the crested newt Triturus cristatus which have been used in this study are: T.c.cristatus (Laurenti), collected in the south of England and supplied by the dealer L. Haig of Newdigate, Surrey; T.c.carnifex (Laurenti), collected near Naples, Italy, and supplied by Dr. P. Dohrn of the Stazione Zoologica; and T.c.karelinii (Strauch), collected near Istanbul and supplied by Dr. A. Sengün.

Portions of ovaries were removed from anaesthetised newts and stored 'dry' in covered glass containers, the covers being sealed in place with a paraffin oil/vaseline mixture.

Although portions of ovaries stored at about 2°C remain in good condition for several days, in this study we have worked almost exclusively with freshly excised ovary fragments. We have as far as possible standardised our material by choosing for study oocytes from within a restricted size range. Such oocytes are casually referred to as 'half-grown', and measure about 1.0 mm. diameter in cristatus, 0.9 mm. diameter in caruifex, and 1.1 mm. diameter in karelinii. In oocytes of these sizes the great majority of the lateral loops of the lampbrush chromosomes are not at their longest - they are longer in smaller oocytes - but the 'landmark' structures are well developed and permit the ready identification of chromosomes and parts of chromosomes.

Details of the methods used for isolation of oocyte nuclei and for removal of nuclear membranes have already been given by Callan & Lloyd (1960b) where Gall's observation chamber for use with an inverted microscope is also described. Only the bare essentials of our routine procedure will be stated here. Each observation chamber consists of a 3" x 1" microscope slide through which a  $\frac{1}{4}$ " diameter hole has been bored; a number 0 coverslip is sealed to one side of the slide with paraffin wax, across the hole, and this forms the floor of the chamber. A nucleus is isolated and freed from cytoplasm and yolk in a five to one mixture of 0.1 M potassium and sodium chlorides, (the potassium/sodium ratio of five to one

being in accordance with unpublished data of Dr. W.T.W. Potts) and is then transferred to Callan and Lloyd's medium C in an observation chamber for removal of its membrane and for the sap to disperse. Medium C consists of seven parts of the five to one 0.1 potassium/sodium chloride mixture, together with three parts of 0.001 molar potassium dihydrogen orthophosphate. This saline, after sterilisation by boiling, has a pH of 6.2.

The observations which we describe in the present paper were all made on freshly isolated nuclei, and the changes induced by the applications of the various agents were all sufficiently rapid to be ascribable to these agents, not to natural deterioration.

When about 50 mm.<sup>3</sup> of medium C is placed in an observation chamber it forms a meniscus convex upwards. Once the nuclear membrane has been removed from a nucleus lying in the chamber, a small square coverslip is dropped in place over the meniscus, care being taken to avoid trapping air bubbles in the chamber. The preparation is now observed with an inverted phase contrast microscope (Cooke, Troughton, & Simms Ltd., York) giving 'dark contrast' (alternatively known as 'bright field'), and further action is delayed for a few minutes until the nuclear sap has sufficiently dispersed

to leave the chromosomes spread out in one plane over the lower coverslip. Nuclei from oocytes in the size range which we have chosen have diameters of the order 0.35 mm., and hence are of volume approximately  $0.022 \text{ mm}^3$ . Thus when the sap has fully dispersed its dilution is more than two thousand fold.

Each agent whose effects we have studied has been applied by pipette as a drop of solution beside the top coverslip; and we have endeavoured to add the same quantity of fluid -  $50 \text{ mm}^3$  - as that previously present in the chamber. The coverslip rises to accommodate the extra fluid, which then mixes with the original medium C. Description of the control over pH, and further technical details, is deferred to the several chapters devoted to particular agents which we have used. In these chapters the term 'concentration' of an agent, unless otherwise qualified, refers to the ultimate concentration reached after mixing with Medium C in the observation chamber.

To record morphological changes in the lampbrush chromosomes, photographs of the primary image formed by the objective lens have been taken by means of a single exposure camera loaded with Ilford Micro-neg Pan film. For certain series of photographs a Xenon flash served as the light source, and this successfully 'stopped' Brownian movement. However

for series attempting to record rapid changes the time lag between successive exposures, necessitated by recharging of the condenser unit which serves the flash, proved too great; and a conventional light source with a "Compur" shutter of 35 mm. aperture on a vibration-free mount interposed between the light and the microscope condenser was therefore used instead, the exposures given being 1/10th or 1/25th of a second. Although such exposures fail to eliminate blurring due to Brownian movement, they proved adequate.

Series of negatives were processed identically, and so were prints taken from negatives. In this way an impression of changes in the refractility of components of the lampbrush chromosomes, albeit imperfect because of the limitations of the phase-contrast system, has been recorded.

The enzymes pepsin and trypsin were purchased in crystalline form from Armour Pharmaceutical Co.Ltd. (Eastbourne). 'Pan-Protease' was kindly provided by Dr. Daniel Mazia, who visited our laboratory in 1959 and suggested that we compare its action to that of trypsin alone. Ribonuclease (RNase) and deoxyribonuclease (DNase) were first obtained from Nutritional Biochemicals Ltd. (Cleveland, Ohio, U.S.A.), later from L. Light & Co. (Colnbrook, Bucks, England). The chelating agent versene

(diamino-ethane-tetra-acetic acid, disodium salt) was obtained from British Drug Houses Ltd.

Measurements of pH were taken with a Cambridge Bench type meter using a glass electrode.

### III SELECTED CHROMOSOME REGIONS

The structural organisation which is common to all lampbrush chromosomes has been discussed in several recent papers, (Callan 1955, Callan 1957, Callan & Lloyd 1960a, Duryee 1950, Gall 1954, Gall 1956), and, more particularly related to this paper, the lampbrush chromosomes of Triturus cristatus have been described by Callan & Lloyd (1960b). In the present paper attention will be confined to those aspects of the structural organisation on which the enzyme work directly bears.

Each oocyte nucleus of T. cristatus contains twelve pairs of lampbrush chromosomes, homologous chromosomes being associated at one or more points so that they form bivalents. There is a series of granules, chromomeres, in each lampbrush chromosome axis, neighbouring granules being connected to one another by a thin, extensible and elastic fibre. Pairs or multiples of pairs of loops project laterally from the overwhelming majority of chromomeres, and most of these lateral loops conform to the type described by Callan & Lloyd as "normal". Such loops have a fine

fibrous texture, the fibres appearing to project radially from a denser loop axis. Throughout the present paper we have applied the term "matrix" to this fibrous material. There is a gradation in the amount of matrix surrounding loop axis as one follows a loop out from its "parent" chromomere and through its entire length back to the same chromomere, one end of the loop being bare of matrix.

When not impeded by nuclear sap and when not denatured, normal lateral loops in saline show violent Brownian movement. Brownian movement is disadvantageous for photographic recording, but in other respects it is an asset; whereas undamaged normal lateral loops retain their linear continuity despite the stresses to which they are evidently exposed, breaks produced by any agency are immediately apparent; and the independent movements of the matrix fibres show that these fibres are each attached to loop axis, not to one another.

Some normal lateral loops are present in all the regions which we have selected for special study, but also included in these regions are other objects having distinct morphologies, objects which have been used by Callan & Lloyd as 'landmarks' for chromosome identification. A working map of the lampbrush chromosomes of T.c.carnifex, showing the relative lengths of the twelve members of the complement, the positions of their centromeres and of the major landmarks is given in Text-fig.1. Where the positions of landmarks are described in terms of units, a unit is defined as being 'one-hundredth part of the length of chromosome V, or its equivalent in length on another

chromosome" (Callan and Lloyd 1960b). On the working map, the longer arm of each chromosome is drawn projecting to the left thus defining the 'left hand end' of each chromosome. The selected chromosome regions will now be detailed.

T.c. carnifex: middle of chromosome II

The left end of this region is defined by 'marker' loops at 56 units, the right end by a large granule, not associated with conspicuous lateral loops, which lies in the chromosome axis at 78.5 units. The marker loops are stiff and granular in texture, and there is a particularly conspicuous granule at the point where they are attached to the chromosome axis. Through the mid region of chromosome II dense 'lumpy' structures hang at intervals laterally from the axis, the largest of these being situated at 61.5 and 69.5 units. The lumpy objects may be fused together in various ways: within the length of one and the same chromosome, producing reflexions of its axis; or between partner chromosomes, not necessarily at homologous loci. The centromere lies between the largest of the lumpy objects, at 65 units, and in carnifex, as in cristatus, it is a smooth, round dense granule which lacks lateral loops.

T.c. carnifex: left arm ends of chromosomes V and VIII

"Spheres" are the striking features of these two regions, there being spheres at 2 and 11 units on chromosome V and a single sphere at 3.5 units on chromosome VIII. These

objects are smooth and round with firm outlines, they may reach up to 15 $\mu$  in diameter, they may be homogeneous in texture or they may contain one or more vacuoles, (figs. 5 and 8 ). During their formation spheres are tightly attached to chromosome axes, but detached spheres are often also present in newt oocyte nuclei and are the most conspicuous and easily recognised of freed chromosome products. Two chromosome sites concerned with sphere formation may both be connected to a single sphere, and such "sphere-fusions" may associate homologous or non-homologous sites. The left arms of chromosomes V and VIII end in telomeres, smooth, round, loop-free structures which are characteristic of the ends of all lampbrush chromosomes. Telomere fusions are common: usually they associate homologous ends, but occasionally the fusions are non-homologous.

T.c. carnifex: giant loops of chromosome XI

These massive structures lie in the left arm of chromosome XI at 24 units. They have well defined but somewhat irregular outlines. They are formed of stiff, highly refractile and often vacuolated material, (figs. 11 and 13). The degree to which they maintain the form of loops is variable from oocyte to oocyte; the loop form may be partially or totally obliterated by fusion, fusions occurring within single loops, between sister loops or between homologous loops. Fusions can also take place between these loops and structures of similar

texture which in certain newts are also present on chromosomes X and XII. In larger oocyte nuclei big, roughly spherical bodies may detach from the giant loops and for a time whilst free in the nuclear sap they conserve the texture of the parent structures; but oocyte nuclei of the size with which we are now concerned generally lack free products from this source.

T.c.cristatus: left arm end of chromosome XII.

The landmark to which we have given particular attention in this chromosome region is a pair of giant granular loops at 9 units. These loops, each of which may exceed 100 $\mu$  in overall extended length, (Figs.41 and 52) are a feature of subspecies cristatus; they are absent from the other subspecies. Each giant granular loop shows a striking gradation in form along its length. At one end a thin strand leaves the chromomere of origin; this becomes thicker and twisted in a spiral; then follows a zone of variable extent where refractile rods are wrapped around, or project radially from, the loop axis; the rest of the loop, two thirds or so of its length, consists of granules or strings of granules attached by very thin fibres to the loop axis, the granules progressively diminishing in size as the other end of the loop is approached. The axis of the giant granular loop is usually not visible in a freshly made preparation, but with continuing dispersal of nuclear sap (or possibly due to the solution of some constituent of the loop itself) the axis becomes evident after several hours. The

granules or strings of granules in this loop show independent Brownian movement but they do not scatter; this is our main justification for claiming that they are attached by fine fibres to the loop axis.

When describing experiments involving the granular loops we have used the term matrix as referring to the fibrous material attaching granules to the loop axis, and to any other diffuse material which may be accumulated in the spaces between fibres and granules. Although the giant granular loops are exceptional and readily identifiable lampbrush loops, they are exceptional in one respect only - their great size. As Gall (1964) found also in Triturus viridescens, many smaller lateral loops of all subspecies of T. cristatus carry refractile granules which show independent Brownian movement, and the thinner insertions of lateral loops of diverse morphologies are not infrequently wound in tight spirals.

The left arm of chromosome XII terminates in a typical telomere; all the telomeres of cristatus chromosomes are larger than the comparable structures of other subspecies, and telomere fusions are more frequent.

T.e. karelinii: centric regions

The centromeres of all karelinii lampbrush chromosomes are flanked by dense "axial bars" which lack lateral loops. This is a feature which karelinii does not share with other

subspecies of T. cristatus, though Gall (1954) has described similar structures in the lampbrush chromosomes of T. viridescens and Amblystoma tigrinum. The axial bars increase in length as oocytes grow in size, and Callan & Lloyd (1960b) have demonstrated that this results from progressive incorporation of the substance of neighbouring chromomeres, which withdraw their lateral loops as they amalgamate with the axial bars. Most chromomeres are so tiny that their reactions to agents are difficult to observe. For our purposes the axial bars of karelinii are conveniently large aggregates of chromomere material, and moreover they betray the positions of the centromere granules, which in other subspecies are often difficult to find. Though we have not observed centromere granule fusions in cristatus or carnifex, centromere fusions are commonly present in karelinii bivalents and the nature of these attachments is of some interest. Furthermore in regions relatively close to the centromeres of karelinii chromosome II, III, IV, V, VI, and VII one frequently encounters fusions between what look like, at first glance, unusually large chromomeres. These structures, called "axial granules" by Callan & Lloyd, may fuse between homologues at homologous sites; but they may also fuse non-homologously, and when two axial granules on the same chromosome are fused to one another a "reflected" portion of the chromosome axis results. We have studied the actions of enzymes on such fusions in an attempt to

discriminate between those which do, and those which do not include chiasmata.

#### IV THE STAINING OF FIXED CHROMOSOMES

Although the fixation and staining of lampbrush chromosomes does not fall within the title limits of this paper, we include a chapter on the subject because it is complementary to the enzyme study.

Gall (1952) has shown that formaldehyde vapour and osmic acid vapour fix isolated lampbrush chromosomes in a reasonably adequate state, though both these agents cause chromosome axes and lateral loops to contract, stiffen and increase in refractility. We have fixed exclusively with formaldehyde vapour. Fig. 1 shows bivalent XI of T.c.karelinii unfixed, and fig.2 shows the same bivalent after 15 minutes exposure to formaldehyde vapour.

Fixation in the literal sense, i.e. attachment to a glass surface, is necessary if isolated lampbrush chromosomes are to be studied as stained preparations. To ensure attachment we have allowed oocyte nuclear sap to disperse for at least thirty minutes in medium C before placing slides in a formaldehyde vapour chamber. During fixation the slides must not be disturbed, otherwise the chromosomes fail to attach to the coverslip below. After one hour in formaldehyde vapour all fixed chromosomes preparations were placed in 10% neutralised formalin solution for twelve hours or more.

A. Feulgen's reagent and light green

After fixation, each observation chamber was washed in water, and then placed in normal hydrochloric acid at 60°C. for 6 minutes hydrolysis. During hydrolysis the coverslip drops off the bored slide and bears the lampbrush chromosomes provided they were firmly attached during fixation. Each coverslip was recovered and placed for 2 hours in Feulgen's reagent (prepared according to Coleman's (1938) recipe), rinsed twice in "SO<sub>2</sub> water", rinsed in water, taken up through alcohols to 70%, counterstained for 3 minutes in 0.2% light green in 70% alcohol, dehydrated, cleared and mounted in DePeX (G. T. Gurr, London).

We find that the chromomeres of lampbrush chromosomes stain with Feulgen's reagent, and thus confirm the observations of Brachet (1940), Dodson (1948), Guyénot and Danon (1953), and Gall (1954). Furthermore we confirm Gall's (1954) finding (made on T. viridescens) that centromere granules and the "axial bars" adjacent to the centromeres of T.c. karelinii are also Feulgen-positive. None of the structures attached laterally to chromosome axes appear Feulgen-positive, though these structures survive the mild hydrolysis prerequisite for the Feulgen reaction and stain with light green. Feulgen/light green staining reveals that telomeres and axial granules are compound structures, despite the fact that when observed in phase contrast unfixed they may appear like large chromomeres.

Each telomere and axial granule consists of a crescent-shaped Feulgen-positive part closely applied to a spherical or nearly spherical mass staining with light green. Figs. 3 and 4 show part of bivalent XI of karelinii (the same bivalent is illustrated unfixed in Fig. 1) from a preparation stained in Feulgen and light green. Figure 3 was photographed using Feulgen's reagent developed with formaldehyde as a filter, and shows the distribution of light green stained material. Figure 4 was photographed using a solution of light green as a filter; although the light green stained lateral loops are visible in the photograph, the Feulgen-positive axial structures (particularly the bars adjacent to the two centromeres) can be distinguished.

B. Toluidine blue with and without previous digestion by ribonuclease

Formalin-fixed slides were washed in water and the coverslips bearing lampbrush chromosomes were then prized off. Some of these preparations were stained for 40 minutes in a 0.2% solution of toluidine blue (G. T. Gurr, London) in 0.01 molar phosphate buffer at pH 5.4, then washed in similar buffer, run quickly through an alcohol series, cleared and mounted in DePeX. In these circumstances all components of the chromosomes, both axial and lateral, stained with toluidine blue, staining intensities being roughly in accordance with the refractivities of the various structures as observed unfixed in phase contrast.

Other preparations prior to staining were incubated

for two hours at 37°C. in ribonuclease (Nutritional Biochemicals) at 0.2 mg./ml. dissolved in 0.01 molar phosphate buffer at pH 6, with controls incubated in buffer only. The staining of control preparations resembled in all particulars that of unincubated preparations. Preparations incubated with ribonuclease showed normal staining of chromomeres and, surprisingly enough, of the spheres of chromosomes V and VIII, but markedly reduced staining of all other lateral structures.

Gall (1954) found that ribonuclease pre-treatment entirely eliminated - not merely reduced - the toluidine blue-staining of lateral loops; this discrepancy between our results is probably due to the use of stains from different sources. Our findings are otherwise in accordance - Gall found that the stainability of the "knobs" of T. viridescens is unaffected by ribonuclease, just as we have found for the spheres of T. cristatus - and they demonstrate that all lateral components of lampbrush chromosomes, spheres (= knobs) excluded, contain RNA.

#### C. Fast green FCF

We have used the method of Alfert and Geschwind (1953) for detecting basic proteins by means of fast green FCF at a pH between 8 and 8.5. Formalin-fixed slides were immersed in boiling trichloroacetic acid for 30 minutes so as to remove both DNA and RNA from the chromosomes. During this procedure the coverslips drop off the bored slides. Each coverslip was recovered, washed in 70% alcohol, rinsed in water and stained

for 30 minutes in a 0.1% solution of fast green FCF (National Aniline, U.S.A., Cert.No.N.G.F.7)\* in 0.2 molar disodium hydrogen phosphate, pH 8.2. Preparations were subsequently washed in buffer, transferred direct to 95% alcohol, dehydrated, cleared and mounted in DePeX. In such preparations only the chromomeres are stained and thus, if the method be sound, basic proteins are restricted to these axial components of lampbrush chromosomes.

#### V. OBSERVATIONS ON UNFIXED CHROMOSOMES

Before considering the actions of special reagents on lampbrush chromosomes, we must take note of how these chromosomes react to variations in saline pH and concentration. Ideally one would wish to start with knowledge of the appearance of lampbrush chromosomes in their natural surroundings, inside intact, living oocytes. Unfortunately lampbrush chromosomes are not visible in living oocytes; they become visible when the nuclear sap is diluted with saline, but we now face the problem of assessing whether their state is life-like. The problem is not so intractable as it at first appears. We can recognise degrees of leaching out of components of lampbrush

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\* We are indebted to Dr. O. L. Miller of the Zoology Department University of Minnesota, for supplying a sample of National Aniline fast green FCF. Dr. Miller (personal communication) has used Alfert & Geschwind's method on the lampbrush chromosomes of T.viridescens with results comparable to our own. Our earlier experiments using G.T. Gurr's fast green gave contradictory results - all chromosome components staining.

chromosomes, when the nuclear sap has been greatly diluted, by reduction in the contrast of their phase contrast images and by enhanced Brownian movement of the lateral loops; moreover we can recognise when lampbrush chromosomes have been fixed (see page 19). We have taken as our criterion of a life-like state that, with the nuclear sap dispersed, the chromosomes should show maximum optical contrast consonant with the maintenance by normal lateral loops of a supple and relaxed form. Though this criterion is not entirely objective, it has practical value.

A. Effects of varying pH

When studying the effects produced by pH alteration, medium C, itself of pH 6.2, was used as the initial sap dispersing saline in each observation chamber. In this medium, lampbrush chromosomes maintain a life-like state for many hours. The pH was subsequently altered by pipetting a "modified" C medium around the top coverglass of the preparation; mixing follows immediately. Modified C media ranging in pH from 6.2 to 8 were prepared by replacing 0.001 M  $\text{KH}_2\text{PO}_4$  with an equal fraction of 0.001 M  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer. Modified C media ranging from 8 to 10 in pH were obtained by adding small quantities of NaOH to ordinary C medium, whilst for pH values from 6.2 to 1.8 small quantities of HCl were similarly added. The quantities of NaOH or of HCl so added were too small to have significantly altered the ionic strengths of these media.

The pH reached after mixing of media was not measured directly within each observation chamber but was predicted from tests of media mixed on an aliquot basis.

As pH is increased within the range 6.2 to 8, lampbrush chromosomes examined under phase contrast show progressively less and less contrast, (figs. 5, 6 and 7) but no other physical change. Once the pH has risen above 7, back adjustment to pH 6.2 fails to restore the original contrast, though in other respects the chromosomes remain life-like. Thus although no visible aggregates detach from lampbrush chromosomes between pH 7 and 8 we may assume that some loop matrix constituents pass into solution.

Above pH 8 there is more evident solution of normal loop matrix. The widths of normal loops are reduced, previously discernable gradations in width are largely obliterated, and increased violence of Brownian movement shows that such loops have lost substance. As their matrix dissolves, normal loops contract in length and instead of projecting from chromosome axes in smooth, sweeping curves they become irregularly bent and twisted, an observation which suggests that in their natural state the loop axes of normal lateral loops are stretched out by accumulating matrix. The denser landmarks are less strikingly altered, though by pH 8.5 they too show significant loss of contrast.

At some pH between 8.5 and 9 there is a sudden fall in

the contrast of lampbrush chromosomes, followed by their disappearance. Once having disappeared from view even fixation in formalin vapour fails to make the chromosomes visible again. We must conclude that they have totally dissolved.

Let us now turn to media more acid than pH 6.2. Between pH 6.2 and 5.8 lampbrush chromosomes do not alter in appearance, but at some pH between 5.8 and 5.4 the chromosomes stiffen, contract and increase sharply in contrast, (Figs.8 and 9). This is the easily recognisable 'fixed' condition, lampbrush chromosomes taking on the same appearance when exposed to formaldehyde or osmic acid vapours, or to calcium ions at molarities above  $10^{-3}$ . At this same pH the nuclear sap, previously dispersed in saline, precipitates. Once fixed by low pH, back adjustment to pH 6.2 fails to restore lampbrush chromosomes to a life-like state. The fixed appearance is maintained in media down to pH 2.5, and to still lower values if the rate of fall of pH is slow. If on the other hand pH is rapidly lowered, then at some value between 2.5 and 2 there is a striking change in the appearance of lampbrush chromosomes. From a previously fixed state, all chromosome components become relaxed, swollen and of reduced contrast, (Figs.9 and 10). The relaxed condition persists provided pH is held below 2, but if pH is raised above 2.5 the fixed state reappears; this is now irreversible.

B. Effects of varying saline concentration

When oocyte nuclei are isolated in an unbuffered 5:1 mixture of molar KCl and NaCl, the nuclear sap clarifies and hydrates and the nucleus swells, whilst a component of the sap investing the chromosomes, called by Callan (1952) the "structural colloid" and by Duryee and Doherty (1954) the "chromogel", sinks to the bottom of the nucleus. After the nuclear membrane has been removed this structural colloid may take up to three hours to disperse. The lampbrush chromosomes first become visible as ragged strands of low contrast: they lack lateral loops and all landmarks, and do not display chromomeric patterns. As dispersal of the structural colloid continues, the chromosomes' axes fragment and three hours after isolation in molar saline a cloud of tiny granules in violent Brownian movement is all that remains of the original chromosome complement.

0.5 molar K/NaCl is slightly less destructive to lampbrush chromosomes. About two hours after isolation of a nucleus, when the structural colloid has dispersed, occasional short lengths of axis show signs of chromomeric pattern, but lack all traces of lateral loops. The spheres of chromosomes V and VIII are the only landmarks remaining recognisable in saline of this strength.

In 0.25 molar K/NaCl the structural colloid disperses within 30 minutes of isolation, revealing unbroken lampbrush

chromosomes of low contrast. The chromosomes carry lateral loops but these have suffered so much loss of substance that the various types, including landmark loops, are indistinguishable from one another. The spheres of chromosomes V and VIII, alone amongst the landmarks, are recognisable. Chromomeres are visible, but swollen. The appearance of lampbrush chromosomes in 0.25 molar saline remains unaltered for several hours after isolation, and the fact that chromosome axes do not break in this medium must be stressed.

In 0.1 molar K/NaCl the nuclear sap remains stiff and slightly opaque for several minutes after isolation. The nuclear membrane can be peeled off and thereafter the sap disperses, allowing the chromosomes to spread out over the lower coverslip. Sap dispersal is well advanced within 20 minutes of removal of the nuclear membrane, but we would emphasise that saline of this strength, in other respects a satisfactory isolation medium, usually fails to bring about complete dispersal of the structural colloid. A thin layer of this material persists between the chromosomes and the coverslip forming the bottom of the observation chamber, interfering with critical observation and preventing the chromosomes from spreading in one focal plane. In 0.1 molar K/NaCl the normal loops of lampbrush chromosomes are of relatively high contrast, the chromomeres are also dense and highly refractile. Lampbrush chromosomes are well preserved in this medium; days may elapse before there is perceptible loss of substance from normal loops.

Medium C has an approximate molarity of 0.07 but unlike other salines discussed in this section it contains a low concentration of  $\text{KH}_2\text{PO}_4$  and has a pH of 6.2 after sterilisation by boiling. Medium C produces conveniently rapid dispersal of the sap, including the structural colloid, after removal of the nuclear membrane. The chromosomes spread and come to lie flat on the bottom of the observation chamber. All their features are well preserved for many hours and we have noticed only slight deterioration in preparations kept in medium C at 2°C for up to two weeks.

We have isolated oocyte nuclei and removed their nuclear membranes in unbuffered K/NaCl solutions of 0.05, 0.005 and 0.001 M. Finally we have examined the responses of nuclei and chromosomes to isolation in distilled water, (the latter is always slightly contaminated by saline pipetted into the observation chamber with the nucleus). As the salt concentration of the medium is lowered the nuclear sap hydrates more and more quickly and components of the lateral loops go into solution. The matrix of normal loops is most sensitive to low saline concentrations, dissolving in media which are less than 0.05 M. The spheres of chromosomes V and VIII and the giant loops of chromosomes X, XI, and XII are the most resistant extra-axial structures. In distilled water solution of normal loop matrix is almost instantaneous and within a few minutes of isolation only chromosome axes, still structurally continuous but of very low

optical contrast, remain. Of all landmarks, spheres alone survive treatment with distilled water. Chromomeres swell but persist as such and fusions involving axial or extra-axial components are unaffected. Dr. J.G.Gall (personal communication) has studied the appearance of isolated lampbrush chromosomes in pure distilled water and has found that more than 24 hours after isolation the chromosome axes are still recognisable and structurally continuous.

### C. Trypsin

The optimum pH-for tryptic digestion is between 7.5 and 8.5. We have worked with trypsin initially dissolved in ordinary C medium at 0.5mg/ml., the pH remaining at 6.2; and dissolved at a like concentration in modified C medium, with pH adjusted to 8.05 by replacement of the  $\text{KH}_2\text{PO}_4$  fraction with  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer at 0.001 molar.. After application to a preparation of chromosomes in ordinary C medium, both solutions give an effective enzyme concentration of 0.25mg/ml. the former at pH 6.2, the latter at pH 7.8.

#### 1. At pH 6.2

The first sign of tryptic action on unfixed lampbrush chromosomes is a breakdown of the matrix of normal and granular loops - a process which we have been able to follow in some detail on the giant granular loop of chromosome XII of cristatus.

During the first three minutes of enzyme action the matrix of this loop is progressively dissolved and the granules released and scattered by Brownian motion. Solution of matrix and release of granules starts at the thick end of the loop and progresses towards the thin end, until an extremely slender but clearly unbroken loop axis, surrounded by a mass of rapidly dispersing granules, is all that remains of the original structure, (figs.41 to 44). The fate of individual granules is uncertain; we have watched them for periods of up to two hours after detachment and although they are reduced in size, they are not totally destroyed. All dense landmarks similarly undergo a slow reduction in size and are rarely completely dissolved, (figs.11 and 12). As a rule, no fusions of any sort are broken by the prolonged action of trypsin at pH 6.2. On four occasions, however, we have witnessed the complete and rapid destruction of the giant loops of chromosome XI of carnifex (figs.13 to 15). On one of these occasions all four giant loops of one bivalent were fused together prior to application of the enzyme. Trypsin totally dissolved the mass of material involved in this fusion and the two chromosome arms drifted apart where they had originally been joined: only shrunken loop axes remained to mark the sites of the original loops. After about 30 minutes of tryptic digestion at pH 6.2 the chromosomes' axes lose some optical contrast. The chromomeres swell and coalesce, giving the axis the appearance of a more or less uniform cylinder.

This effect is considered in more detail in the chapter on pan-protease.

## 2. At pH 7.8

The effect of trypsin at alkaline pH is similar to its action at pH 6.2, but more rapid and more drastic. Application of trypsin solution is followed in a matter of seconds by an explosive disruption of the matrix of normal and granular loops, accompanied by an equally rapid disappearance of all granules. Loop axes, however, remain unbroken. Dense landmark structures are slowly reduced in size but are not completely dissolved, (Figs. 17 to 26). After an hour the chromosomes are almost invisible (Figs. 21 and 26) but their linear continuity can be demonstrated by adding a drop of 4% formaldehyde to the preparation, so increasing their refractility (Fig. 22). We have not observed breakage of any sort of fusion by the action of trypsin at pH 7.8.

Roth (1960), in a study of the interaction of proteolytic enzymes and ribonucleoprotein, found that all samples of trypsin which he tested were contaminated with RNase. The action of RNase on lampbrush chromosomes can be distinguished from the action of trypsin and since RNase, unlike trypsin, retains its activity after boiling, we were able to test our sample of trypsin for RNase contamination. C medium containing trypsin at 0.5 mg./ml. was boiled for 15 minutes, allowed to cool and its pH checked (it remained at 6.2), and was then applied to preparations of lampbrush chromosomes. The test gave unequivocal evidence of RNase contamination, which we judge to be of the order 1-5% by comparison with the rates of action of known concentrations of RNase on similar material.

#### D. Pan-protease

This enzyme preparation is listed in pre-1959 Worthington catalogues as "crude protease". More recently it has been given the name "pan-protease" and has been assayed to contain 4,000 chymotryptic units and 7,000 tryptic units per milligram (using benzoyl-L-arginine-ethyl ester as substrate). As with trypsin, we have studied the action of pan-protease at an effective concentration of 0.25mg./ml. dissolved in ordinary C medium at pH 6.2 or in C medium at pH 7.8.

##### 1. At pH 6.2

The action of pan-protease on lampbrush chromosomes is just like that of trypsin. During the first ten minutes of enzyme action the matrix of normal and granular loops is dissolved, loop granules are scattered by Brownian motion, whilst dense landmark structures are slowly reduced in size. Each chromosome takes on a "fuzzy" appearance. After about 50 minutes the chromomeres begin to lose their refractility, they swell and coalesce with a consequent loss of the normal chromomeric pattern.

##### 2. At pH 7.8

pan-protease works more rapidly at alkaline pH and after one hour lampbrush chromosomes reach a stable, almost invisible, state. Their chromomeres hydrate, like those of salivary gland chromosomes digested by trypsin in the presence of electrolytes (Kaufmann 1952), and as though in life they owe their compactness to some bonding material which is removed by pan-protease. The axial bars flanking the centromeres of karelinii lampbrush chromosomes

provide convenient material for studying this process, and Figures 27 to 32 show the main features of pan-protease action at pH 7.8 on the centric regions of bivalent XII. When all but the last two photographs of this series were taken, the chromosomes were floating free in the medium and were thus subjected to violent Brownian bombardment. During pan-protease digestion the axial bars show some elongation as well as transverse swelling (Text-fig.2), whilst the centromere granules remain relatively unaffected. In the later stages of digestion all that remains of the axial bars is a fine fibrous material, which nevertheless still maintains its lengthwise coherence. When fixed in formaldehyde vapour after two hours of pan-protease digestion this fibrous material and the adjacent centromere granule give a faint but decidedly positive Feulgen reaction. Centromere fusions in karelinii persist even after prolonged treatment with pan-protease. Fusions between giant loops, spheres, axial granules and telomeres also persist though these bodies may be reduced to less than 5% of their original volumes. No interchromomeric breaks are produced by pan-protease. Moreover we have noted that in chromosome regions which were accidentally stretched during preparation, and which remained under tension due to occasional attachments to the glass surface below, this enzyme failed to weaken intrachromomeric bonding, the mechanical disruption of which normally leads to the production of "double bridges" (Callan, 1955). As with trypsin, tests with boiled pan-protease showed that this enzyme preparation is contaminated with RNase.

E. Pepsin

The optimum pH for peptic action is between 1.5 and 2.5. Our test solutions were made up to give an effective enzyme concentration of 0.25mg/ml. in ordinary C medium at pH 6.2 and in C medium adjusted to pH 1.8.

1. At pH 6.2

The action of pepsin at pH 6.2 on lampbrush chromosomes is mild in comparison with that of trypsin or pan-protease. A rapid disintegration of normal and granular loop matrix with a dispersal of loop granules by Brownian motion, is followed by a sbv reduction in the sizes of dense landmarks. At this pH pepsin appears to have no effect upon chromomeres or upon the axial bars of karelinii.

2. At pH 1.8

Our observations on the effects of pH variation alone need to be borne in mind whilst assessing the action of pepsin at pH 1.8. To recapitulate, a drop in pH much below 6.2 produces an initial contraction and sharp increase in optical contrast of lampbrush chromosomes followed, at pH 2, by an extension and loss of refractility. The latter state persists provided the pH remains low. For convenience we have used the word "fixed" to describe the contracted, refractile state, and the word "relaxed" to describe the state of extension with low optical contrast.

The sequence of events accompanying a drop in pH from 6.2 to 2 is different if pepsin is present. The relaxed condition sets in as expected, but it lasts for only a few seconds. Thereafter the chromosomes contract again, stiffen, stick to the glass bottom of the chamber, and become highly refractile. Peptic digestion begins at this point and all structures attached laterally to lampbrush chromosomes are slowly reduced in volume (Figs. 33 and 34). After six hours of peptic digestion at pH 1.8 there may be long stretches of chromosome consisting only of chromomeres and a tenuous interchromomeric thread. The thread is not always visible but its presence can be inferred when two chromomeres are attached to the glass and have one or more unattached chromomeres between them. The unattached chromomeres show Brownian motion but remain anchored to their neighbours (Text. fig. 3).

Under these experimental conditions our primary aim is being frustrated, and we are forced to witness the action of pepsin on fixed chromosomes. In an attempt to circumvent this difficulty we have adopted a two-stage approach. Pepsin in C medium at pH 6.2 is allowed to act for 10 minutes in a preparation of lampbrush chromosomes, and only thereafter is pH dropped to 1.8. During a relaxed phase, which lasts for about five seconds after the drop in pH, there is a spectacular solution of gene products. Spheres, axial granules and the matrices of

all lateral loops are stripped from the chromosomes. Yet in spite of the violence of peptic digestion when allowed to proceed in this way, the axes of lampbrush chromosomes remain unbroken. Moreover, intrachromomeric breaks are not produced.

Under these conditions the fate of fusions between lampbrush chromosomes is of particular interest. Sphere fusions on chromosomes V and VIII of carnifex are always broken (Figs. 35 to 38), each place where a sphere was originally located being later recognisable as a gap in the chromosome axis traversed by a thin fibre.

Fusions between axial granules fall into two categories which can often be discriminated in fresh undigested preparations. In one type (Text-fig.4a) the two chromosomes are associated by a granule which they share in common, but their axes are not otherwise in contact. Such fusions are disrupted by pepsin at low pH. In the other type of fusion (Text-fig.4b) the two chromosomes may or may not share a common axial granule but their axes are intimately associated. These latter are not disrupted by pepsin even though the granules are dissolved: in all probability chiasmata are involved in such fusions.

Telomere fusions are not disrupted by peptic digestion, whether the associated telomeres be homologous or not. This is a rather surprising observation since in fresh undigested preparations it is often possible to see two terminal chromomeres

apparently separate from one another but both imbedded in a round mass of telomere material. The telomere material is dissolved by pepsin, but a thin thread joining the terminal chromomeres and bearing a tiny thickening midway along its length is left exposed (Figs. 39 and 40).

Centromere fusions in karelinii are also not broken by pepsin. In the case of bivalents from relatively large oocytes the fused centromere granules are themselves large and are usually so imbedded in axial bar material that the whole association is a stiff and compact structure. It remains so throughout the duration of peptic digestion. In the case of bivalents from small oocytes, where constrictions separate the axial bars from the centromere granule, the constrictions may lengthen during peptic digestion, (Text-fig.3), yet they do not break. When the medium containing such a bivalent is disturbed, all four chromosome arms may move relative to one another but they remain firmly attached at the fused centromeres.

Reflected fusions, such as occur so frequently on chromosomes III, IV, and VI of carnifex, likewise resist peptic digestion. This is another surprising observation, since such fusions are necessarily between genetically non-homologous structures and thus presumably of gene product rather than of primary genetic material.

The role played by pepsin, when this enzyme is applied

before lowering pH, can be gauged by comparing its action with that of trypsin similarly applied. After tryptic digestion for a short period at pH 6.2, lowering of pH to 2 likewise results in stripping of the matrices of all lateral loops and the breakage of sphere fusions, but the chromosomes remain relaxed. It is unlikely that trypsin has much enzymatic activity at pH 2, hence we must assume that the effect of lowering pH is direct i.e. that both peptic and tryptic digestion of lampbrush chromosomes at pH 6.2 renders loop matrices and other gene products acid-soluble.

Unlike trypsin and pan-protease, boiled pepsin proved to be inactive, and hence we can infer that our sample was not contaminated with RNase.

#### F. Ribonuclease

The optimum pH for RNase action is 7.7 (Kunitz 1940) but spontaneous degradation of the enzyme takes place at this pH. RNase is nevertheless active at pH 6.2 and we have therefore used this enzyme, at an effective concentration of 0.25mg./ml. in ordinary C medium.

The first observable effect of RNase at pH 6.2 on the giant granular loop of chromosome XII of cristatus is similar to that of trypsin, pepsin or pan-protease at the same pH. Figures 41 to 44 are part of a photographic record to show the

first 6 minutes of RNase action on this loop. The series would equally serve to demonstrate the early action of a proteolytic enzyme on this structure. Fig.42 was taken 3 minutes after the application of RNase solution. The enzyme has begun to act upon the loop matrix and two granules, attached to one another by a thin fibril have come adrift. The loop axis can be seen at the thick end of one loop. In Fig.43, taken 2 minutes later, more loop matrix has gone into solution, granules are being scattered by Brownian motion, and the loop axis is clearly visible. Fig.44 was taken 6 minutes after the start of RNase action. The loop axis has been stripped of its matrix and granules for over half its length. "Stripping" progresses towards the thick end of the loop, leaving the axis bare and limp, but unbroken. In the long run, RNase seems to remove less material from normal loops and from landmarks than does trypsin, pepsin or pan-protease (cf. Figs.46 and 12). The giant loops of chromosomes K, XI, and XII of carnifex first become increasingly vacuolated; later they slowly shrink to about half their original volumes. As normal loop matrices are dissolved by RNase, loop axes are exposed; these contract and increase in refractility. There is also some contraction of the main axes of chromosomes, but no loss of chromomeric pattern and no disruption of fusions even if pH is subsequently lowered to 2. The linear continuity of lampbrush chromosomes remains undisturbed by RNase, and is equally maintained if tryptic digestion follows RNase.

The rate of RNase action on lampbrush chromosomes appears roughly proportional to the concentration at which the enzyme is used. The final state reached by the chromosomes is the same over a range of RNase concentrations from 0.25mg. to 0.000025mg./ml. Some evidence of RNase action can be seen even at a concentration as low as  $10^{-6}$  mg./ml.

In accordance with expectation, tests made with RNase which had been boiled for 15 minutes showed that enzymatic activity is retained.

#### G. Deoxyribonuclease

In the preceding chapters we have described how trypsin, pan-protease, pepsin and ribonuclease affect lateral loop matrices, spheres and other landmarks. We have also demonstrated that none of these enzymes break the axes of lampbrush chromosomes, no matter how the enzymes are applied. Taken by itself, this is evidence that the linear continuity of lampbrush chromosomes does not depend either on protein, on RNA, or on a combination of these materials. The other known constituent of lampbrush chromosomes is DNA, and we will now present the complementary evidence which demonstrates that it is this substance which holds lampbrush chromosomes together.

DNase was used at effective concentrations of 0.125 or 0.0125 mg/ml dissolved in C medium at pH 6.2. In some experiments  $\text{MgSO}_4$  was dissolved in the enzyme solution at 0.005% to function as activator, but its presence made no significant difference to the results. DNase was used at lower concentrations than the other enzymes in order to slow down its action and thus allow its dramatic effects to be recorded photographically.

A few minutes after application of DNase solution lampbrush chromosomes break at several places along their axes, each break occurring across the slender fibril connecting adjacent chromomeres (Figs.49 and 50). The first breaks generally occur in accidentally stretched regions. As time passes more and more axial breaks occur, but soon breaks in the lateral loops also become evident. Lateral loops are rapidly broken into smaller and smaller fragments, each fragment retaining, for a time at least, its original fine morphology (Figs.48 and 51). Brownian motion so scatters the products of this continuing disruption that after 15 to 20 minutes the entire chromosome group is no longer recognisable.

For purposes of comparison we have followed the action of DNase on the giant granular loop of chromosome XII of cristatus. Besides breaking the axis of this loop, DNase mimics the action of ribonuclease or a proteolytic enzyme in that the matrix of the loop is dissolved and the granules released (Figs.52 to 55).

This led us to wonder whether our sample of DNase was perhaps contaminated with other enzymes. DNase solution after being boiled for 15 minutes proved entirely inactive, hence we can rule out the possibility of RNase contamination. We cannot absolutely rule out the possibility of contamination by a proteolytic enzyme, hence the "stripping" action of our sample of DNase on the giant granular loop of chromosome XII of cristatus remains an inconclusive observation.

The crucial result of our test with DNase, however, is the discovery that this enzyme breaks the main axes and the lateral loops of lampbrush chromosomes. Possible contamination with proteolytic enzymes is here inconsequential, since we have already shown that proteolytic enzymes are unable to produce such breaks.

## II. Versene

In a paper published in 1954, Mazia described the results of certain experiments on grasshopper mitotic and meiotic chromosomes and on salivary gland chromosomes of Drosophila, from which he concluded that these chromosomes may consist of particulate "macromolecular complexes of nucleic acid and proteins .... linked together by bridges of divalent ions". The basis for Mazia's claim was that in material frozen by solid CO<sub>2</sub>, extracted in distilled water, and then either treated with

sodium citrate followed by further extraction in distilled water or treated with the chelating agent Versene, the chromosomes were totally dissolved or at least partly fragmented. In Masia's opinion "chromosomes may be dispersed without splitting peptide bonds if two conditions are met: (1) treatment with an agent capable of binding Ca or Mg ions, and (2) provision of a medium of sufficiently low ionic strength". In view of Masia's findings we decided to extend the scope of our research and study whatever action Versene might have on lampbrush chromosomes.

First a 0.005 M solution of Versene was prepared in ordinary C medium, and pH readjusted to 6.2 by addition of a trace of NaOH. When this solution was applied to a fresh preparation of carrifox lampbrush chromosomes lying in C medium it had no discernable effect.

Second, carrifox oocyte nuclei were isolated in 5:1 K/NaCl at 0.1 M, transferred to observation chambers containing 0.005 M Versene in C medium at pH 6.2, and their membranes removed. The nuclear sap failed to disperse, but the chromosomes appeared life-like. Identical results obtained when Versene at pH 6.7 was used.

Third, oocyte nuclei were isolated in 5:1 K/NaCl at 0.1 M, rinsed in distilled water, transferred to observation chambers containing distilled water, and their membranes removed.

After fifteen minutes the chromosomes were located and kept under observation whilst a 0.001 M solution of Versene in distilled water (with pH readjusted to 7 by addition of a trace of NaOH) was added. A slight increase in the refractility of the chromosomes resulted, but their state was otherwise unaltered. The conditions of this experiment closely approach those used by Mazia - except for the omission of freezing with solid CO<sub>2</sub> - and we observed no breakage of chromosomes whatsoever.

Fourth, lampbrush chromosomes were prepared in C medium, and trypsin at an initial concentration of 0.5 mg./ml. in C medium adjusted to pH 7.8 was added. The chromosomes were kept under observation until they neared the limits of visibility, and a 0.005 M solution of Versene in C medium adjusted to pH 7.8 was then added. No change in the appearance of the chromosomes resulted. After three hours the preparations were fixed with formaldehyde vapour; near the limits of resolution unbroken chromosomes could be discerned.

Fifth, the above experiment was repeated after pre-treatment for 30 minutes with ribonuclease at 0.25 mg./ml. in ordinary C medium. The result was identical: unbroken chromosomes.

In passing, we may mention the result of one further experiment. To fresh preparations of lampbrush chromosomes lying in C medium, C medium containing CaCl<sub>2</sub> at 0.01 was added.

The chromosomes became stiff, contracted and highly refractile, showing their typical reaction to calcium ions as first recorded by Duryee (1941). After fifteen minutes a 0.01 M solution of Versene in C medium at pH 6.2 was added in excess. There was no resultant change in the appearance of the chromosomes.

## VI. DISCUSSION

Jørgensen (1913) was the first person to study the action of an enzyme on giant chromosomes. In a long paper mostly concerned with the growth of oocytes, Jørgensen recorded that whereas "basichromatischen Chromosome" (chromosomes during mitosis) withstand peptic digestion, "oxychromatischen Chromosome" (lampbrush chromosomes) are rapidly and entirely dissolved. Jørgensen worked with alcohol-fixed sections of oocytes from various animals, including Salamandra maculosa, and concluded from his experience that chromosomes during oocyte growth lack nucleic acids. More than twenty years elapsed before giant chromosomes were again tested with enzymes, by Caspersson (1936) working with dipteran salivary gland material. Caspersson sought for chemical corroboration of the inferences which he had drawn from studies of ultra violet light absorption. He squashed Drosophila salivary glands in 45% acetic acid, separated coverslip from slide and fixed in Carnoy's fluid, then exposed the squashed material to tryptic digestion. Caspersson

found that trypsin caused the stainable cross-bands to fall apart from one another; though they became greatly swollen they did not completely dissolve.

Working with Chironomus Caspersson further observed that the structural degradation produced by trypsin could be largely inhibited if lanthanum ions were present in the enzyme solution, an action which he ascribed to precipitation of nucleic acid as this substance became disengaged from protein. Caspersson concluded from these observations that salivary gland chromosomes have throughout a structural framework of protein, with which nucleic acid is combined in the stainable cross-bands.

In 1939 Mazia & Jaeger described the actions of pepsin, trypsin and nucleases on unfixed salivary gland chromosomes of Drosophila. They confirmed Caspersson's observations that trypsin (in 0.05 molar  $K_2HPO_4$ ) disintegrates salivary gland chromosomes, they found that pepsin reduces chromosome volume without causing disintegration, and that crude beef spleen nuclease digests away the Feulgen-staining material from cross-bands, again without disintegrating the chromosomes. Two years later Mazia (1941) recorded that, since even in moderately alkaline solutions, without enzymes, salivary gland chromosomes disperse into a fibrous mass, he had repeated his experiment with trypsin, this time at pH 5. Even at this pH, far from its optimum, trypsin caused total disintegration of the chromosomes.

Mazia worked with the salivary glands of Sciara, Drosophila, and Chironomus, and also with artificial fibres prepared from thymus nuclei. He again found that pepsin reduced salivary gland chromosome volume without destroying continuity, that papain failed to digest these chromosomes, and that crude protaminase from pancreas removed Feulgen-staining material from the cross-bands but left the chromosomes otherwise intact. From these observations Mazia concluded that, since trypsin splits peptide links near free basic groups (and hence would be effective against histone), whereas pepsin splits peptide links near free acidic groups (and hence would be ineffective against histone), and since trypsin disintegrates salivary gland chromosomes, whereas pepsin does not, chromosomes must be held together lengthwise by histone. Indeed Mazia contrasts "matrix" non-histone protein with "skeletal" histone when discussing salivary gland chromosome structure.

In 1944 Prolova, working with alcohol-fixed sections and aceto-carmines squash preparations of Chironomus salivary glands, confirmed that beef spleen nuclease removed Feulgen-stainable material from cross-bands without causing other structural degradation, but he further found that subsequent treatment of nuclease digested chromosomes with pepsin caused total disintegration. Prolova also reported that RNase caused slight shrinkage of salivary gland chromosomes, but no other noticeable change in their appearance.

Three years later two further papers were published on this subject. . . Mazia, Hayashi & Yudowitch (1947) reported once again the capacity of trypsin to disintegrate salivary gland chromosomes, contrasting with the inability of pepsin and cathepsin to act likewise. . . Catcheside & Holmes (1947), in a paper mainly concerned with the actions of various nuclease preparations, confirmed that trypsin destroyed salivary gland chromosomes of Drosophila which had been fixed in 45% acetic acid; they were unable to work with unfixed material, since the unfixed chromosomes "lysed and lost their structure" independently of enzyme treatment. . . They further reported that thymonuclease (DNase) prepared from pancreas removed Feulgen-stainable material from the cross-bands, but interpreted this result with caution because they found that several other preparations, inactive on their own, were also capable of removing Feulgen-stainable material if the chromosomes were first exposed to RNase. . . Catcheside & Holmes confirmed Frolova's observation that RNase acting alone caused slight shrinkage of salivary gland chromosomes but no other recognisable change.

This generally consistent story was challenged by Kaufmann and various co-workers in a series of papers, the first of which appeared in 1946. . . Kaufmann, Gay & McDonald (1950) demonstrated that the apparent total digestion of onion root-tip and salivary gland chromosomes by trypsin depends on the presence of electrolytes in the enzyme solution. . . Salivary

gland chromosomes of Chironomus were fixed and squashed in 45% acetic acid, then digested with salt-free trypsin solution. When examined by phase contrast, Kaufmann, Gay & McDonald found that: "Such treatment did not lead to any perceptible distortion of the pattern of banding of the chromosomes. When phosphate buffer replaced the trypsin solution, there was an immediate swelling of the chromosomes and lateral separation of the bands into component chromomeres". Kaufmann, Gay & McDonald stated further that: "The dissolution usually attributed to the specific action of trypsin has been shown to be due to the removal by water of degradation products produced by the action of trypsin in combination with electrolytes". Having observed that chromosomes are destroyed by successive treatments with proteases and nuclease, or with proteases and hot trichloroacetic acid, but not by any of these agents when used alone, Kaufmann, Gay & McDonald concluded that: "...the chromosome represents an integrated fabric, in which no single protein or nucleic acid may be regarded as the primary structural component". In later papers Kaufmann (1952, 1953); Kaufmann, Pennoyer & Rowan (1953); and Kaufmann & McDonald (1956) the results of further experiments with enzymes are claimed to support this conclusion.

Whilst these studies on salivary gland chromosomes were in progress, other cytologists had been experimenting with lampbrush chromosomes. In a paper mainly concerned with the oocyte "nucleoli" of Rana spp. Gersch (1940) observed that pepsin failed

to destroy the lengthwise integrity of unfixed lampbrush chromosomes. Gersch also experimented with trypsin, but his observations with this enzyme were inconclusive.

Duryee (1941) worked with pepsin, trypsin and a variety of electrolytes; he found that both these enzymes, electrolytes at pH greater than 8.4 and acids at concentrations above 0.7 molar all "removed" the lateral loops from lampbrush chromosomes of Rana and Triturus spp. Although Duryee does not make the point distinctly, it would appear that none of the reagents which he tested broke the axes of lampbrush chromosomes, though the chromosomes were found to dissolve in 0.01 molar sodium hydroxide.

In 1948 Dodson gave an account of his studies on the lampbrush chromosomes of Amphiuma means and Squalus suckleyi. Dodson worked throughout on fixed and sectioned oocytes, exposing 10 $\mu$  sections to enzymes and staining thereafter to assess what actions had occurred. He reported that 3 days digestion with thymonuclease (DNase, possibly contaminated with RNase), though producing Feulgen-negative preparations, left chromosomes which could be stained by iron haematoxylin. He found that pepsin caused shrinkage of the lateral loops, but that chromosomes' axes survived peptic digestion and could later be stained by Feulgen's reagent. Trypsin, on the contrary, dissolved the lampbrush chromosomes entirely. After twelve hours digestion even iron haematoxylin staining failed to reveal any residual

chromosome material. Dodson reached a conclusion substantially in agreement with Mazia & Jaeger. He stated: "The structural skeleton of the (lampbrush) chromosome appears to be histone, while the nucleic acids are attached to this skeleton through protamine".

In 1954 Gall, working with Triturus viridescens, demonstrated conclusively that although the lateral loops of unfixed lampbrush chromosomes are "removed" by trypsin this enzyme fails to destroy the lengthwise coherence of chromosome axes. Gall claimed that Dodson's contrary observations were probably due to the thin chromosome axis being mechanically removed, "washed out", from sections after their loops had been digested. Gall further reported that pepsin at low pH "removes" the lateral loops from both fixed and unfixed lampbrush chromosomes, but that pepsin, like trypsin, fails to destroy chromosome axes.

Having observed the "double loop bridges" which are regularly produced when lampbrush chromosomes are stretched and their axes broken, Callan (1955) predicted that each lateral loop must have a fibrillar core, comparable in elasticity and tensile strength to the fibre running between adjacent chromomeres in the chromosome axes, and around which the products of synthesis accumulate. Gall (1956) put this theory to test by fixing lampbrush chromosomes to a glass slide by formaldehyde vapour; he then digested such preparations with pepsin, made shadow-cast Formvar replicas, and observed the replicas with an electron

microscope. Gall showed that there is indeed a loop axis which resists peptic digestion whilst the bulk of the loop material dissolves, and that its diameter is roughly equal to that of the fibre running between adjacent chromomeres. Gall's observations shed new light on the structure of lampbrush chromosomes; he inferred that the loop axis fibril may be continuous with the interchromomeric fibril, and that both may contain DNA. With this background of information and speculation the enzyme studies described in the present paper were begun.

We have found that proteolytic enzymes and RNase dissolve matrix from lateral loops. Since we have not worked with an electron microscope we do not know whether loop axes withstand trypsin and pan-protease digestion, but they certainly resist DNase. Neither of these enzymes break the axes of lampbrush chromosomes, nor does Versene. Our critical observation is that DNase, on the contrary, does break chromosome axes. This enzyme breaks not only the fibril between adjacent chromomeres; it also breaks lateral loops, and the appearance of the loop fragments so produced indicates that DNase acts directly upon loop axis. By breaking lampbrush chromosomes in this manner DNase accomplishes something which is uncommonly hard to do, even mechanically. We conclude that DNA, not protein, is responsible for the linear integrity of lampbrush chromosomes.

We now have to face the problem of reconciling the above conclusion with that of Kaufmann and co-workers drawn from studies on salivary gland chromosomes.

The action of DNase on lampbrush chromosomes was briefly reported by Callan & Macgregor in 1958. Dutt & Kaufmann (1959) have recently described the action of DNase on unfixed salivary gland chromosomes of Drosophila and on testicular chromosomes of two grasshopper species, and they have criticised our 1958 conclusions. Dutt & Kaufmann have shown that DNase may lead to loss of DNA from salivary gland chromosomes without causing marked deformation, or to structural alteration of the chromosomes without complete removal of DNA. They found testicular chromosomes more refractory than salivary gland chromosomes, and conclude: "that DNase does not cause deformation of chromosomes by removal of DNA that is essential for the maintenance of structural integrity. Deformation is attributable to modification of the properties of the complex nucleoprotein fabric of the chromosome".

This may indeed be the appropriate negative conclusion to draw from observations on salivary and testicular chromosomes, but we have drawn a positive conclusion from work on different material. Dutt & Kaufmann claim that in our 1958 paper we: "emphasize the difference in response of fixed and unfixed chromosomes to enzymatic hydrolysis" as though this might explain

our different results. At least for lampbrush chromosomes the emphasis is justified. If a lampbrush chromosome, fixed and attached to a glass slide, is digested by DNase, it appears unbroken when viewed with a light microscope. This observation does not signify that interchromomeric fibrils or lateral loop axes are intact; all it signifies is that in lampbrush chromosomes there are constituents other than DNA remaining attached to the slide. Dutt & Kaufmann further claim that: "... dissociation in itself is not a critical index to the selective action of a given agent on a specific chromosomal component..... A more reliable criterion of the digestion of chromosomal DNA by DNase is the loss of Feulgen stainability, whether this is associated with structural deformation or not". Yet fixed lampbrush chromosomes can be digested with DNase until all their Feulgen stainability has disappeared; thereafter they still appear unbroken. As we have already described in this paper, unfixed lampbrush chromosomes behave otherwise. They are broken by DNase, not by proteolytic enzymes, RNase or Versene, and because they are not attached to a slide Brownian movement reveals that they have been broken.

Dutt & Kaufmann suggest and prefer an alternative explanation: "... that the meiotic lampbrush chromosomes differ from the animal and plant chromosomes with which we (Dutt and Kaufmann) have worked in regard to the nature and distribution

of their essential structural components". Whilst we would agree that lampbrush chromosomes differ in many respects from dipteran salivary gland and grasshopper testicular chromosomes, such differences are more likely to concern the arrangement than the nature of the primary genetic material, and the extent to which gene product is associated with this material.

The main conclusion which we have drawn from our observations is that the primary genetic constituent of a chromosome is an uninterrupted DNA fibre. In those parts of lampbrush chromosomes where the DNA concentration per unit of length is high, and where histone is also demonstrable, i.e. in the chromomeres and, more strikingly, in the axial bars near the centromeres of karelinii, DNase fails to produce breaks. We do not know how DNA and histone are arranged in such regions, but trypsin and pan-protease digestion reveals that when histone is withdrawn the remaining DNA preserves its lengthwise coherence though it fails to maintain a compact form. Such parts of lampbrush chromosomes may well resemble in structural organisation the cross-bands of salivary gland chromosomes and the compact chromosomes of metaphase, which from our experience with lampbrush chromosomes we would expect to withstand DNase digestion. We might, on the other hand, expect DNase to break the inter-band regions of salivary gland chromosomes. However since salivary gland chromosomes are polytene, clean breaks would only be demonstrable if several hundred DNA fibres were all broken

at the same interband, and provided the cross-bands on either side separated to reveal the break. In our opinion these are the main reasons why Kaufmann and his co-workers have reached conclusions different from our own.

#### SUMMARY

The chromomeres of lampbrush chromosomes of Triturus cristatus are Feulgen-positive and hence they contain DNA. After removal of their DNA by trichloroacetic acid the chromomeres stain with fast green at alkaline pH and hence they contain basic protein. The lateral loops are Feulgen-negative. They stain with toluidine blue at acid pH, but much less intensely following RNase digestion, hence they contain RNA. The spheres of chromosomes V and VIII do not contain RNA.

Unfixed lampbrush chromosomes retain a life-like appearance in 0.07 molar K/NaCl at pH 6.2; in this medium the nuclear sap dissolves. As pH is raised to 8.5 the matrices of lateral loops dissolve but chromosome axes remain unbroken. Above pH 8.5 lampbrush chromosomes dissolve. As pH is lowered from 6.2, at between 5.8 and 5.4 fixation occurs. If pH is rapidly reduced still further, a persistent relaxed condition sets in between 2.5 and 2.

In concentrations of K/NaCl above 0.5 molar lampbrush

chromosomes dissolve. Lateral loop matrices dissolve in 0.25 molar saline but chromosome axes remain unbroken. In concentrations of K/NaCl below 0.05 molar lateral loop matrices dissolve, but chromosome axes remain unbroken, even in distilled water.

Trypsin both at pH 6.2 and 7.8 strips the matrices from lateral loops and occasionally breaks matrix fusions. It causes chromomeres to swell and coalesce, but fails to break chromosome axes. The action of "pan-protease" resembles that of trypsin in all respects.

Pepsin at pH 6.2 strips the matrices from lateral loops, but does not attack chromomeres. At low pH peptic digestion is slow, since it is attacking fixed chromosomes; but if peptic digestion precedes a lowering of pH the overall outcome is a rapid solution of loop matrix, and under these conditions matrix and sphere fusions are broken. If trypsin or "pan-protease" digestion precedes a lowering of pH there is a similarly rapid solution of loop matrix; thus the action is not specifically referable to pepsin. Under no conditions does pepsin break the axes of lampbrush chromosomes.

RNase at pH 6.2 strips the matrices from lateral loops; this action is detectable even at extreme dilution. RNase does not attack chromomeres, nor does it break chromosome axes. If tryptic digestion follows RNase digestion this too fails to break chromosome axes.

Unlike the proteolytic enzymes and RNase, DNase at pH 6.2 breaks the axial fibril between adjacent chromomeres, and it also breaks the axes of lateral loops.

Contrary to Mazia's experience with salivary gland chromosomes, Versene does not break the axes of lampbrush chromosomes even when applied in media of low electrolyte concentration.

These results indicate that uninterrupted fibres of DNA run throughout the lengths of lampbrush chromosomes. We consider these fibres to be the primary genetic components of lampbrush chromosomes.

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- APPENDIX II -

- THE BEHAVIOUR OF ISOLATED NUCLEI -

by

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Much has been learned about the ultrastructure of nuclear membranes during the past ten years, yet little is known about their physical and chemical properties. Is the membrane passive and sieve-like, merely separating particulate components of the cytoplasm from those of the nucleus; or does it behave as a simple semipermeable membrane through which molecules pass or do not pass in strict accordance with their sizes and shapes; or is the membrane actively involved in nucleo-cytoplasmic exchange?

There is no doubt that the nuclear membrane is permeable to water. This was originally proved by Hamburger in 1902 whose observations have since been confirmed by Beck & Shapiro (1936), Churney (1942), Callan (1949), and Goldstein & Harding (1950). The membranes surrounding nuclei in isolation have also been shown permeable to physiological electrolytes, (Callan 1949, Goldstein & Harding 1950) and to judge from the report of Abelson & Duryee (1949), the nuclear membrane of a living, intact amphibian oocyte is similarly permeable to salts. With regard to the passage of complex organic molecules the evidence is less convincing. Holtfreter (1954) has shown that germinal vesicle nuclei isolated from frog oocytes are permeable to haemoglobins, and Anderson (1953) has shown that nucleases, beef serum albumin (BSA), partially hydrolysed gelatin, and haemoglobin, all penetrate the membranes of freshly isolated nuclei from rat liver cells.

However, my own observations suggest that information derived from isolated cell nuclei is suspect.

Recent reports which bear directly on the subject of the present paper are those of Battin (1958), Harding & Feldherr (1959) and Hunter & Hunter (1961). These authors are all concerned with osmotic properties of amphibian oocyte nuclei. Battin (1958) isolated oocyte nuclei of Triturus viridescens in various salt solutions. Like Callan (1949) he found that the nuclei swelled even in media which were hypertonic to whole oocytes; but that by adding BSA to salines used for isolation, the swelling of nuclei could be prevented. He concluded that the nuclear membrane is permeable to water and salts, and impermeable to molecules as large as those of BSA, which according to Creeth (1952) has a molecular weight of 65,360. Battin considers that the swelling of isolated nuclei is due to colloid-osmotic pressure of the nuclear sap and he invokes the Donnan law to account for differences in degree of swelling in media of different ionic strengths. Battin recognises the possibility that swelling of the nucleus may alter the permeability of its membrane, and so lead to false evidence concerning its osmotic properties.

Harding & Feldherr (1959) injected BSA and the synthetic polymer (PVP) of molecular weight 30 to 50 thousand, into small (200 to 300 $\mu$  diameter) transparent Rana pipiens oocytes.

Their evidence suggests that the membrane of the germinal vesicle is impermeable to substances of molecular weights greater than 40,000. Harding & Feldherr's conclusions are in agreement with those of Dattin; they consider nuclear swelling in response to injections of hypertonic solutions of BSA and PVP to be a consequence of the colloidsmotic pressure of the nuclear sap.

Hunter & Hunter (1961) have recently determined the rate of swelling of frog germinal vesicles isolated in various solutions of simple electrolytes and non-electrolytes. From their results they conclude that the nuclear membrane is permeable to salts and water; however, they also surprisingly claim that the rate of swelling of isolated germinal vesicles is inversely proportional to the concentration of salts in the external medium, and hence that swelling cannot be due to colloidsmotic pressure of the nuclear sap.

Controversy such as that which has arisen from the studies of Dattin (1958) and Hunter & Hunter (1961) bespeaks a lack of knowledge concerning the events which cause and accompany the swelling of isolated nuclei. It is surely unlikely that an isolated nucleus will behave as a simple osmometer. It is just as unlikely that we shall learn much about nuclei by studying one aspect of their behaviour under different conditions. I have recorded the volume changes in isolated nuclei in relation to dry mass, and time. Moreover

I have tried to simplify matters by using only 2 types of media for isolation; one was colloid free, the other contained protein.

In the present work nuclei were obtained from medium sized oocytes (0.8 to 0.9 mm. diameter) taken from a single female newt of the species Triturus cristatus carnifex (Laurenti). Each nucleus was isolated manually, cleaned of adherent yolk by pumping in and out of a fine drawn glass pipette, and then transferred to an observation chamber of the type designed by Gall (1954). Media used for isolation are specified below. Immediately after transfer of a nucleus a cover glass was placed over the top of the observation chamber. The nucleus was then examined in monochromatic light (wavelength 5710Å) using a Baker Interference Microscope fitted with a x 10 shearing objective and a half shade eyepiece. The eyepiece contained a micrometer. The delay between puncturing an oocyte and examining its nucleus was never more than 35 seconds.

Nuclei were isolated in unbuffered salines consisting of 5 parts of KCl and one part of NaCl; both chlorides were used at the same molar strength. The 5:1 ratio is justified by the unpublished determinations of Dr. W. T. W. Potts, who showed that this is the approximate proportion of K to Na in carnifex oocytes. The pH of all isolation media was between 6 and 6.3 as measured by glass electrode.

Thirty-five seconds after isolation in 0.3 molar\* 5:1 K/NaCl a carnifex germinal vesicle shows 7, 8, or 9 concentric dark fringes when viewed with the interference microscope (Fig.1). About 5 minutes after isolation the nucleus is swollen and it shows only 4 or 5 dark fringes (Figs.3 and 4). The mean changes in volume of five comparable nuclei isolated in 0.3 molar saline are shown graphically in Fig.7. Fig.8 illustrates the rate at which dark fringes disappear from similarly isolated nuclei. From Fig.7 it is clear that a nucleus isolated in 0.3 molar saline swells to a maximum size and then deflates. After 20 minutes in 0.3 molar saline although a nucleus is still turgid, the optical retardation measured through its axis has fallen to less than 1.5 wavelengths, (Figs.5 and 6). This evidence proves that nuclear sap leaches out from an isolated nucleus much more rapidly than had hitherto been suspected. We may enquire whether leaching starts at the moment of isolation or after the membrane has been stretched beyond a certain limit. This problem can be solved as follows:- if M be the dry mass of the nucleus in grams, V the nuclear volume in centimeters<sup>3</sup>, C the concentration

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\* The oocyte nuclei from this particular newt burst within a few minutes if isolated in colloid free salines more dilute than 0.3 molar. Between newts, individual differences exist in this respect. Callan (1952) was able to work with salines down to 0.05 molar.

of organic matter in the nucleus in grams/cm<sup>3</sup>,  $n_i$  the refractive index of the nuclear sap,  $n_o$  the refractive index of the external medium,  $a$  the specific refractive increment (0.0018),  $f$  the optical path difference in centimeters, and  $r$  the radius of the nucleus in centimeters (representing half its thickness) then:-

$$\begin{aligned}
 M &= C \times V & \text{and } f &= 2r (n_i - n_o) \\
 & & \text{but } f &\text{ also } = 2r (100a \times C) \\
 & & &= 2r (100a \times \frac{M}{V}) \\
 & & &= 2r ( \frac{100a \times 3M}{4\pi r^3} ) \\
 & & &= \frac{150 Ma}{\pi r^2} \\
 & & &= 8.6 \times 10^{-2} (\frac{M}{r^2})
 \end{aligned}$$

Discarding constants,  $f$  is therefore proportional to  $\frac{M}{r^2}$ . Thus if the dry mass of a nucleus remains constant, i.e. no organic material leaches out, then a plot of  $f$  against  $\frac{1}{r^2}$  will give a straight line. In Fig.9  $f$  is plotted against  $\frac{1}{r^2}$ . The graph shows that for about three minutes after isolation of a nucleus the decrease in  $f$  is due to swelling but thereafter material leaches out.

Callan (1949) found that a 15% solution of BSA is isosmotic with carnifex oocyte nuclei, whereas Battin (1958) stated that for T.viridescens nuclei the comparable concentration is 3.5%. If we assume that a freshly isolated carnifex nucleus

400 $\mu$  in diameter produces an optical retardation of 9 wavelengths, then according to the formula  $M = \frac{f \times r^2}{8.6 \times 10^{-2}}$  the dry mass of such a nucleus is 2.39 $\mu$ g, about equivalent to a 7% solution of protein. I have isolated nuclei in 3.5, 5, 6, 7, and 10% solutions of crystalline BSA (Armour Pharmaceutical Co.Ltd., Eastbourne) dissolved in 0.2 molar 5:1 K/NaCl, and lightly buffered by phosphate to pH 6. In 3.5% BSA a carnifex nucleus swells slowly to about 120% of its original volume. Thereafter its sap begins to leach out, but the nucleus does not deflate. About 30 minutes after isolation, the nucleus reaches a stable condition, producing an optical retardation of between  $\frac{1}{4}$  and  $\frac{1}{2}$  a wavelength. These events are illustrated in Fig.10 (cf. Fig.9). The behaviour of nuclei isolated in more concentrated solutions of BSA is harder to understand and will not be considered in detail. They swell slightly in 5% BSA; they do not swell or shrink nor does their dry mass change perceptibly in 6% BSA; they shrink and become flaccid in 7% and in 10% BSA. The isosmotic medium has a lower refractive index than the nuclear sap, a difference to be expected if salt concentrations in the external medium and in the sap are unequal. A 9 to 11% solution of BSA gives optical equilibrium.

On the basis of these observations I would suggest that the membrane of an amphibian germinal vesicle in its natural state is permeable to salts and water, but not to protein.

However, whilst an isolated nucleus swells its membrane becomes more permeable.

### Summary

A nucleus from a medium sized newt oocyte, if isolated in a colloid-free salt solution, swells rapidly up to a certain size without losing substance. Further swelling is accompanied by loss of nuclear sap through the nuclear membrane although no physical breaks in the membrane are apparent. After a time the nucleus deflates slowly but remains turgid and does not return to its original volume. Within an hour of isolation its dry mass has decreased by more than 80%.

Incorporation of beef serum albumin in isolation media alleviates nuclear swelling. The degree of swelling is roughly proportional to the rate of leaching of nuclear sap. The nuclei used in the present study did not swell nor lose substance in a 6% protein solution. They shrank and became flaccid in protein solutions more concentrated than 6%.

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ST. ANDREWS.

Dear Professor Callan,

I have read the thesis "The Amphibian Oocyte Nucleus" submitted by Mr. H. C. Macgregor with great pleasure. I think Mr. McGregor is to be congratulated on the substance and presentation of the thesis and I recommend that it should be sent to an external examiner.

Yours sincerely,

A handwritten signature in cursive script that reads "G. R. Tristram".

G. R. Tristram.

REPORT ON THESIS

"The Amphibian Oocyte Nucleus"

submitted by Herbert Cecil Macgregor

Dr. G.R. Tristram, Dr. A.R. Sanderson and I examined Macgregor's thesis, and we had no hesitation in sending it to an external examiner, Professor C.H. Waddington. We considered that Macgregor had shown great initiative and skill in carrying out his research, that he had made several contributions to a subject of fundamental biological significance, and that he had presented the outcome of his work in an agreeable style.

Professor Waddington writes as follows:-

"The thesis gives an account of two closely connected series of experiments and observations. The first of these deals with the changes in the appearances of nuclear structures in newt oocytes which follow the administration of pituitary hormones or the removal of the pituitary gland. The second section records observations, made both with the light microscope and the electron microscope, on the so-called "nucleoli" of these nuclei. Both parts of the thesis are copiously illustrated with remarkably clear and convincing photographs; only the electron-micrographs are of rather low standard. From the evidence presented, it becomes apparent that the

candidate is a careful and industrious worker, who has been able to observe his material closely and to describe it accurately. In my opinion the experimental and observational work which forms the basis of these two sections is of great merit.

"The descriptive sections of the thesis are followed by a discussion of the main points which emerge from the observations. It is clear that the candidate has a good knowledge of the literature, both that on lampbrush chromosomes, and on related topics such as polytene chromosomes, nucleoli in other types of nucleus, etc., including more of the older cytological literature than many present-day students seem to feel worth their attention. . . . .

"The first Appendix, written in collaboration with Professor Callan, records observations of the greatest interest, and they are again very convincingly demonstrated and clearly expounded and discussed. . . . .

"In my opinion the candidate's work possesses both originality and merit, and I shall be surprised if he does not continue to carry out successful research in the future. Much of the work recorded in the main thesis merits publication more or less as it stands.

"I recommend that the degree of Ph.D. be awarded to the candidate without further examination."

The internal examining committee agree with what Professor Waddington has to say.

Report on Thesis

The Amphibian Oocyte Nucleus,

submitted by H.C. MacGregor.

The candidate's main thesis is accompanied by two Appendices; one of considerable length, written in conjunction with Professor Callan, and a shorter one written by the candidate alone. I shall in this report devote most attention to the main thesis, which describes the candidate's own observations and thoughts.

A The thesis gives an account of two closely connected series of experiments and observations. The first of these deals with the changes in the appearances of nuclear structures in newt oocytes which follow the administration of pituitary hormones or the removal of the pituitary gland. The second section records observations, made both with the light microscope and the electron microscope, on the so-called "nucleoli" of these nuclei.

only the electron-micrographs are of rather low standard. Both parts of the thesis are copiously illustrated with remarkably clear and convincing photographs. From the evidence presented, ~~in these~~, it becomes apparent that the candidate is a careful and industrious worker, who has been able to observe his material closely and to describe it accurately. In my opinion the experimental and observational work which forms the basis of these two sections is of great merit.

B The descriptive sections of the thesis are followed by a discussion of the main points which emerge from the observations. It is clear that the candidate has a good knowledge of the literature, both that on lampbrush chromosomes, and on related topics such as polytene chromosomes, nucleoli in other types of nucleus, etc., including more of the older cytological literature than many present-day students seem to feel worth their attention.

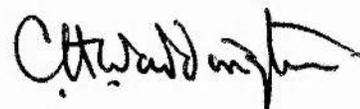
His remarks and evaluations show considerable originality and a capacity to think for himself, although not all of his opinions can be regarded as fully substantiated as yet.

C The first Appendix, written in collaboration with Professor Callan, records observations of the greatest interest, and they are again very convincingly demonstrated and clearly expounded and discussed. Some of the value of this part of the work is, of course, to be attributed to the senior author.

The second Appendix is comparatively slight. It records the behaviour (swelling or diminishing in volume, changing in optical retardation etc.) of oocyte nuclei isolated in various solutions. It is to be regarded, perhaps, more as the opening up of a new line of study than as a finished work. It is valuable, however, in demonstrating that the candidate can tackle problems of a biophysical nature as well as those of a more purely cytological kind.

D In my opinion the candidate's work possesses both originality and merit, and I shall be surprised if he does not continue to carry out successful research in the future. Much of the work recorded in the main thesis merits publication more or less as it stands.

E I recommend that the degree of Ph.D. be awarded to the candidate without further examination.



Professor of Animal Genetics,  
Edinburgh University

31st May, 1961

**- THE AMPHIBIAN OOCYTE NUCLEUS -**

**VOLUME II : ILLUSTRATIONS**

A Thesis presented for the degree

of

Doctor of Philosophy

to

The University of St. Andrews

by

Herbert Cecil Macgregor

Department of Natural History

St. Salvator's College

University of St. Andrews.



- THE AMERICAN SOCIETY OF ANTHROPOLOGY -  
- THE UNIVERSITY OF CHICAGO -

The following are listed with their respective  
names and addresses as they appear in the  
files of the American Society of Anthropology  
at the University of Chicago. The names are  
given in the order in which they appear in  
the files. The names of those who have  
been deceased are indicated by an asterisk.  
(1900) (1901) (1902) (1903) (1904) (1905)

to

Department of Anthropology

ms 2187.

to

The University of Chicago

to

Report Cecil M. Lewis

Department of Natural History

The University of Chicago

University of Chicago



- INTRODUCTORY REMARKS -

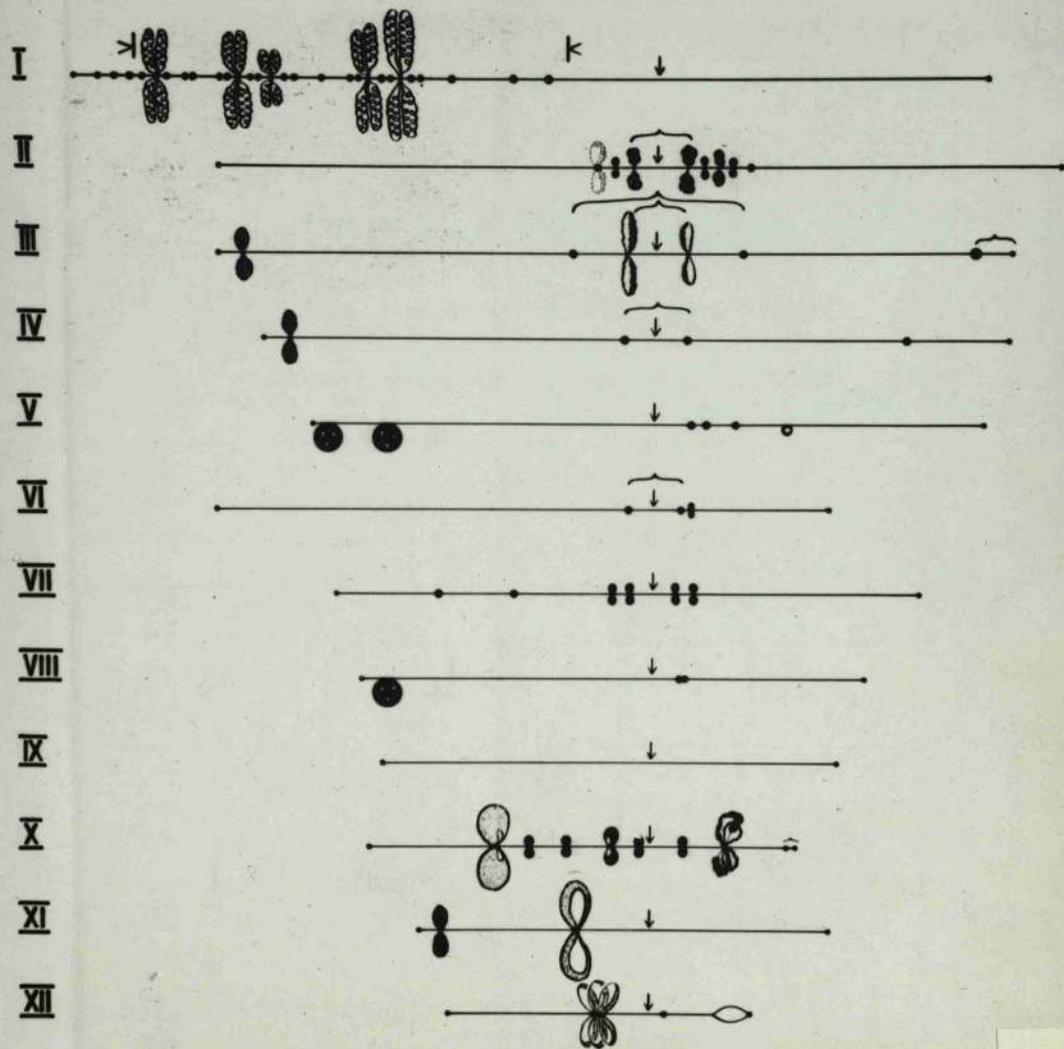
The Figures in this thesis are set out in the approximate order in which they are mentioned in the text. Newts which are referred to by an identity number only belong to sub-species carnifex. Unless otherwise stated, the photographed chromosomes and "nucleoli" were isolated in Callan and Lloyd's (1960) "medium C".

---

Figure 1

(Also Text Figure 1 of Appendix I)

Working map of the lampbrush chromosomes of T.c.carnifex showing the relative lengths of the twelve chromosomes and the main landmarks used for identification. Centromere positions are indicated by vertically aligned arrows. Structures which often show "reflected fusion" are linked together by brackets. The limits of the heteromorphic region of chromosome I are marked by > and K (Courtesy of Professor H. G. Callan).



Figures 2 to 16.

Photographed with the IPCM, x 10 objective, and x 40 phase annulus (see page 41, Chapter II). Lampbrush chromosomes, free granules, and oocyte "nucleoli", from standard-size oocytes of carnifex females.

Each preparation which was photographed was chosen as being representative at the time of all standard-size-oocyte nuclei of the respective newt.

Photographs labelled (a) are of the contents of oocyte nuclei taken from newts at the start of an experiment; photographs labelled (b) are of the contents of oocyte nuclei taken from the corresponding animals at the end of the experimental period.

Gonadotrophin-treated newts

(2a) Newt 35, before treatment.

(2b) Newt 35, after treatment with 400 I.U. CG (Swiss)

(3a) Newt 35, before treatment.

(3b) Newt 35, after treatment with 400 I.U. CG (Swiss).

(4a) Newt 51, before treatment.

(4b) Newt 51, after treatment with 400 I.U. CG + PMS.

Scale = 0.5mm

Controls to gonadotrophin-treated newts.

- (8a) Newt 51C, at the start of the experiment.
- (8b) Newt 51C, 9 days later; after it had received injections of sterile water.
- 
- (9a) Newt 35C, at the start of the experiment.
- (9b) Newt 35C, 9 days later; after it had received injections of sterile water.
- 
- (10a) Newt 48C, at the start of the experiment.
- (10b) Newt 48C, 9 days later; after it had received injections of sterile water. In this picture the chromosomes are blotted out by large numbers of free granules and a few "nucleoli".

Scale = 0.5mm

Hypophysectomised newts.

(11a) Newt 24, before hypophysectomy

(11b) Newt 24, 30 days after hypophysectomy.  
Note the lack of "nucleoli" in this preparation,  
and the shortened and thickened appearance of  
the chromosomes. The latter condition is even  
more evident in figure 12b.

(12a) Newt 27, before hypophysectomy.

(12b) Newt 27, 46 days after hypophysectomy.

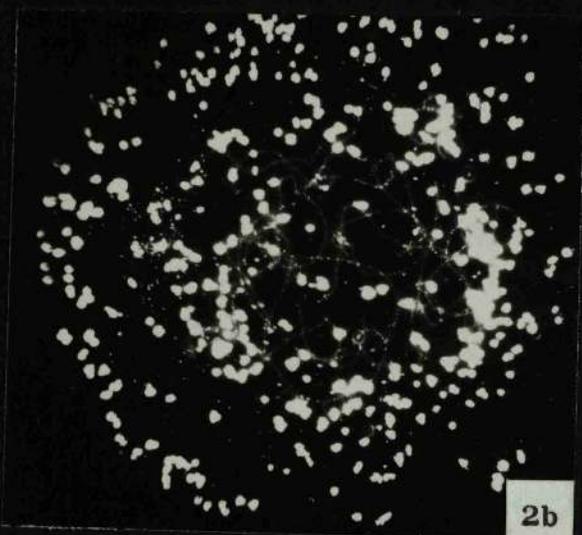
(13a) Newt 30, before hypophysectomy.

(13b) Newt 30, 21 days after hypophysectomy,

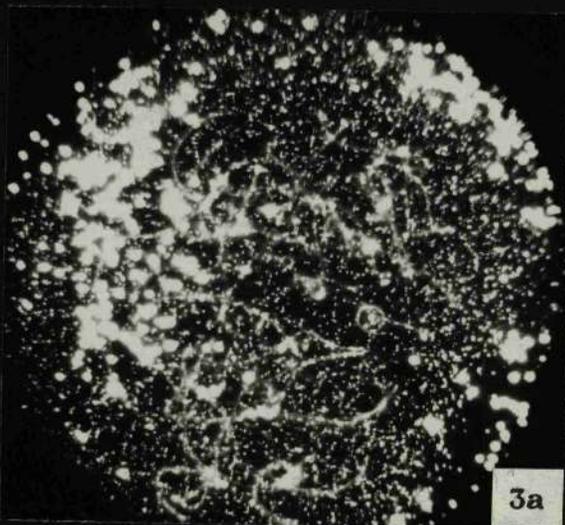
Scale = 0.5mm



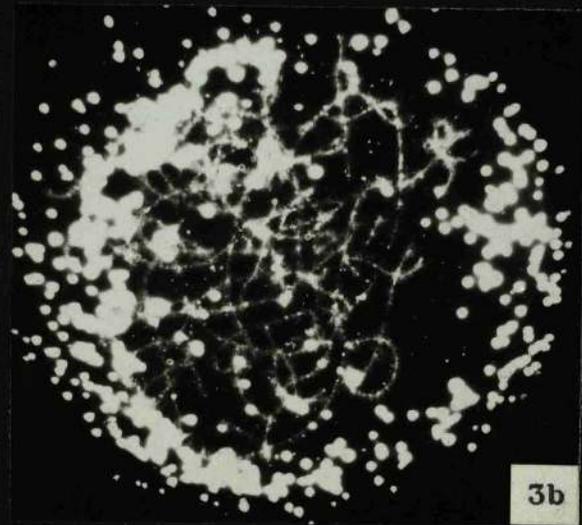
2a



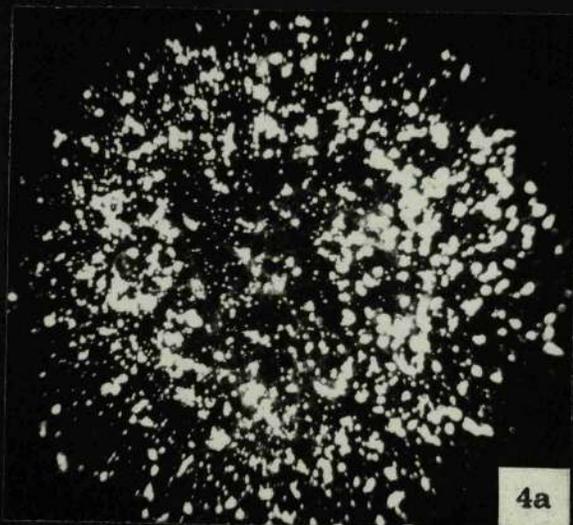
2b



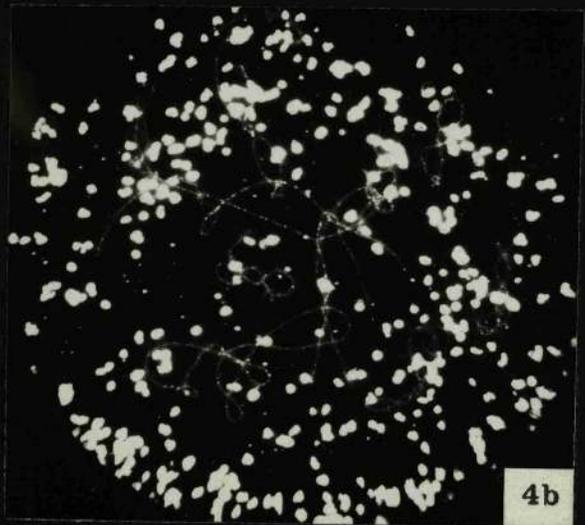
3a



3b



4a



4b



Gonadotrophin-treated newts

(5a) Newt 55, before treatment

(5b) Newt 55, after treatment with 400 I.U. CG (Organon)  
Large numbers of small granules clustered around  
the chromosome group.

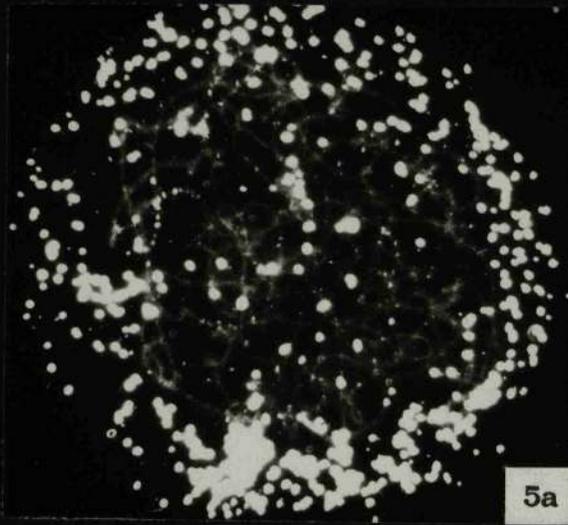
(6a) Newt 52, before treatment.

(6b) Newt 52, after treatment with 400 I.U. CG + PMS.

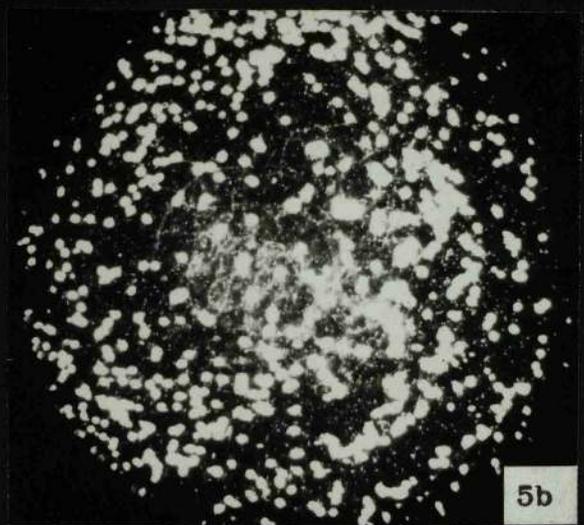
(7a) Newt 54, before treatment

(7b) Newt 54, after treatment with 400 I.U. PMS.

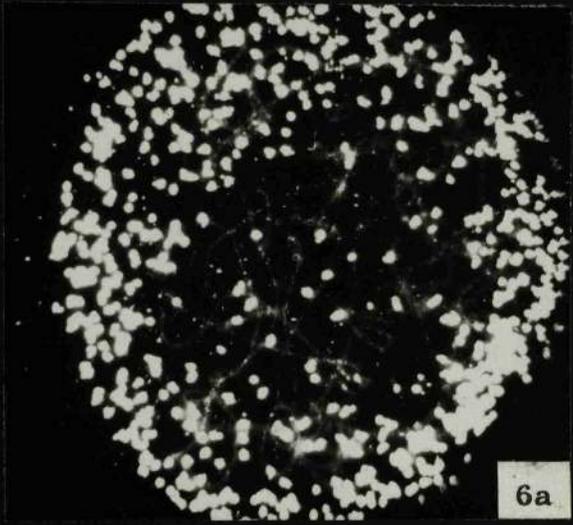
Scale = 0.5mm



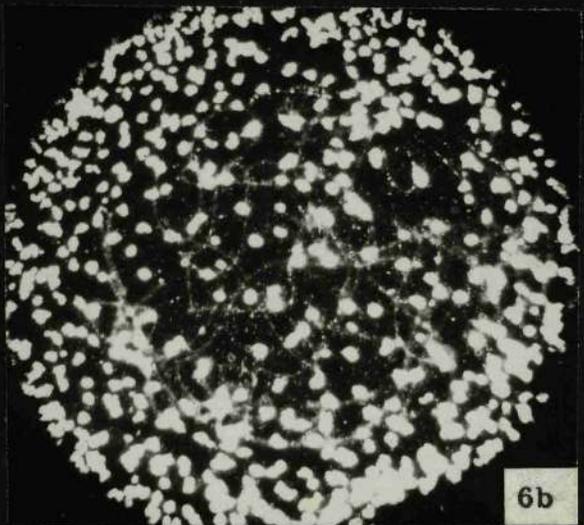
5a



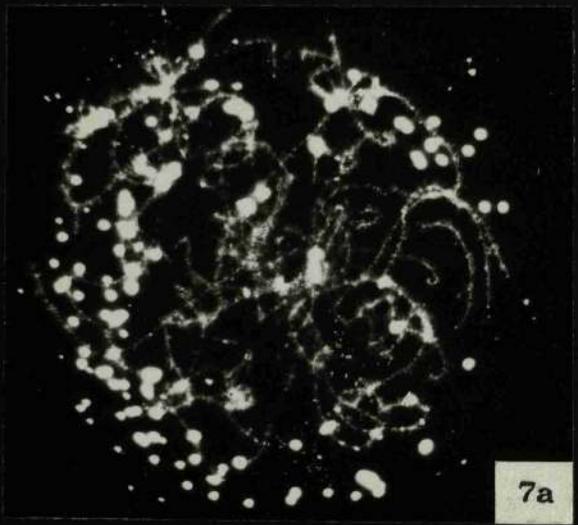
5b



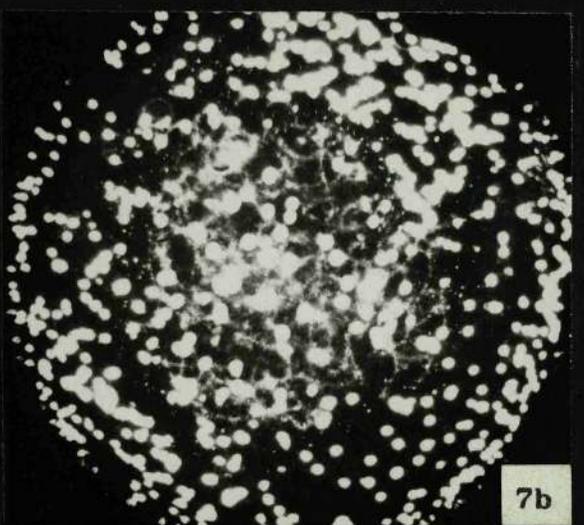
6a



6b

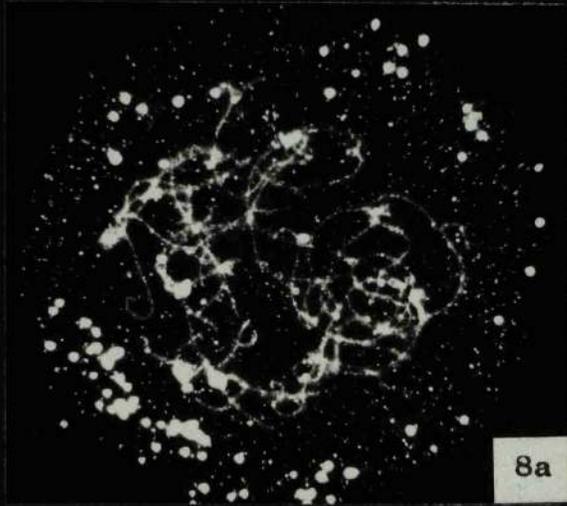


7a



7b

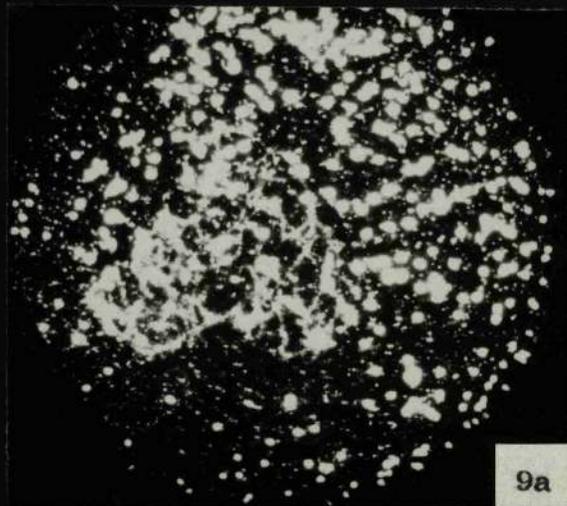




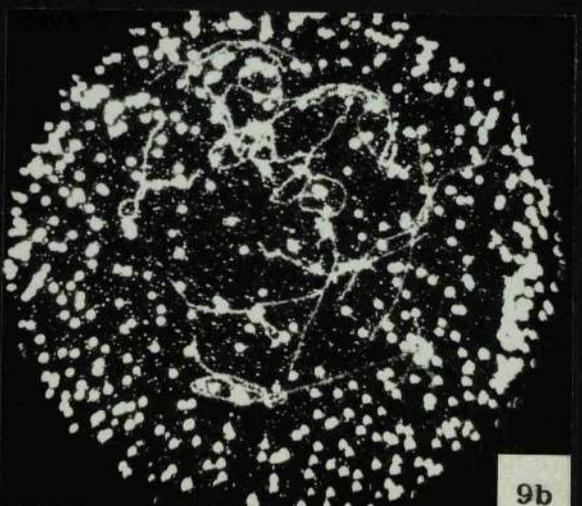
8a



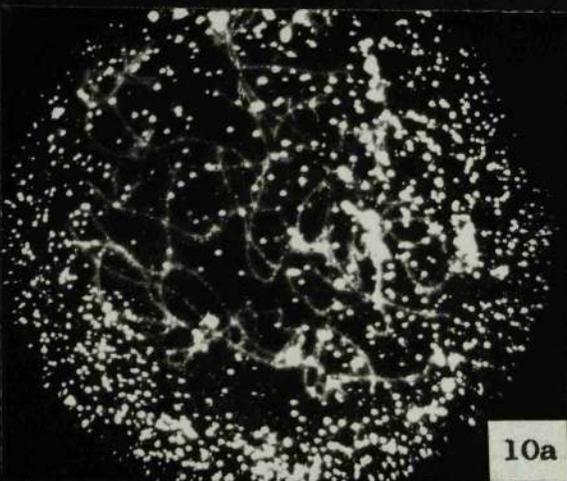
8b



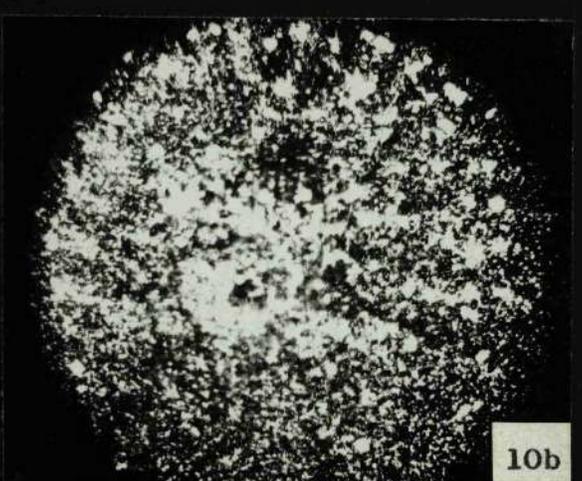
9a



9b

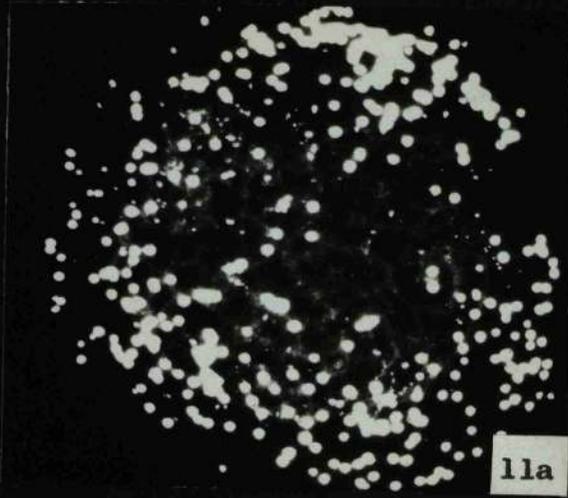


10a

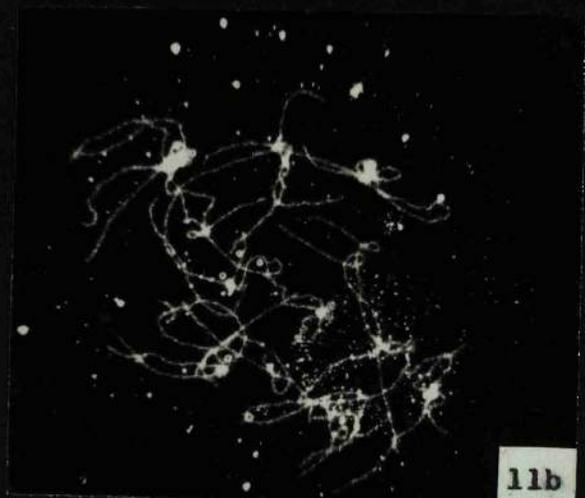


10b

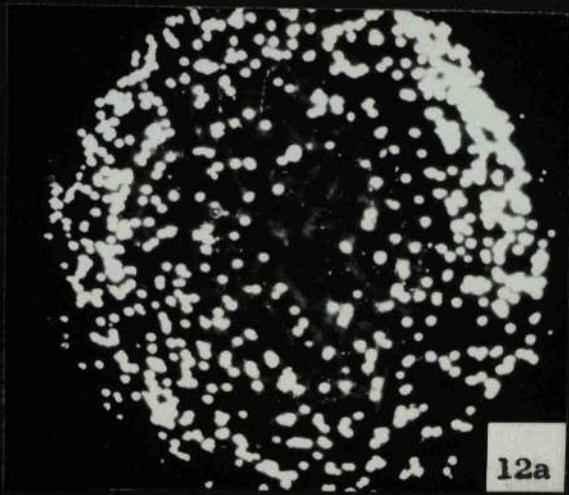




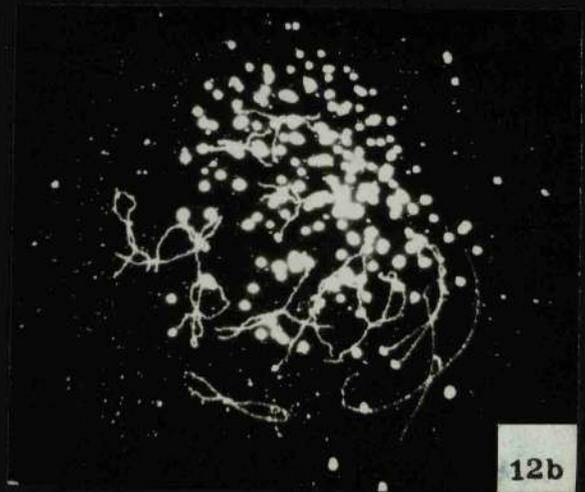
11a



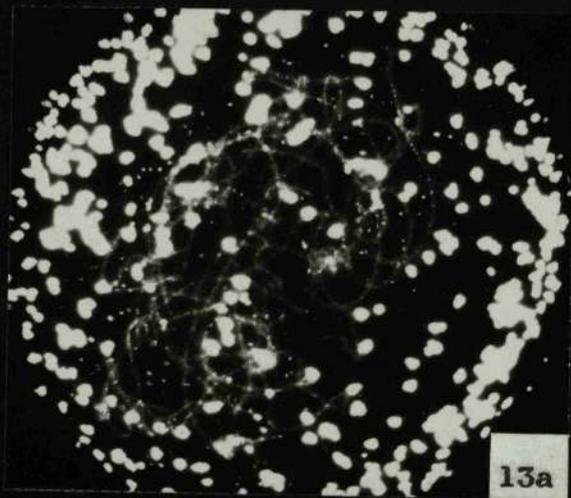
11b



12a



12b



13a



13b



Controls to hypophysectomised newts.

(14a) Newt 24C, before mock hypophysectomy.

(14b) Newt 24C, 30 days after mock hypophysectomy.

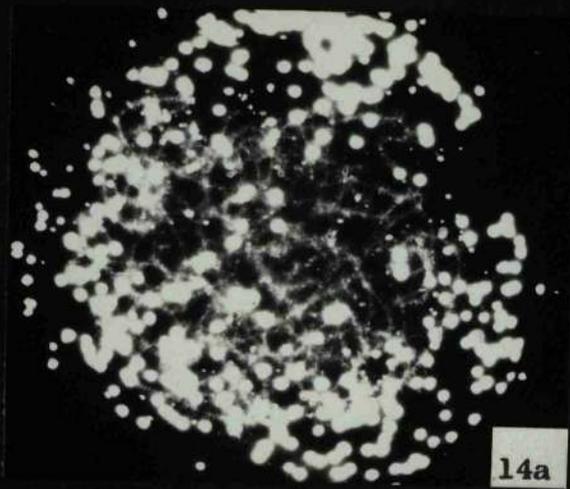
(15a) Newt 27C, before mock hypophysectomy.

(15b) Newt 27C, 46 days after mock hypophysectomy.

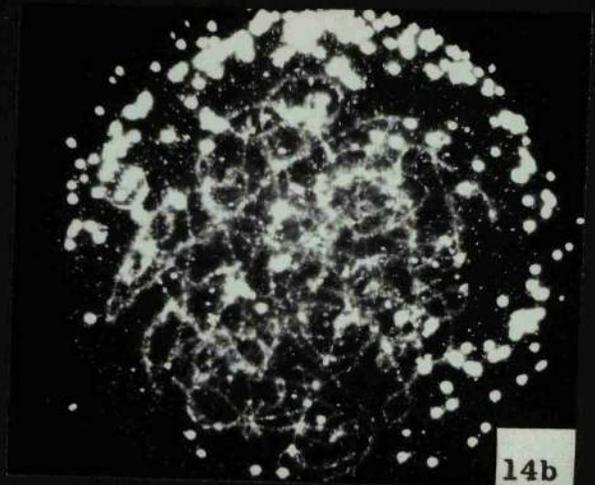
(16a) Newt 30C, before mock hypophysectomy.

(16b) Newt 30C, 21 days after mock hypophysectomy.

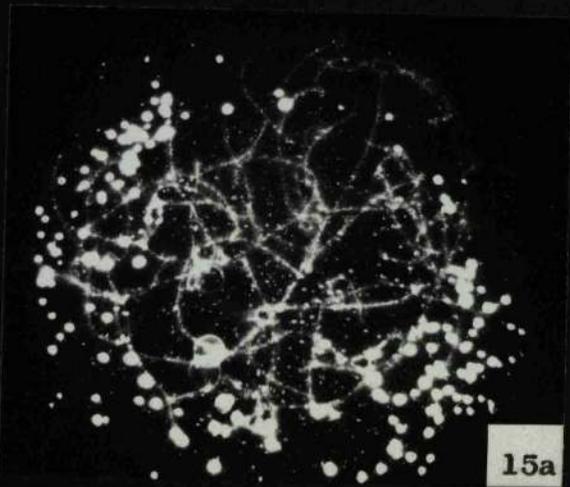
Scale = 0.5mm



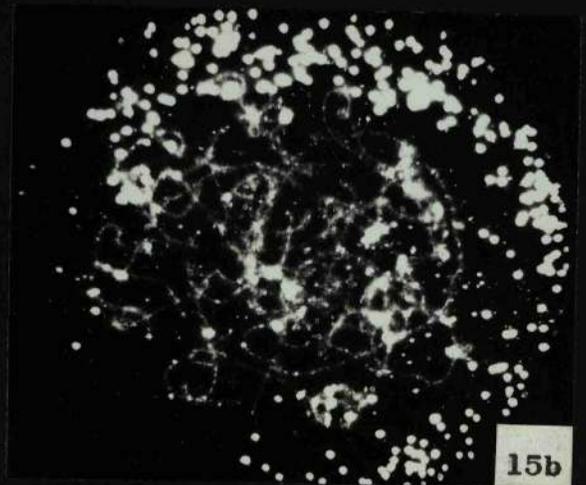
14a



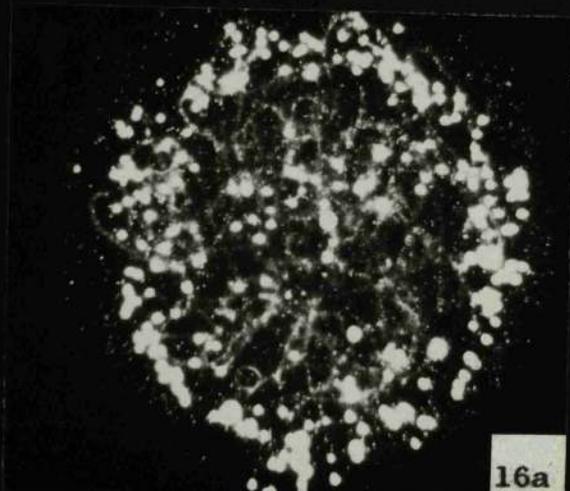
14b



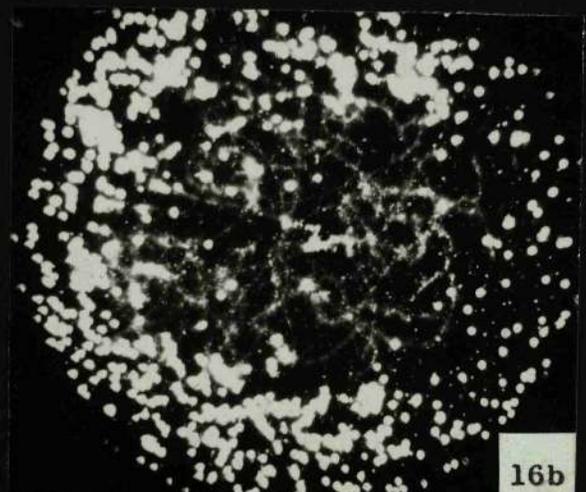
15a



15b



16a



16b



Figure 17.

Photographed with the IPCM and x 95 objective. Illustrating the sizes and character of free granules in standard-size-oocyte nuclei taken from newt 22 35 days after this animal had been hypophysectomised.

Scale = 25 $\mu$

Figures 18 to 25.

Camera lucida drawings of the giant loops of bivalents X, XI, and XII, carnifex. For these drawings the IPCM was fitted with a x 95 objective, x 10 eyepieces, and a camera lucida attached to an inclined monocular tube. Those loops which were drawn were chosen as being representative of the giant loops of standard-size oocytes from the respective newt. Roman numbers indicate the bivalents of which the loops were a part. Figures labelled (a) show giant loops at the start of the experiment; figures labelled (b) show giant loops from corresponding animals at the end of the experimental period.

Gonadotrophin treatment

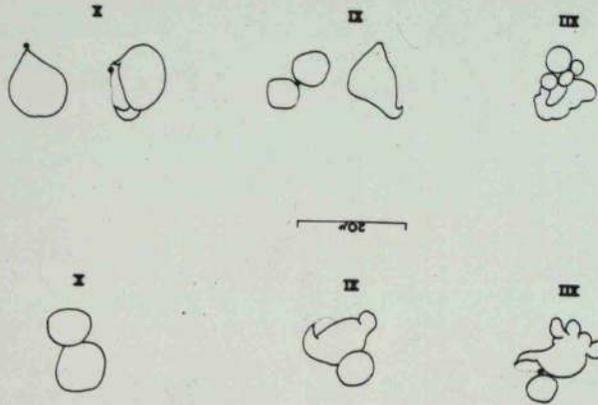
(18a) Newt 36, before treatment.

(18b) Newt 36, after treatment with 400 I.U. CG (Swiss).

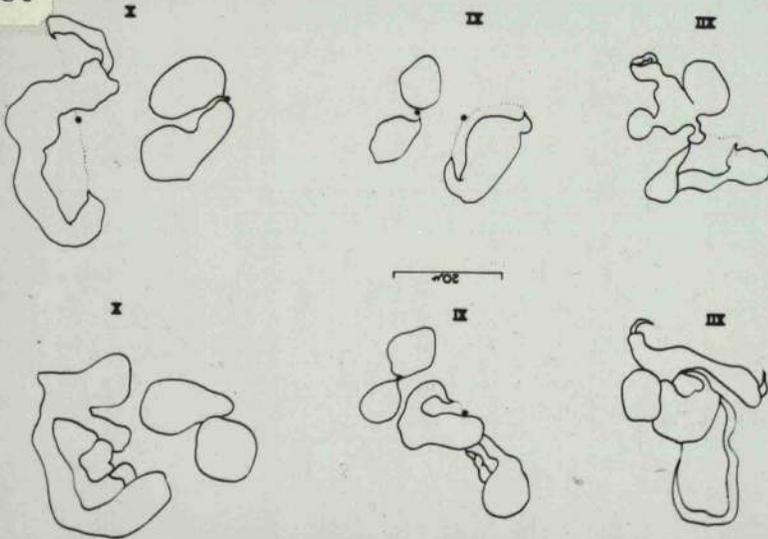
Giant loops characteristically present in newt 36:-

X + +  
XI + +  
XII + +

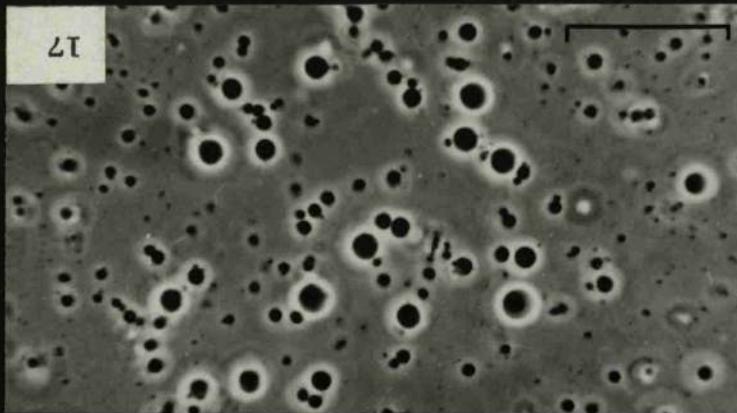
18b



18a



17



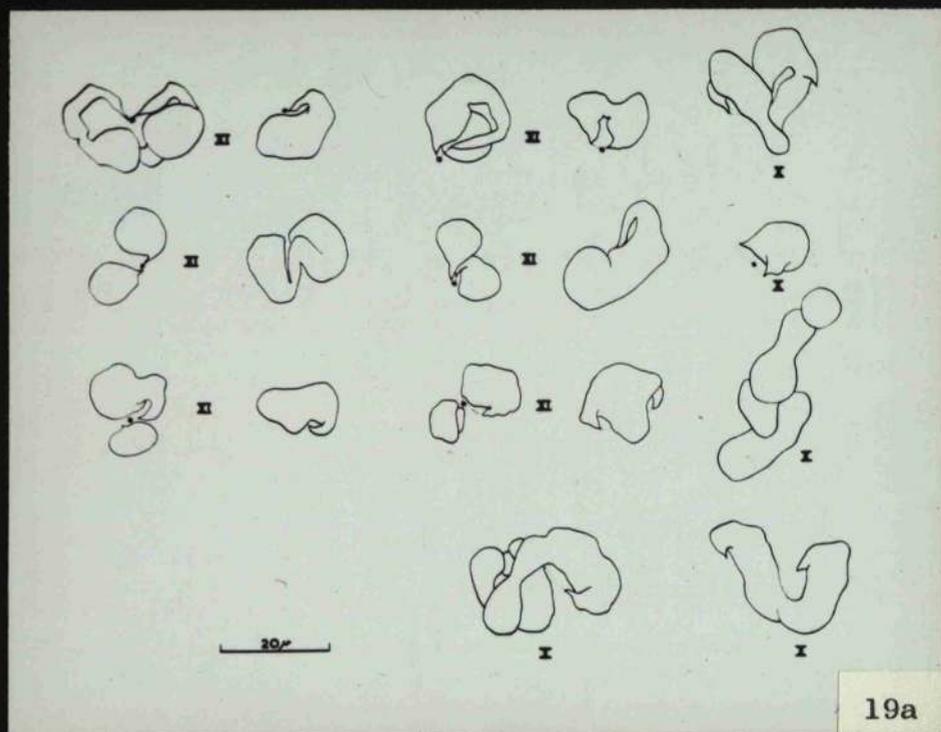
Gonadotrophin treatment

(19a) Newt 58, before treatment.

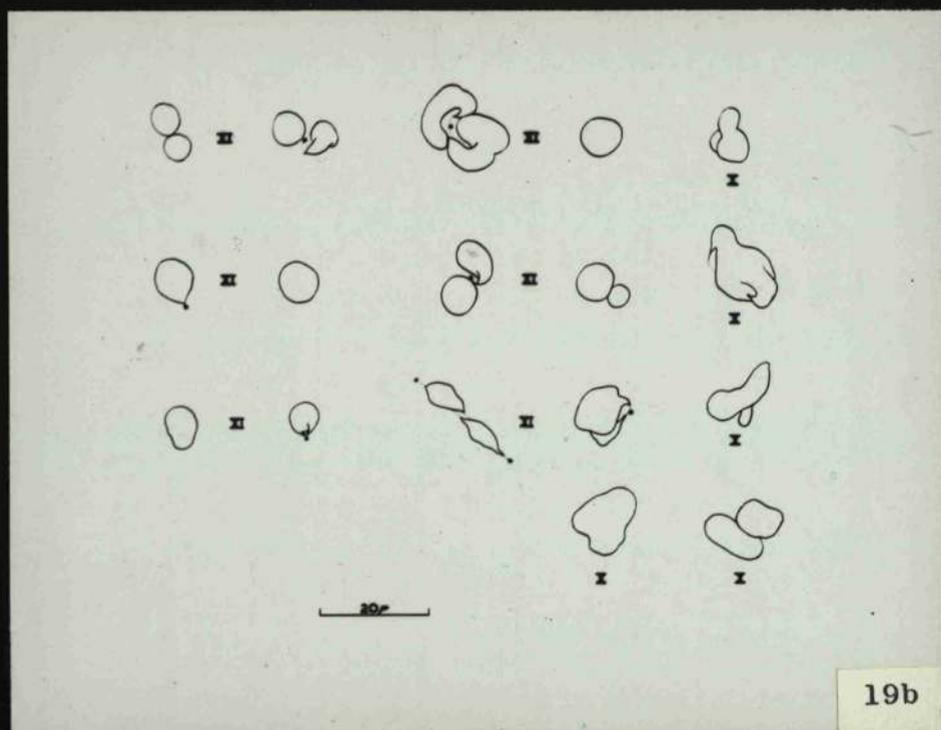
(19b) Newt 58, after treatment with 400 I.U. CG + PMS.

Giant loops characteristically present in newt 58:-

X	+ -
XI	+ +
XII	- -



19a



19b

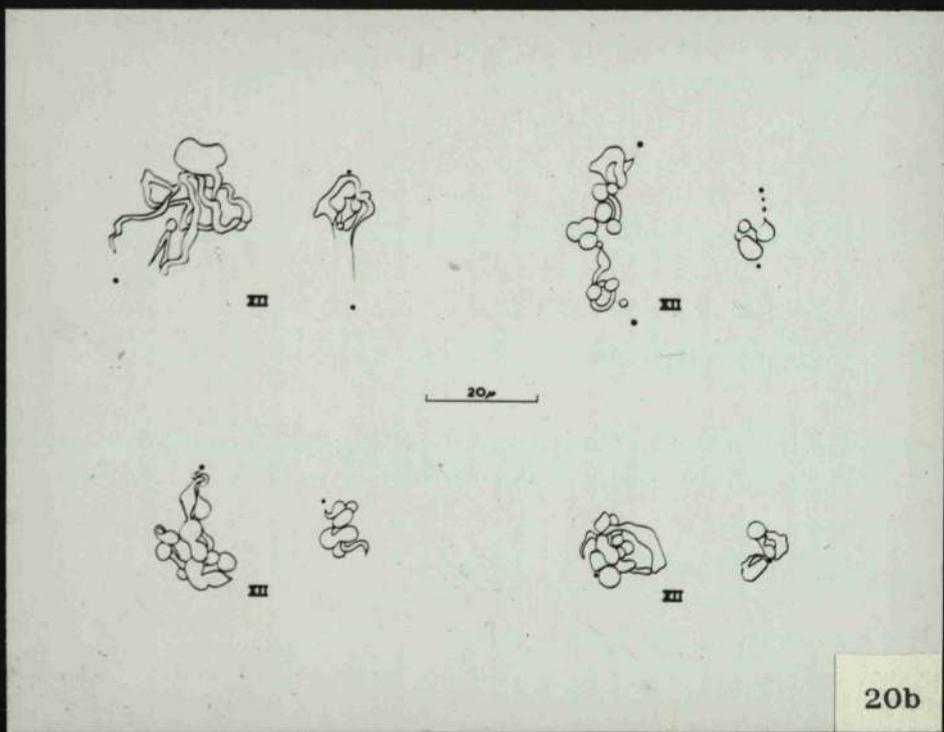
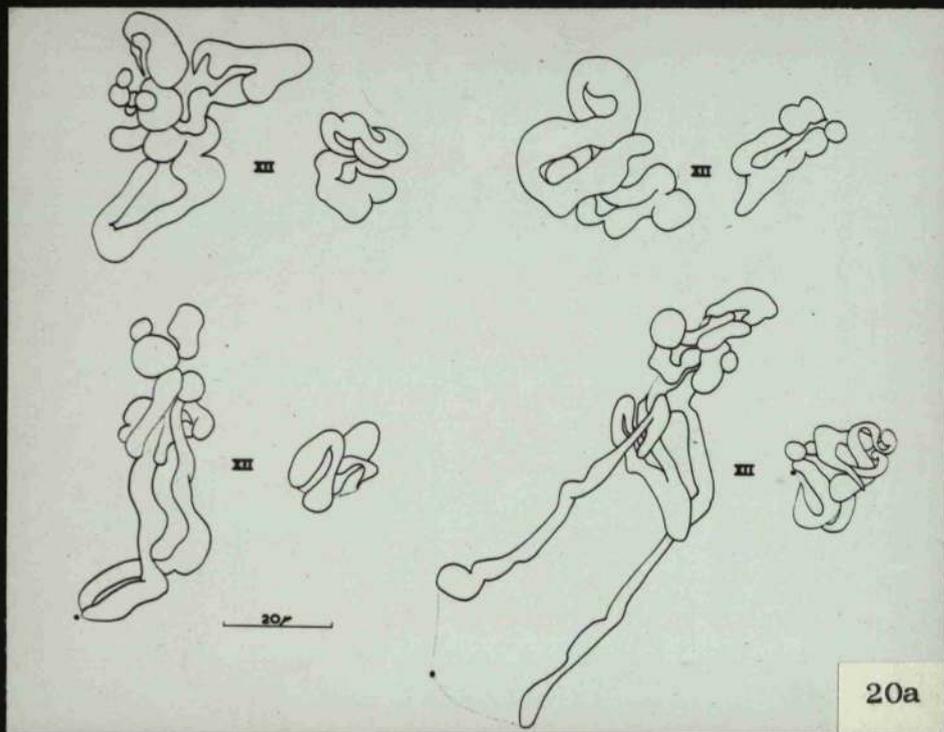
Gonadotrophin treatment.

(20a) Newt 59, before treatment: (bivalent XII)

(20b) Newt 59, after treatment with 400 I.U. CG + PMS.

Giant loops characteristically present in newt 59:-

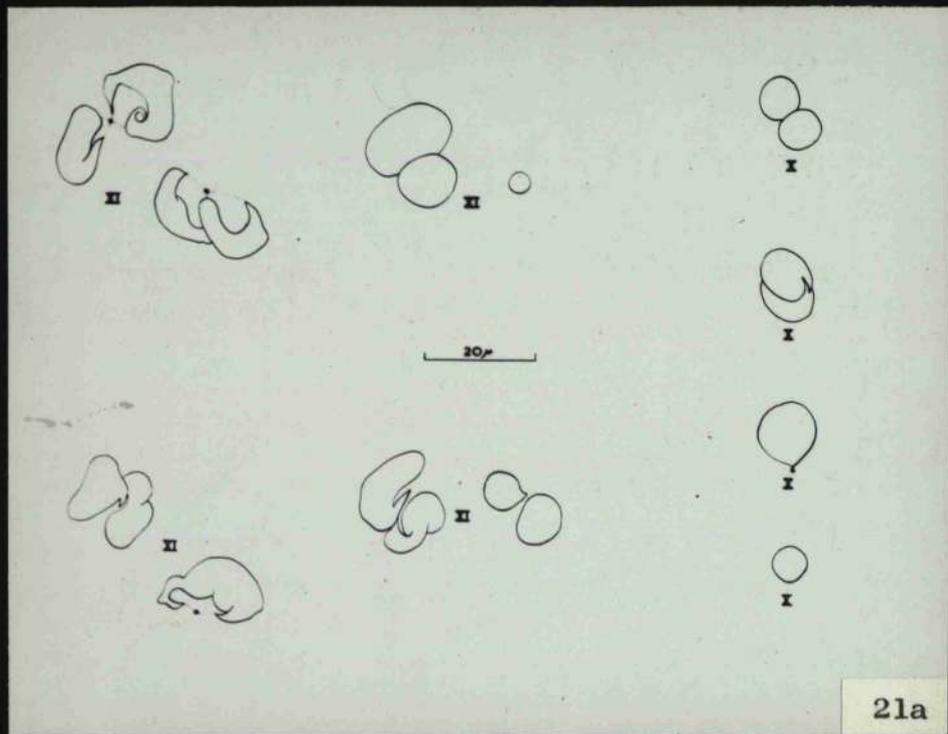
X	+	-
XI	+	+
XII	+	+



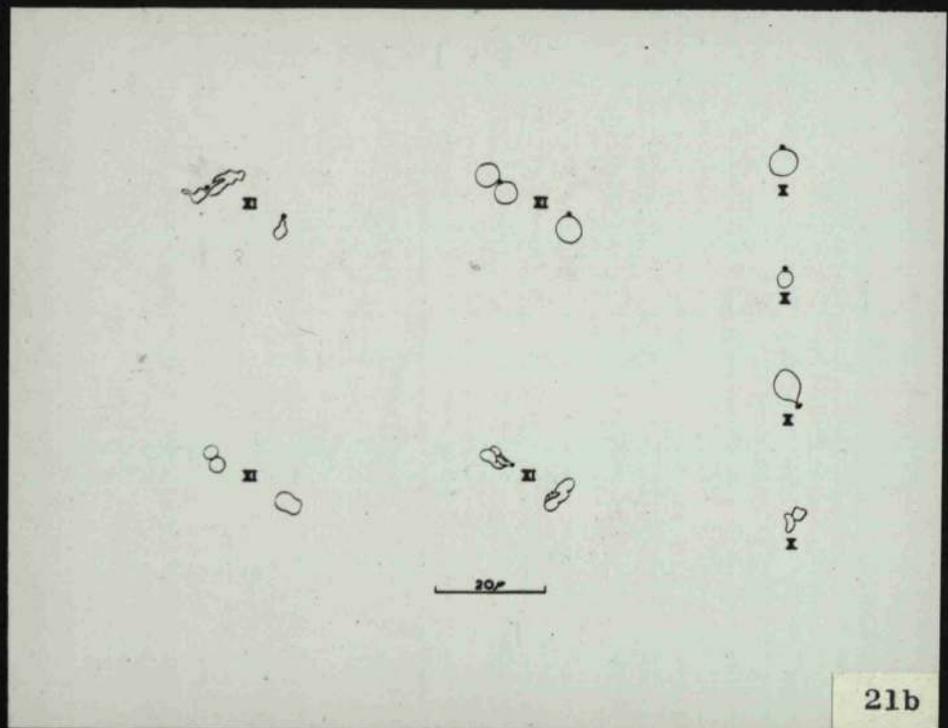
Gonadotrophin treatment

(21a) Newt 59, before treatment: (bivalents X and XI)

(21b) Newt 59, after treatment with 400 I.U. CG + PMS



21a



21b

Controls to gonadotrophin-treated newts

- (22a) Newt 36C, at the start of the experiment.
- (22b) Newt 36C, 9 days later; after it had received injections of sterile water.

Giant loops characteristically present in newt 36C:-

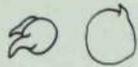
X + +  
XI + +  
XII + -



III



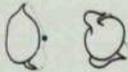
II



I



III



II



I

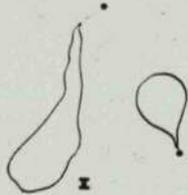
22a



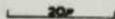
III



II



I



III



II



I

22b

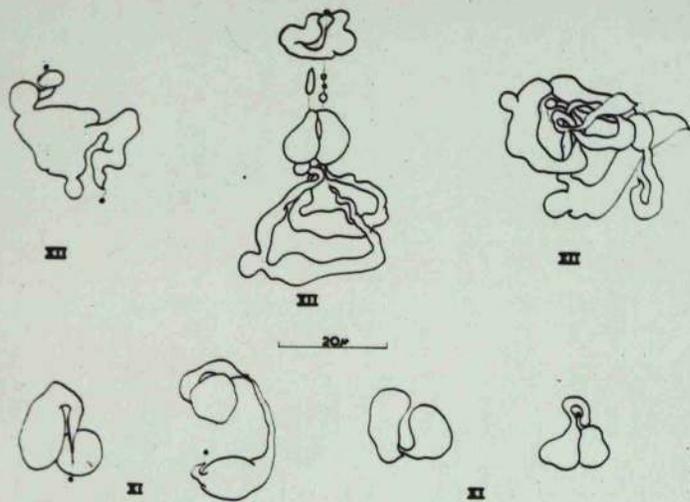
Controls to gonadotrophin-treated newts.

(23a) Newt 59C, at the start of the experiment.

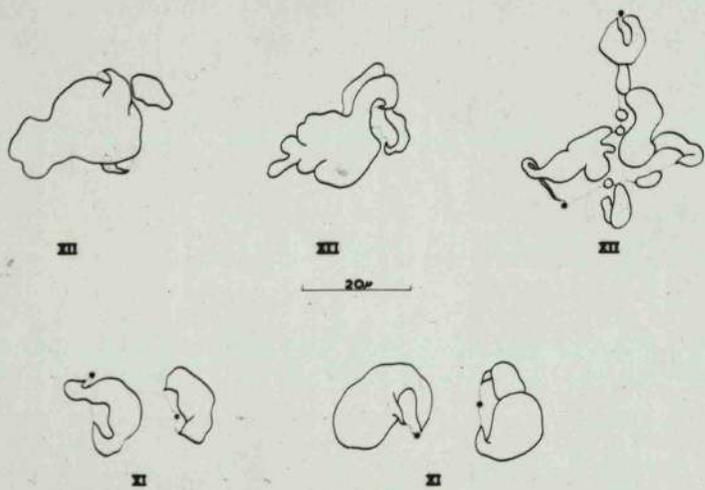
(23b) Newt 59C, 9 days later; after it had received injections of sterile water.

Giant loops characteristically present in newt 59C

X	-	-
XI	+	+
XII	+	-



23a



23b

Hypophysectomised newts.

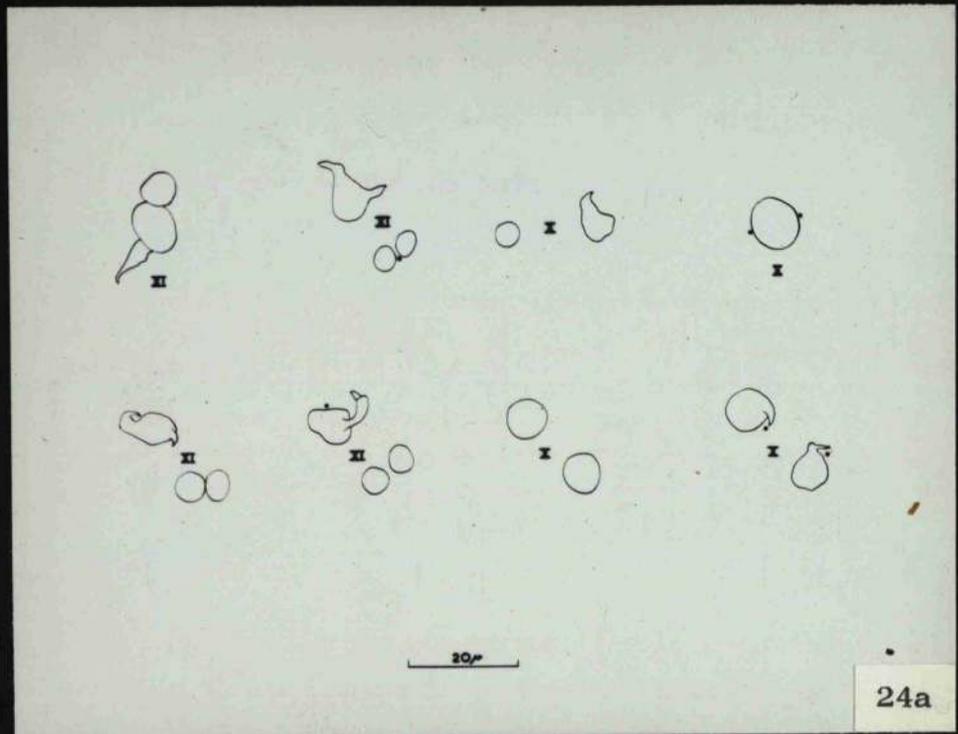
(24a) Newt 13, before hypophysectomy

(24b) Newt 13, 22 days after hypophysectomy.

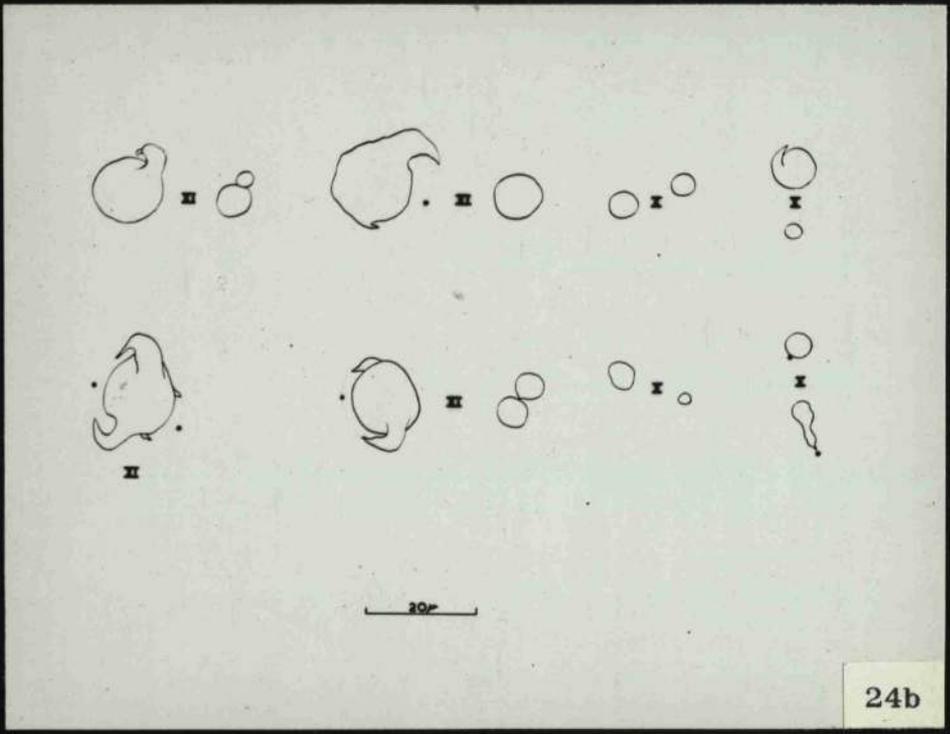
The giant loops on one homologue of bivalent XI increased in size after hypophysectomy.

Giant loops characteristically present in newt 13:-

X	+	+
XI	+	+
XII	-	-



24a



24b

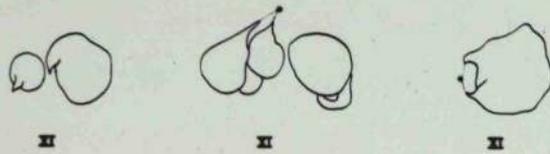
Controls to hypophysectomised newts.

(25a) Newt 13C, before mock hypophysectomy,

(25b) Newt 13C, 22 days after mock hypophysectomy.

Giant loops characteristically present in newt 13C:-

X	-	-
XI	+	+
XII	-	-



20 $\mu$



25a



20 $\mu$



25b

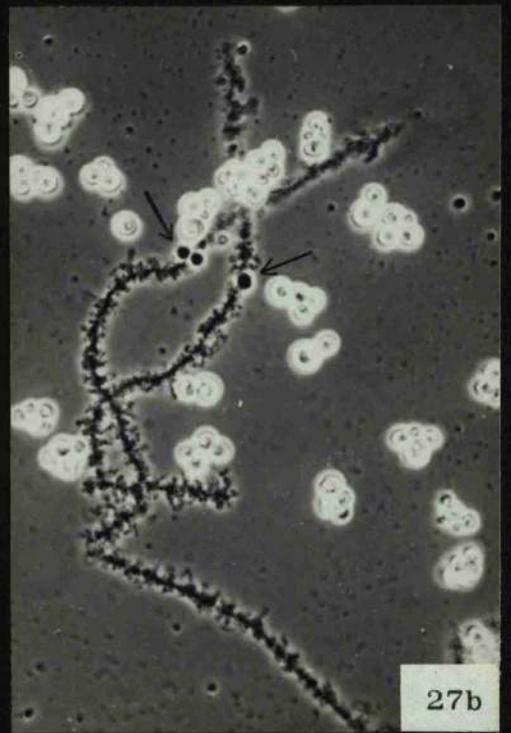
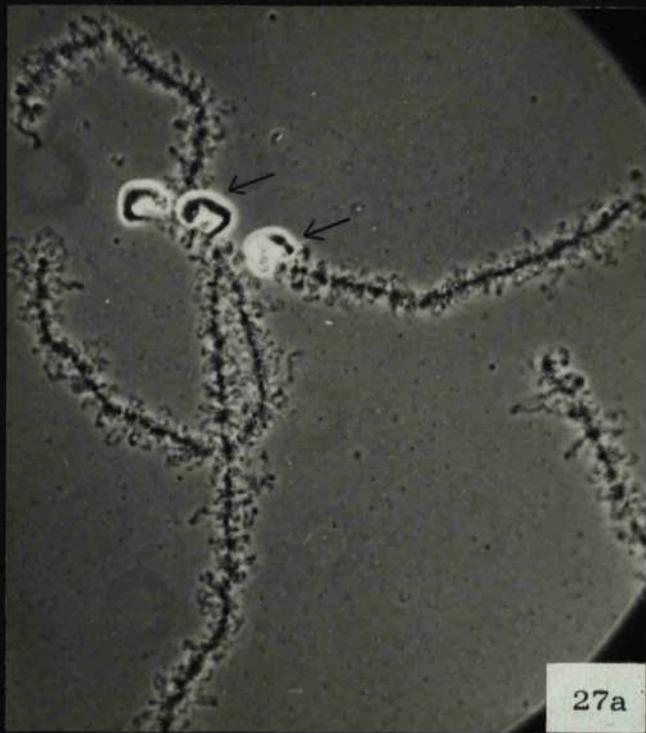
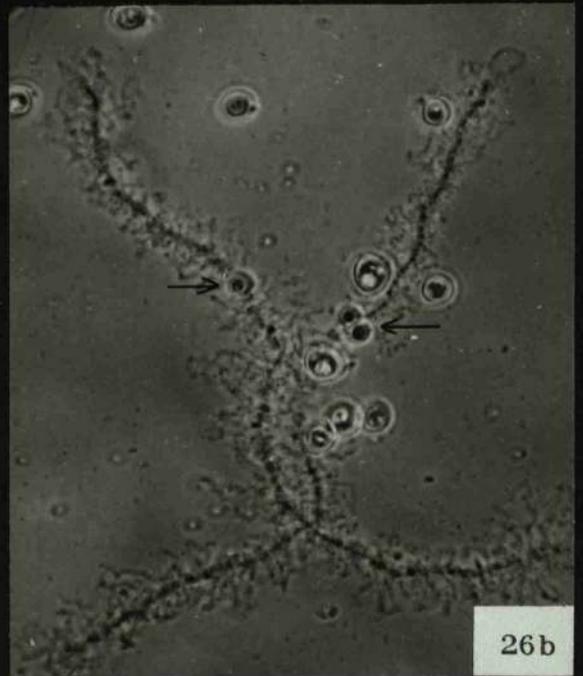
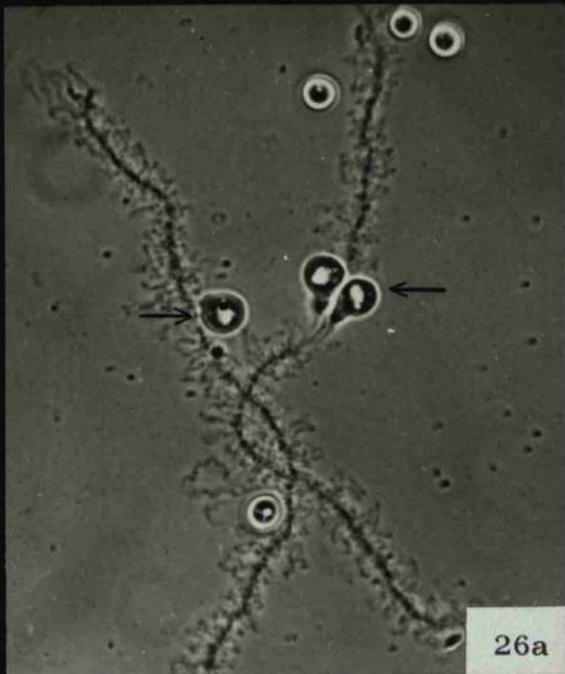
Figures 26 and 27.

Photographed with the IPCM and x 40 objective.

- (26a) Newt 32, before treatment. Bivalent XI entire with conspicuous giant loops (arrowed).
- (26b) Newt 32, after treatment with 400 I.U. CG (Swiss). Bivalent XI entire showing small dense giant loops (arrowed).
- (27a) Newt 58, before treatment. Bivalent XI entire with conspicuous giant loops (arrowed).
- (27b) Newt 58, after treatment with 400 I.U. CG + PMS. Bivalent XI entire showing small dense giant loops, (arrowed).

In all lampbrush preparations made from standard-size oocytes of newt 32 and 58 before treatment, sister giant loops were fused on one homologue of bivalent XI but separate on the other. Gonadotrophin treatment did not alter this feature.

Scale = 80 $\mu$



Figures 28 to 30.

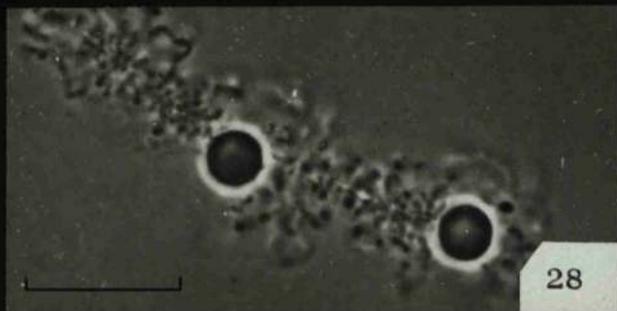
Photographed with the IPCM and x 95 objective.

- (28) carnifex anon. End of the left arm of chromosome V showing "typical" homogeneous spheres at 2 and 11 units.  
Scale = 20 $\mu$
- (29) Newt 59, after treatment with 400 I.U. CG (Organon). End of the left arm of chromosome V showing a large vacuolated sub-terminal sphere and a sphere at 11 units which is probably in the process of dividing.  
Scale = 20 $\mu$
- (30a) Newt 13, before hypophysectomy. Ends of the left arms of bivalent VIII showing a single fused sub-terminal sphere containing two internal refractile bodies (IRBS).  
Scale = 20 $\mu$
- (30b) Newt 13, 22 days after hypophysectomy. Ends of the left arms of bivalent VIII with a small fused sub-terminal sphere. Objects marked "N" are free "nucleoli".  
Scale = 20 $\mu$

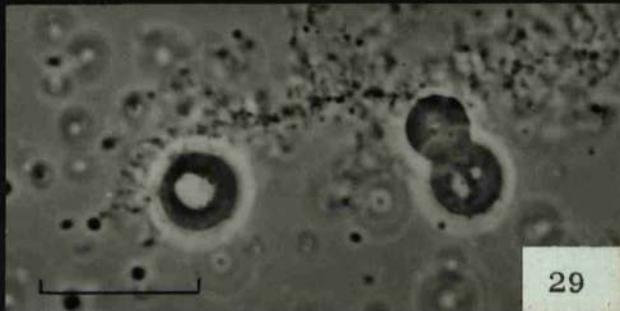
Figures 31a and 31b.

Photographed with the IPCM and x 40 objective.

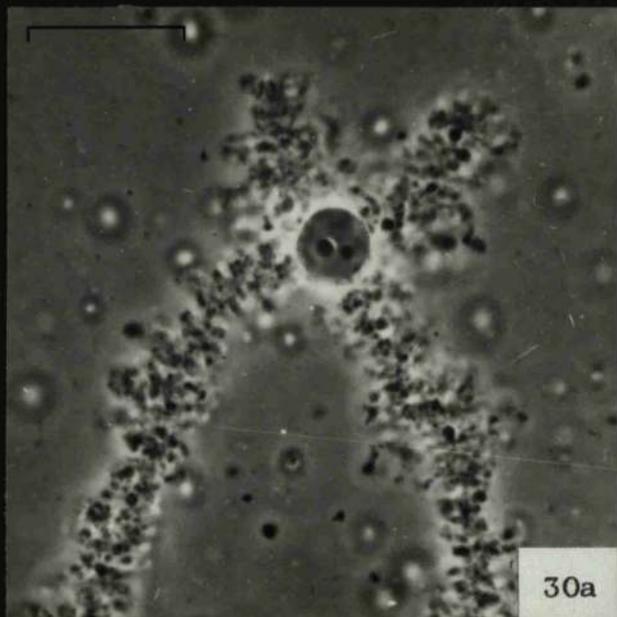
- (31a) Newt 36, before treatment. Bivalent XI entire with giant loops and conspicuous currant buns (arrowed)  
Scale = 40 $\mu$
- (31b) Newt 36, after treatment with 400 I.U. CG (Swiss). Bivalent XI entire. Currant buns are no longer evident.  
Scale = 40 $\mu$



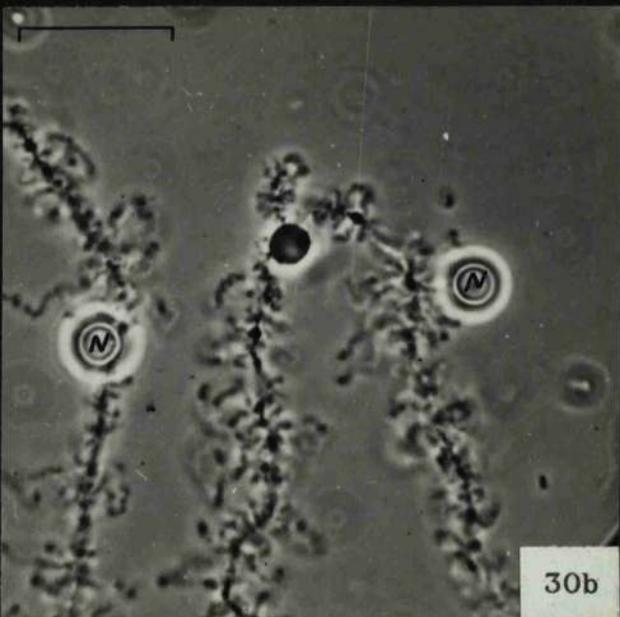
28



29



30a



30b



31a



31b

Figures 32 to 34.

Photographed with the IPCM and x 95 objective.

- (32) Newt 52, after treatment with 400 I.U. CG + PMS. Part of the heteromorphic arm of chromosome I (see Page 52, Chapter II) showing bunching at axial granule loci. Arrows point to sites where bunching is particularly evident. The large bright objects are free "nucleoli". Note the resemblance between free granules (FG) and the granules attached to the chromosomes.
- (33a) Newt 13, before hypophysectomy; Part of the heteromorphic arm of chromosome I with conspicuous axial granules.
- (33b) Newt 13, 22 days after hypophysectomy. Part of the heteromorphic arm of chromosome I. Axial granules are small and scarcely distinguishable from nearby chromomeres.
- (34a) Newt 10, before hypophysectomy. Double axis ends of bivalent XII.
- (34b) Newt 10, 35 days after hypophysectomy. Double axis ends of bivalent XII.

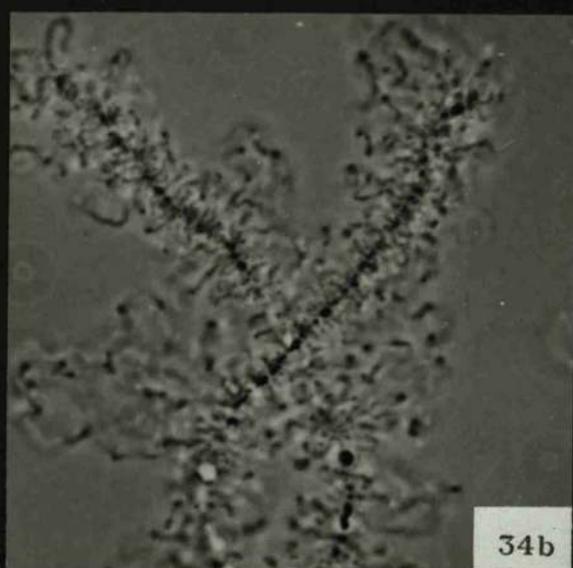
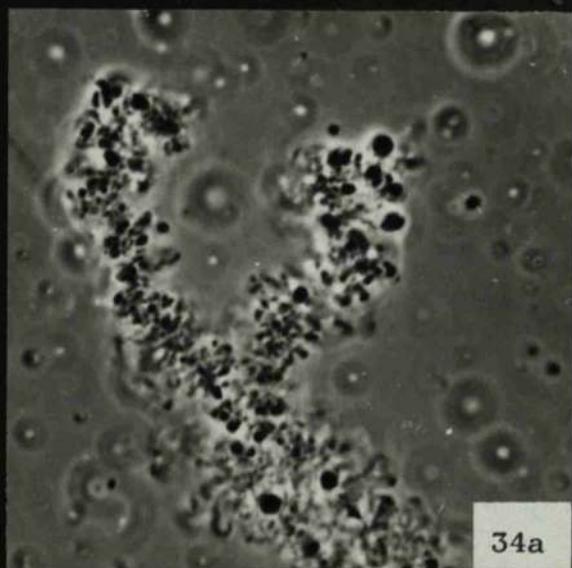
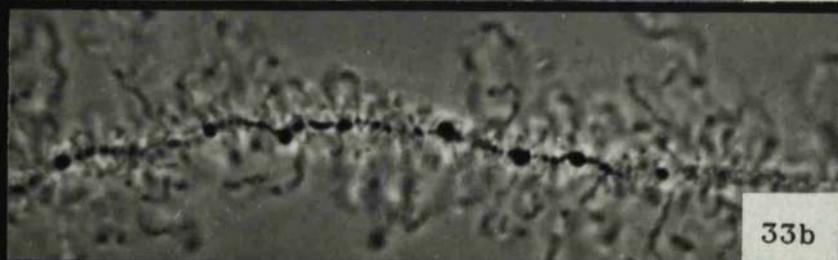
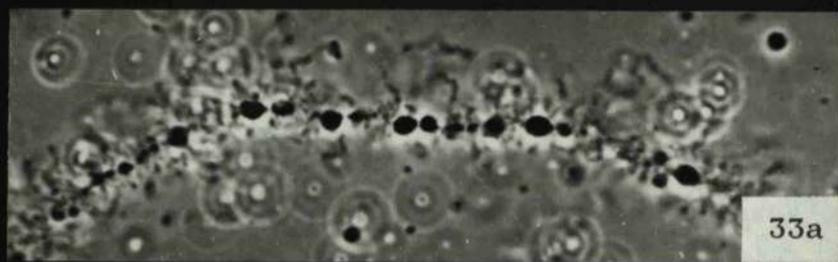
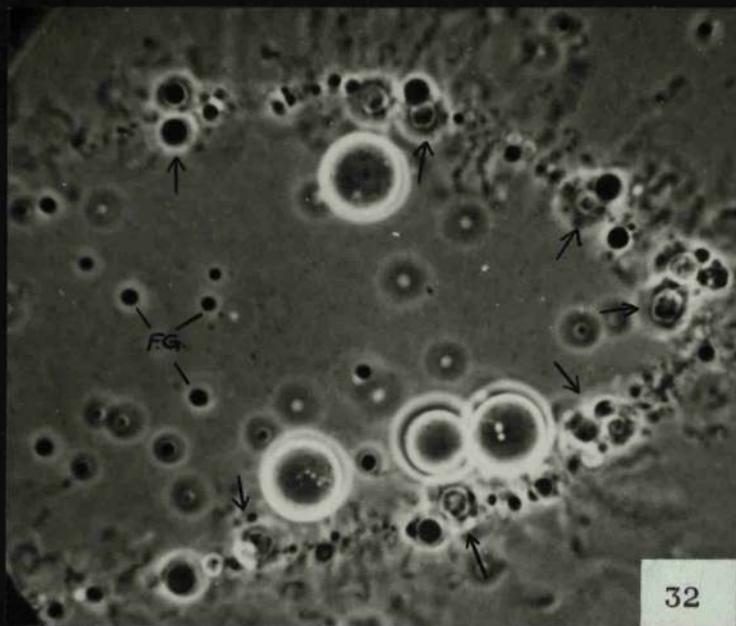
Scale = 40 $\mu$

Figures 32 to 34.

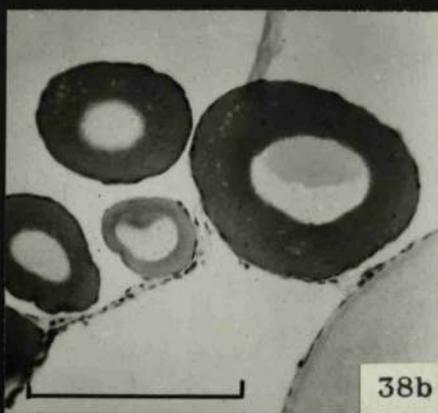
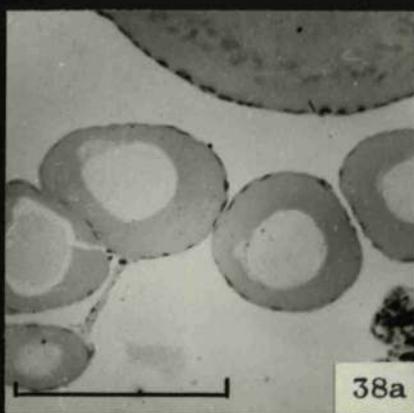
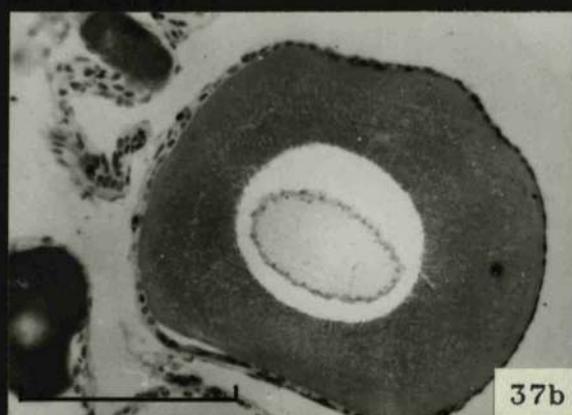
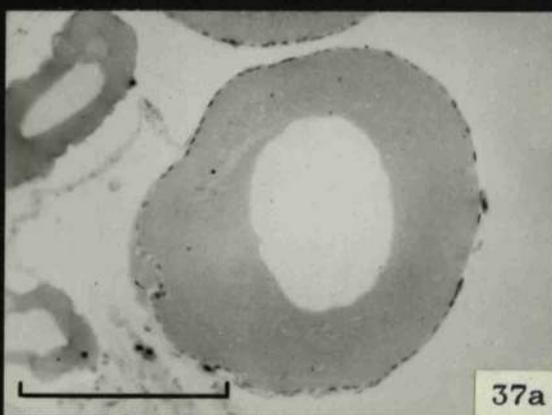
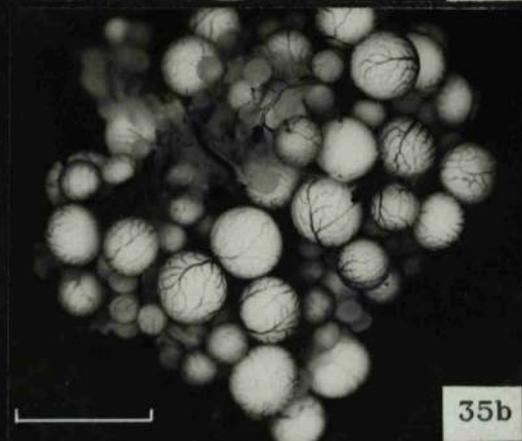
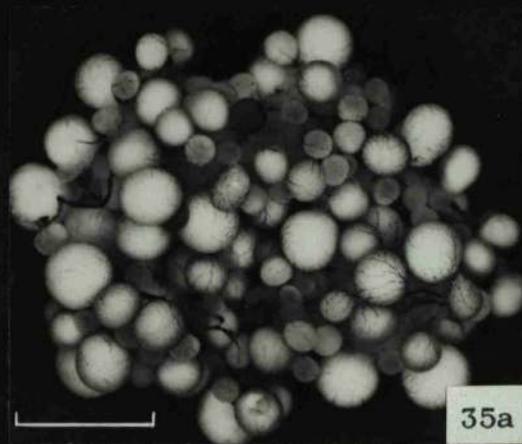
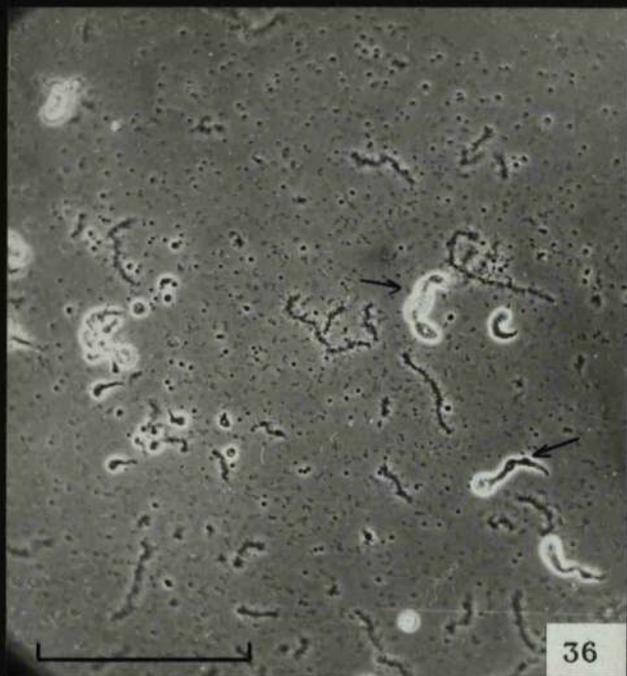
Photographed with the IPCM and x 95 objective.

- (32) Newt 52, after treatment with 400 I.U. CG + PMS. Part of the heteromorphic arm of chromosome I (see Page 52, Chapter II) showing bunching at axial granule loci. Arrows point to sites where bunching is particularly evident. The large bright objects are free "nucleoli". Note the resemblance between free granules (FG) and the granules attached to the chromosomes.
- (33a) Newt 13, before hypophysectomy; Part of the heteromorphic arm of chromosome I with conspicuous axial granules.
- (33b) Newt 13, 22 days after hypophysectomy. Part of the heteromorphic arm of chromosome I. Axial granules are small and scarcely distinguishable from nearby chromomeres.
- (34a) Newt 10, before hypophysectomy. Double axis ends of bivalent XII.
- (34b) Newt 10, 35 days after hypophysectomy. Double axis ends of bivalent XII.

Scale = 40 $\mu$







Figures 43 to 47.

Diagrams constructed as described on page 68 (Chapter II) to show the effects of different experimental treatments upon the rate of uptake of  $^{32}\text{P}$  into oocytes of different sizes.

The height of each individual column was determined by the mean of the SRs of at least 3 oocytes.

Columns	a	represent	the	mean	SR	of	oocytes	of	0.03	to	0.25	mm <sup>3</sup>
"	<u>b</u>	"	"	"	"	"	"	"	0.26	to	0.6	mm <sup>3</sup>
"	<u>c</u>	"	"	"	"	"	"	"	0.61	to	1.0	mm <sup>3</sup>
"	<u>d</u>	"	"	"	"	"	"	"	over	1	mm <sup>3</sup> .	

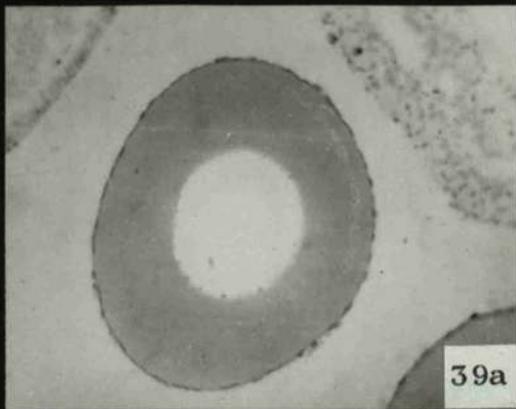
Figure 43.

The effect of treatment with 400 I.U. CG + PMS.

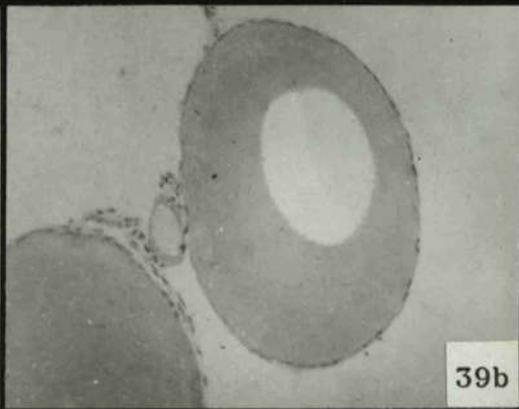
Experimental newts - 48, 49, 51, 52.

Control newts - 48C, 49C, 51C, 52C.

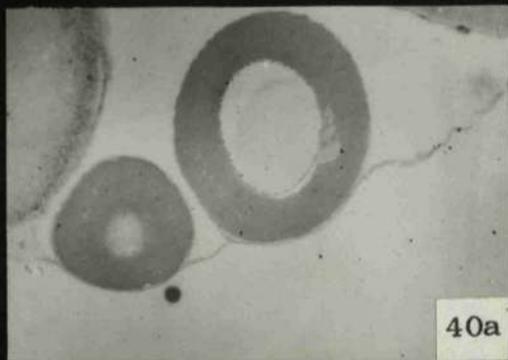
(b' refers to oocytes of 0.26 to 0.4 mm<sup>3</sup>; b'' to oocytes of 0.4 to 0.6 mm<sup>3</sup>.)



39a



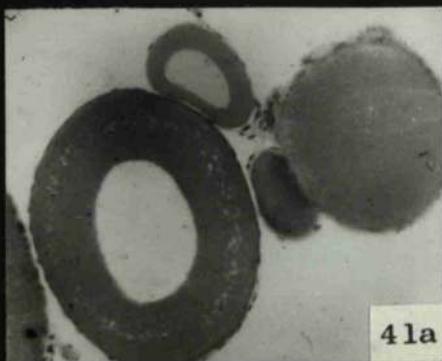
39b



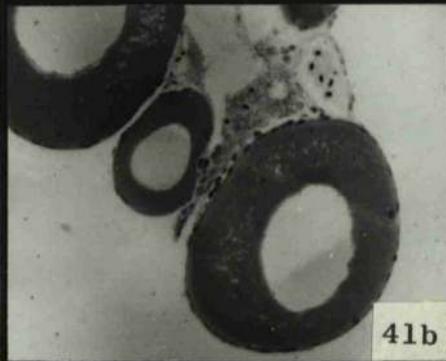
40a



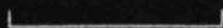
40b



41a



41b



(39a) Newt 35C, at the start of the experiment.

(39b) Newt 35C, 9 days later, after it had received injections of sterile water.

Figures 40 and 41.

Photographed with a conventional microscope system and x 10 objective.

Sections of newt oocytes fixed in Bouin, impregnated with celloidin, embedded in wax, and stained with pyronine at pH 4.7.

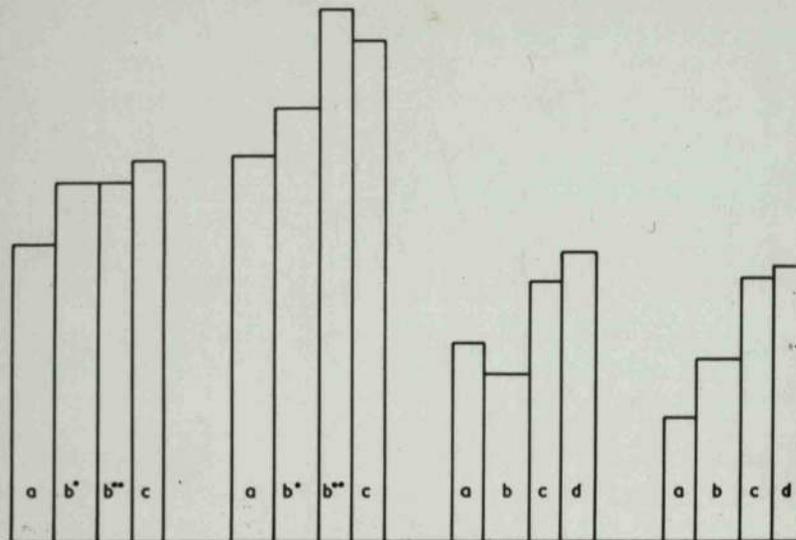
(40a) Newt 51, before treatment.

(40b) Newt 51, after treatment with 400 I.U. CG + PMS.

(41a) Newt 51C, at the start of the experiment.

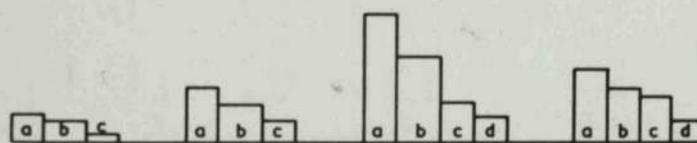
(41b) Newt 51C, 9 days later, after it had received injections of sterile water.

Scale = 0.4mm.



EXPERIMENTALS

C.G.+PMS.



CONTROLS

Figure 44.

The effect of treatment with 400 I.U. CG (Organon).

Experimental newts - 55 and 57.

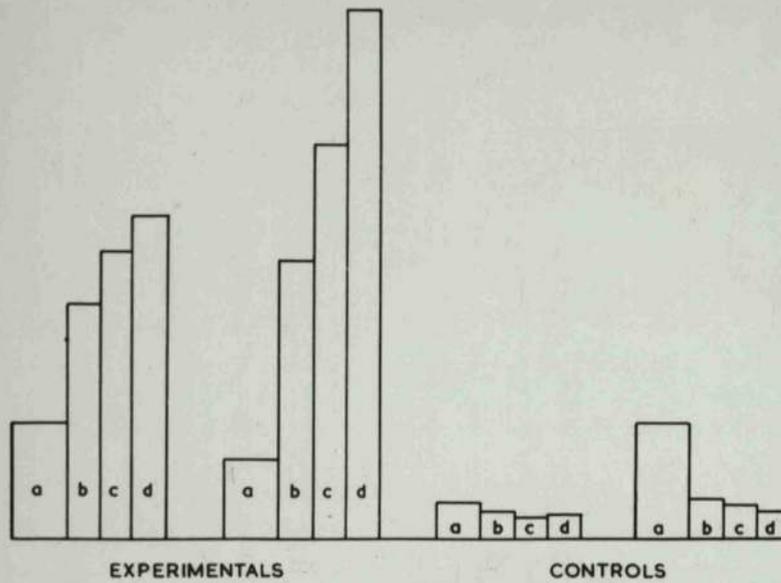
Control newts - 55C and 57C.

Figure 45.

The effects of treatment with 400 I.U. PMS (Organon).

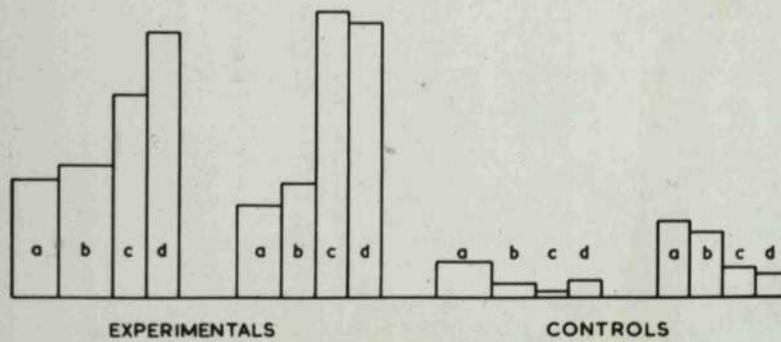
Experimental newts - 54 and 53.

Control newts - 54C and 53C.



C.G. ONLY

44



P.M.S. ONLY

45

Figure 46.

The effects of hypophysectomy.

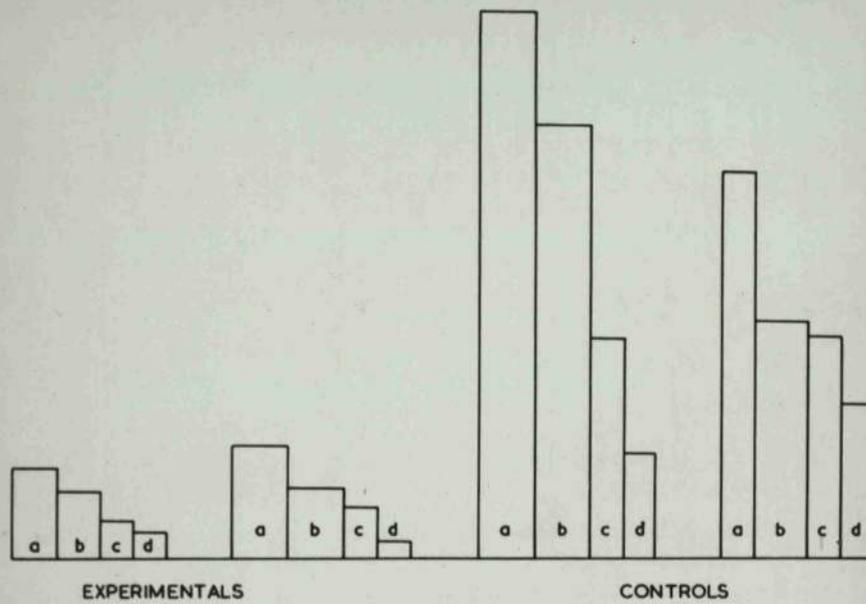
VERTICAL SCALE x 10

Experimental newts - 22 (35 day exp.period); 24 (30 day)

Control newts - 22C, 24C.

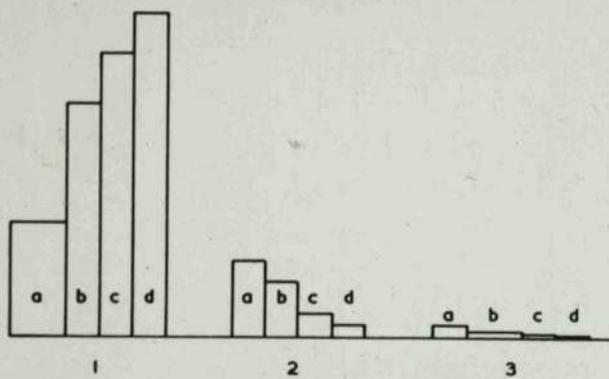
Figure 47.

Constructed on the same scale as Figs.43 to 45, showing different patterns of  $^{32}\text{P}$  uptake into the oocytes of three newts, one of which was gonadotrophin-treated, one untreated, and one hypophysectomised.



HYPOPHYSECTOMY

46



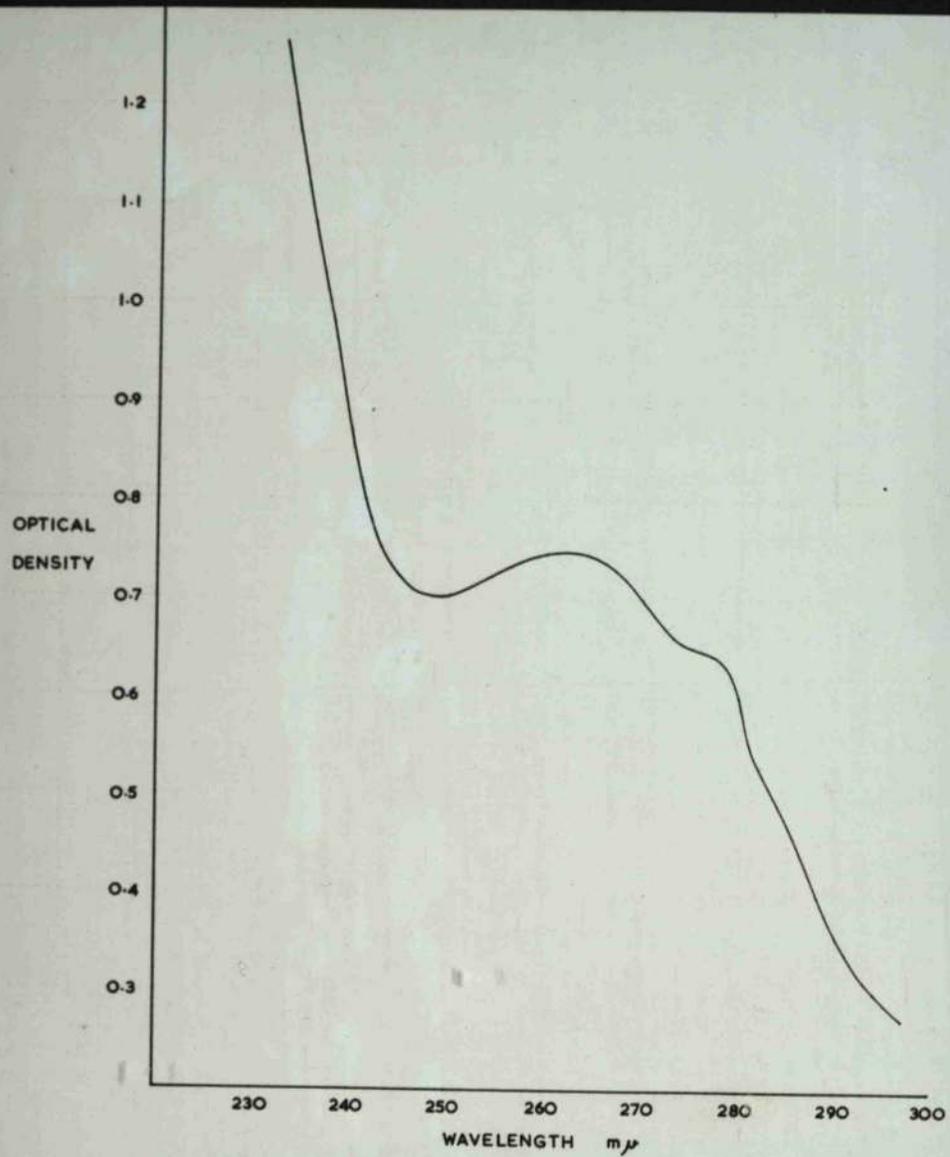
1. INJECTION OF C.G.
2. UNTREATED
3. HYPOPHYSECTOMISED 20 days.

47

Figure 48.

U.V. absorption curve for a colloidal solution of the sap of 100 standard-size-oocyte nuclei in water adjusted to pH 9 by addition of NaOH. The nuclei were obtained from a gonadotrophin-treated newt whose sap factor\* was 10+.

\* See Page 39, Chapter II for definition.



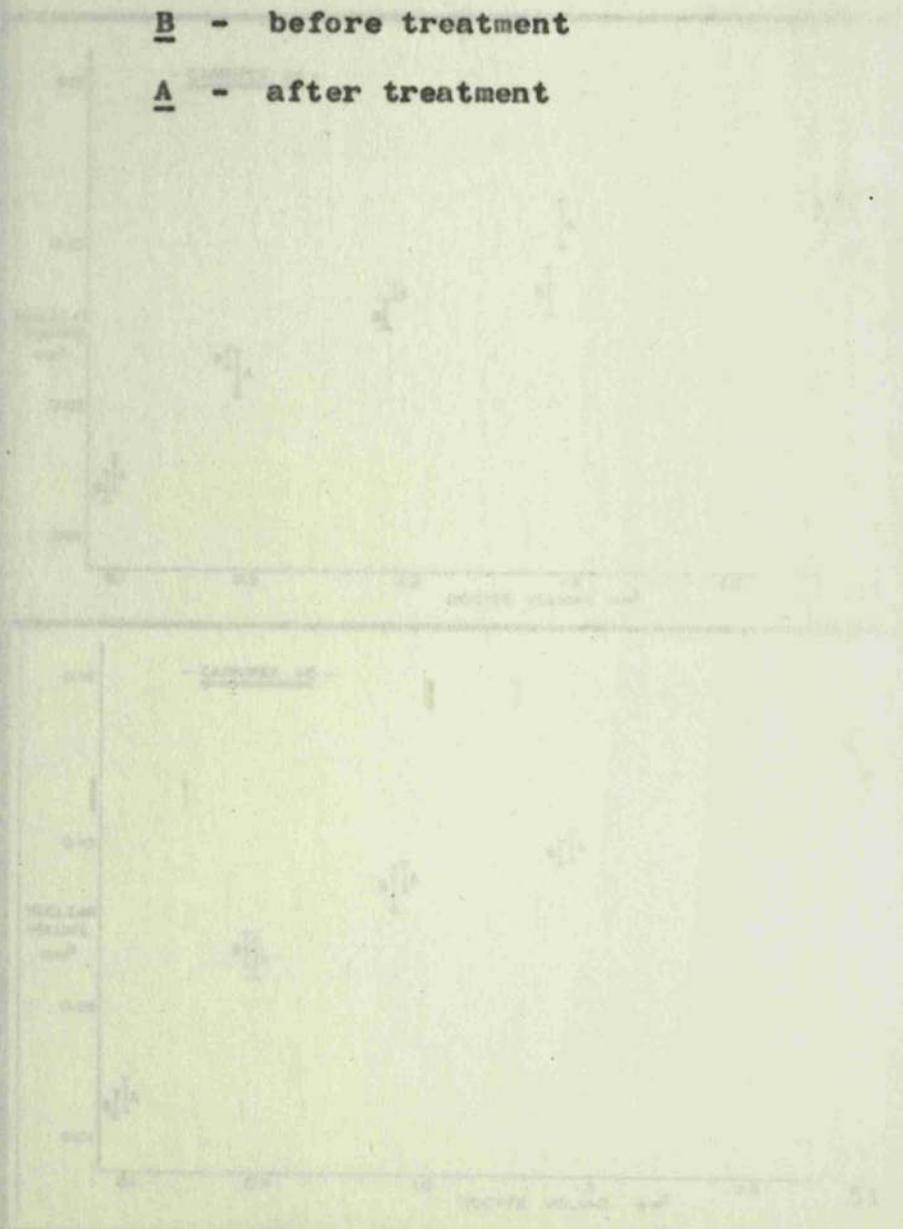
Figures 49, 50, and 51.

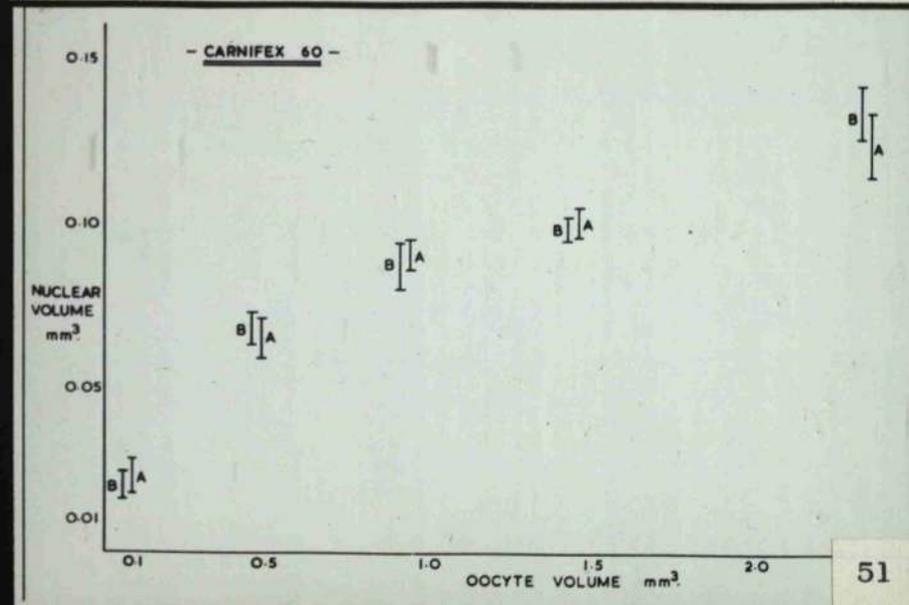
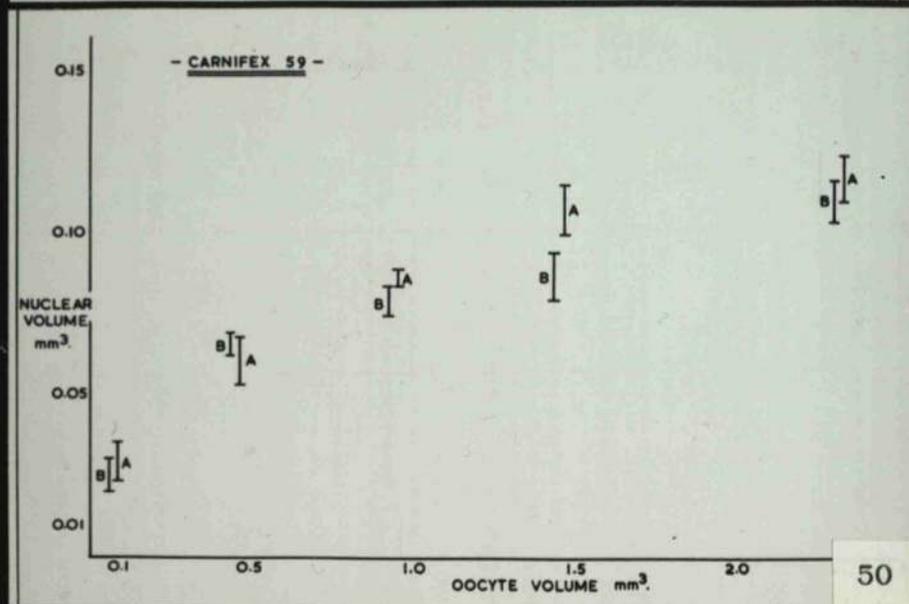
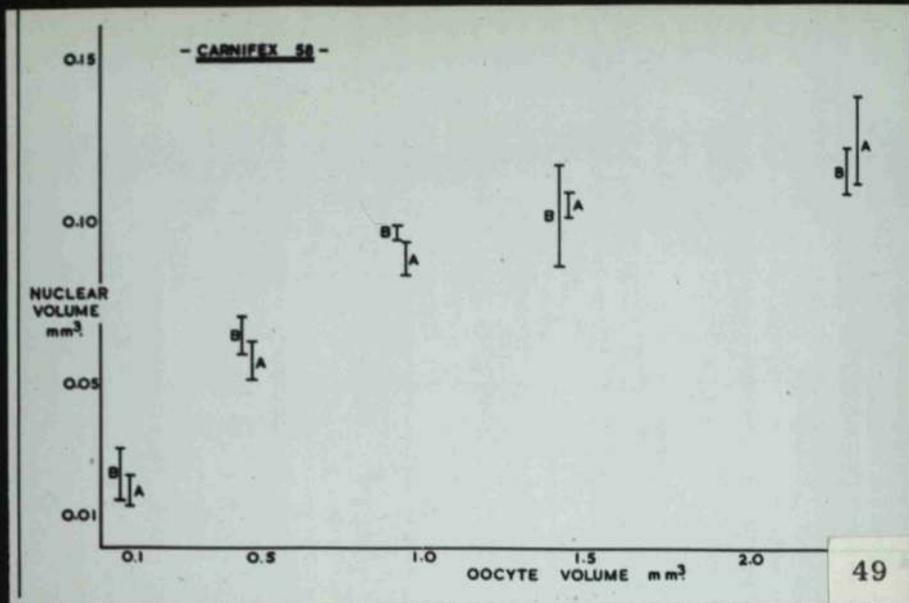
Diagrams representing the results of a survey which was designed to detect changes in nuclear volume with respect to oocyte volume following - though not necessarily resulting from - gonadotrophin treatment, (see Page 74, Chapter II).

Each value is the mean and standard deviation of the volumes of the nuclei of 5 like-sized oocytes.

B - before treatment

A - after treatment





Figures 52, 53, and 54.

Photographed with the IPCM and x 40 objective.

- (52) carnifex 33, bivalent X entire, homozygous for giant loops (arrowed). On both homologues the matrices of sister giant loops have fused. N = free "nucleolus".
- (53) carnifex 9, bivalent XI entire, homozygous for giant loops (arrowed). There is no fusion between sister giant loops. N = free "nucleolus".
- (54) carnifex 32, bivalent XII entire, homozygous for massive multiple giant loops. Double bridges have formed at the giant loop loci on both homologues. N = free "nucleolus".

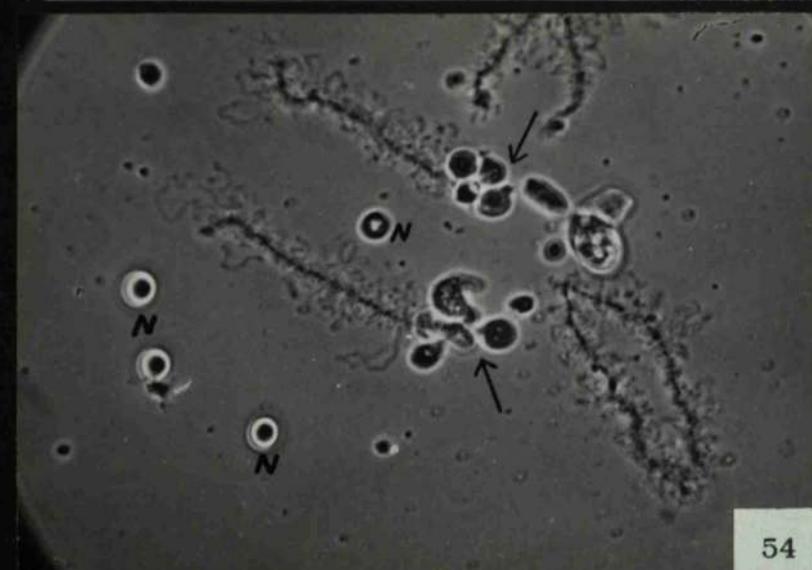
Scale = 80 $\mu$



52



53



54

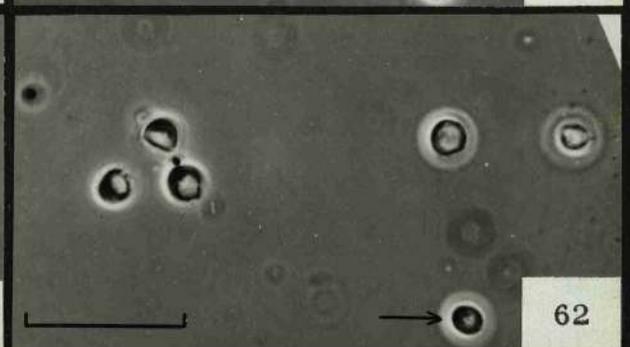
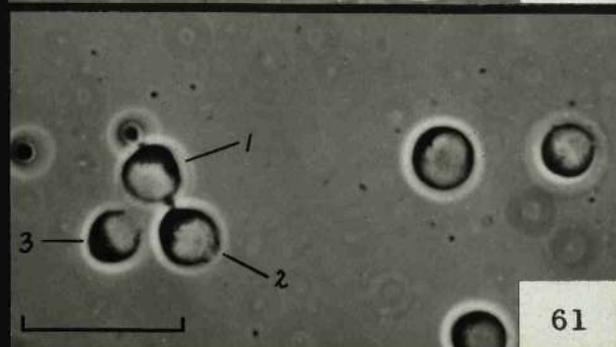
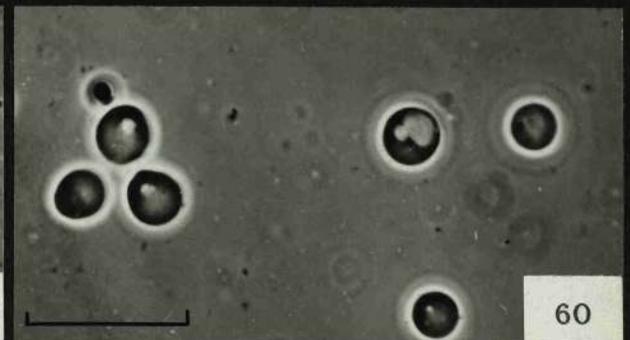
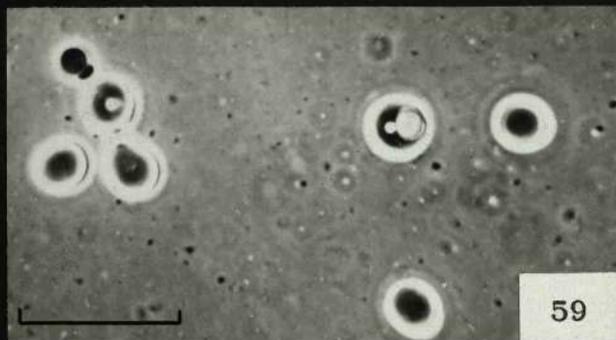
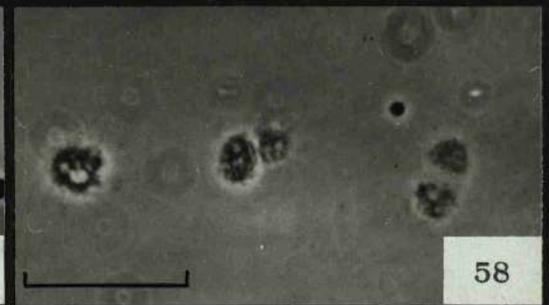
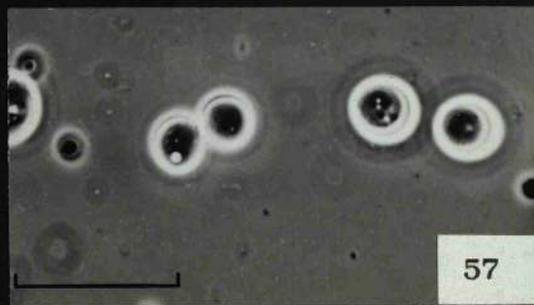
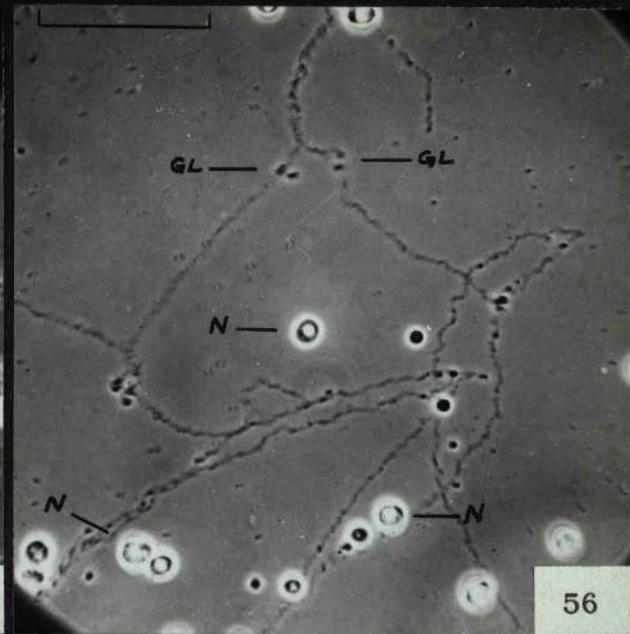
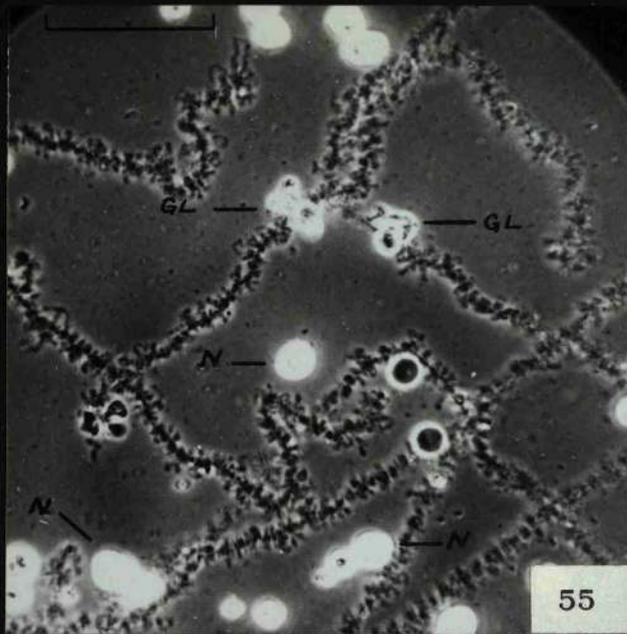


Figures 55 to 62.

The action of trypsin on oocyte "nucleoli".

- (55) Photographed with the IPCM and x 40 objective.  
Part of a preparation of carnifex lampbrush chromosomes.  
C medium, pH 6.2  
N = free "nucleolus".  
GL = giant loops on bivalent XI. Scale = 50 $\mu$
- (56) Same as (55) 30 minutes after application of a solution of trypsin in C medium (pH 6.2). The giant loops are greatly reduced in size and refractility: "nucleoli" are noticeably smaller but they have retained their spherical shape and brightness. Scale = 50 $\mu$
- (57) Photographed with the IPCM and x 95 objective.  
carnifex free "nucleoli" in C medium at pH 6.2. Scale = 25 $\mu$
- (58) Same as (57) 3 minutes after application of a solution of trypsin in modified C medium (pH 7.8). 30 seconds after this photograph was taken the "nucleoli" had disappeared. Scale = 25 $\mu$
- (59) Photographed with the IPCM x 95 objective.  
carnifex free "nucleoli" in C medium at pH 6.2. Scale = 25 $\mu$
- (60) Same as (59) 5 minutes after application of a solution of T2\* in C medium, (pH 6.2). Scale = 25 $\mu$
- (61) Same as (59) 12 minutes after application of the enzyme solution. "Nucleoli" labelled 1, 2, and 3 show three stages in rupture of the "nucleolar" shell. The "nucleolus" on the extreme right is on the point of bursting. Scale = 25 $\mu$
- (62) Same as (59) 30 minutes after application of the enzyme solution, showing collapsed empty shells of five "nucleoli". One "nucleolus" (arrowed) in this picture did not burst. Scale = 25 $\mu$

\* For a definition of T2, see Page 86 of Chapter III.



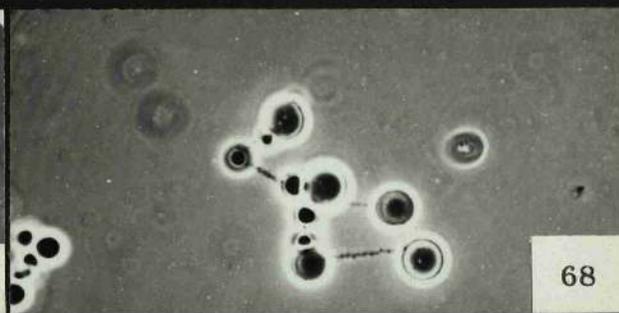
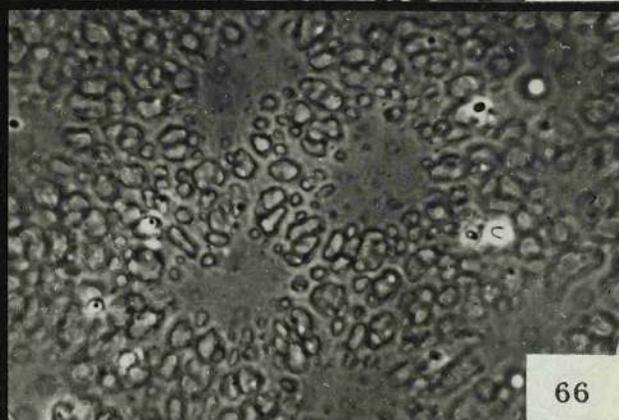
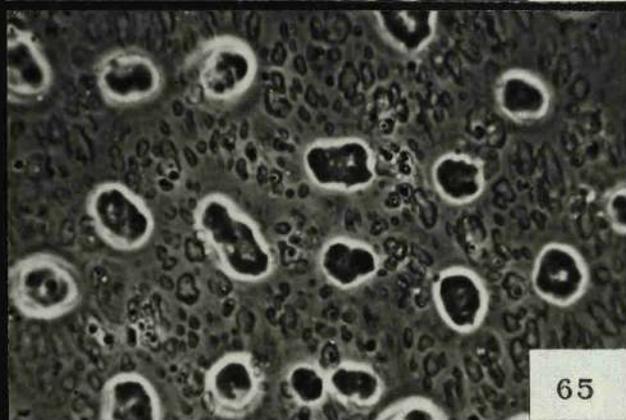
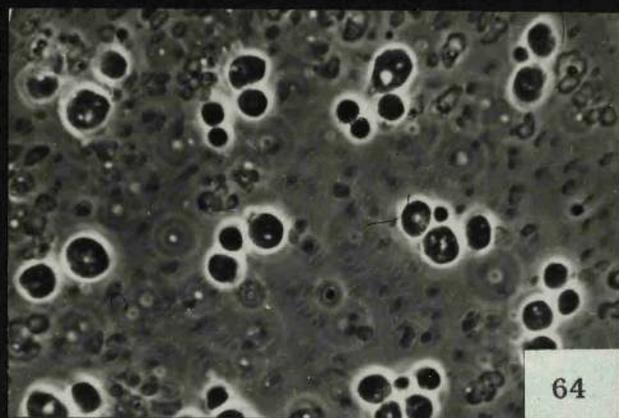
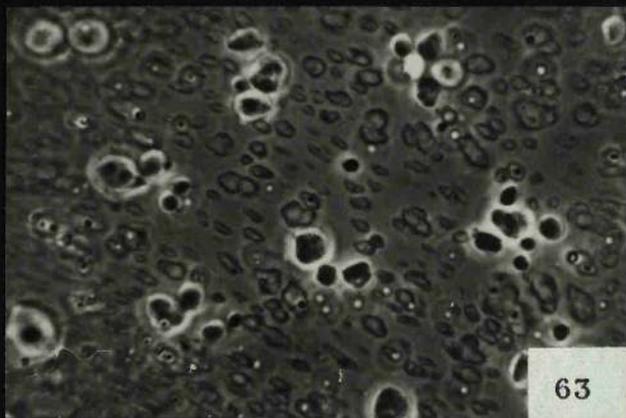
Figures 63 to 66

Photographed with the IPCM and x 95 objective.

Parts of the membranes of standard-size-oocytes nuclei. Each membrane was spread out over the bottom of an observation chamber containing C medium.

- (63) Newt 24, 30 days after hypophysectomy. Small "nucleoli" arranged in groups of 3 to 6, all firmly attached to the nuclear membrane.
- (64) Newt 27, 46 days after hypophysectomy. "Nucleoli" arranged in groups and all firmly attached to the nuclear membrane.
- (65) Newt 26, 37 days after hypophysectomy. Solitary flattened "nucleoli" attached to the nuclear membrane.
- (66) Newt 58, after treatment with 400 I.U. CG + PMS. There were no "nucleoli" attached to the nuclear membranes of standard-size oocytes in this newt. The picture illustrates "pocketing" of the nuclear membrane which was particularly evident in nuclei from gonadotrophin-treated newts.
- (67) Photographed with the IPCM and x 95 objective.
- (68) Free "nucleoli" attached to one another by "strands" (see Page 96, Chapter III).

Scale = 25 $\mu$



Figures 69 to 75.

Drawings made with the IPCM, x 95 objective, x 10 eyepiece, and camera lucida (see Page 95, Chapter III): showing the sizes and distribution of primary and secondary "nucleoli" on the nuclear membranes of carnifex (Figs.69 to 72) and danubialis (Figs.73 to 75) oocytes. Nuclei isolated in 0.2 M 5:1 K/NaCl.

(69) carnifex 62, primary "nucleoli".

Oocyte diameter = 0.13mm  
Nuclear diameter = 89 $\mu$

(70) carnifex 62, flattened secondary "nucleoli" with pseudopodia-like processes.

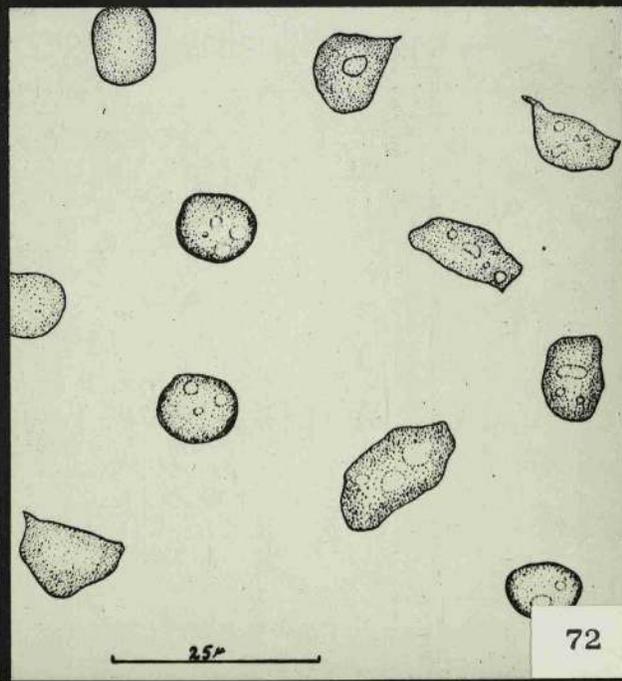
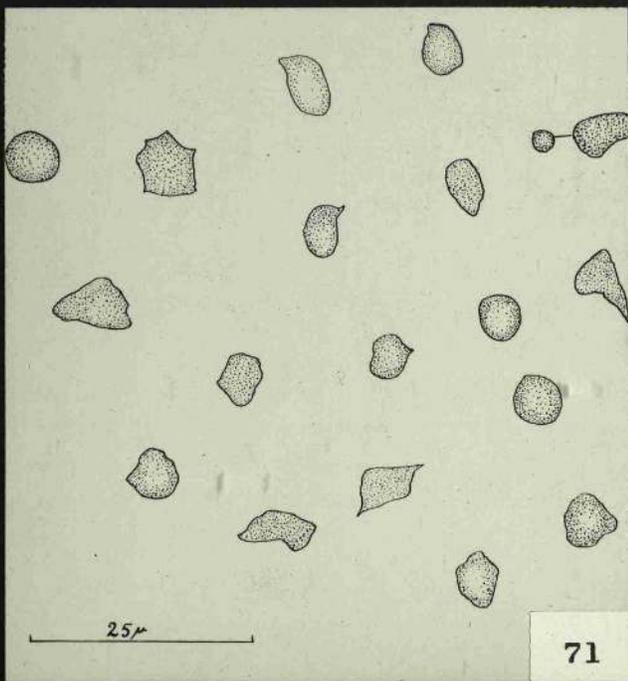
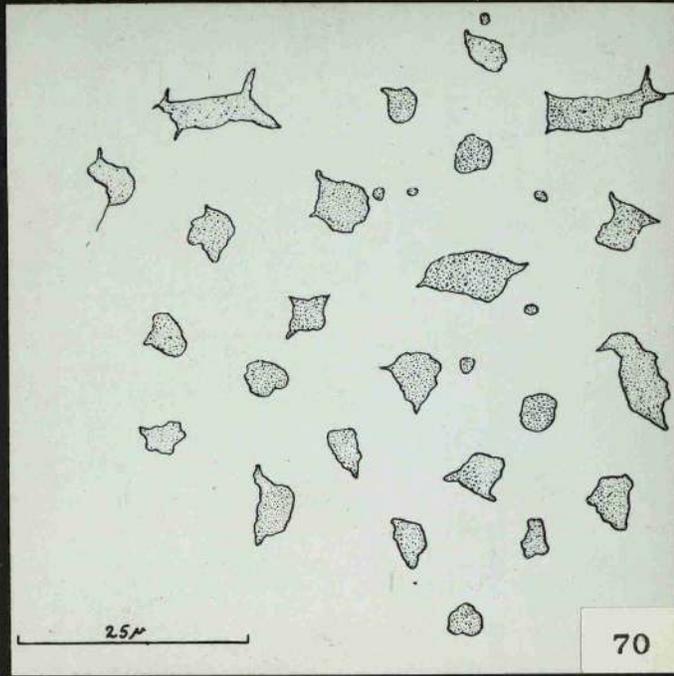
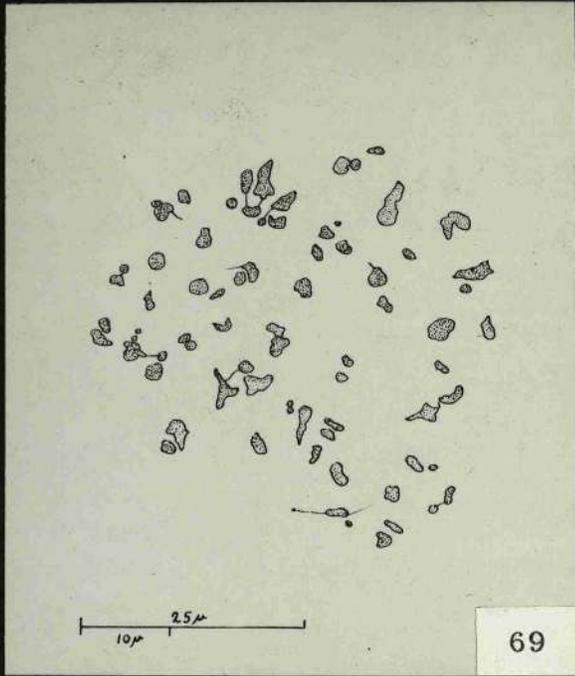
Oocyte diameter = 0.26mm  
Nuclear diameter = 176 $\mu$

(71) carnifex 62, secondary "nucleoli", some of which have rounded off prior to detachment from the nuclear membrane.

Oocyte diameter = 0.42mm  
Nuclear diameter = 272 $\mu$

(72) carnifex 62, some vacuolated secondary "nucleoli"  
All "nucleoli" in this preparation were firmly attached to the nuclear membrane although a few were almost round.

Oocyte diameter = 0.59mm  
Nuclear diameter = 340 $\mu$



- (73) danubialis 66, patches of the sort shown in this drawing were distributed over the entire inner surface of the nuclear membrane.

Oocyte diameter = 0.13mm  
Nuclear diameter = 100 $\mu$

- (74) danubialis 66, patches more numerous but less bizarre in shape.

Oocyte diameter = 0.17mm  
Nuclear diameter = 129 $\mu$

- (75) danubialis 66, groups of bodies which resemble the primary "nucleoli" of carnifex oocytes.

Oocyte diameter = 0.23mm  
Nuclear diameter = 155 $\mu$

Figures 76 to 79.

Photographed with the IPCM and x 95 objective. Inner surfaces of the membranes of whole nuclei from carnifex oocytes (see Page 105, footnote, Chapter III); showing the sizes, distribution, and character of primary and secondary "nucleoli". Nuclei isolated in 0.1 M 5:1 K/NaCl.

- (76) carnifex 63, primary "nucleoli".

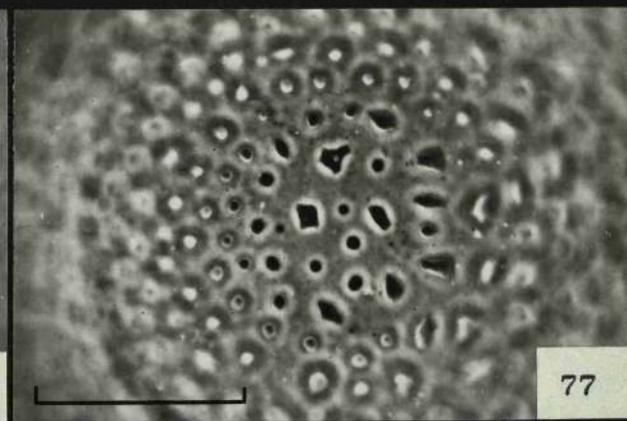
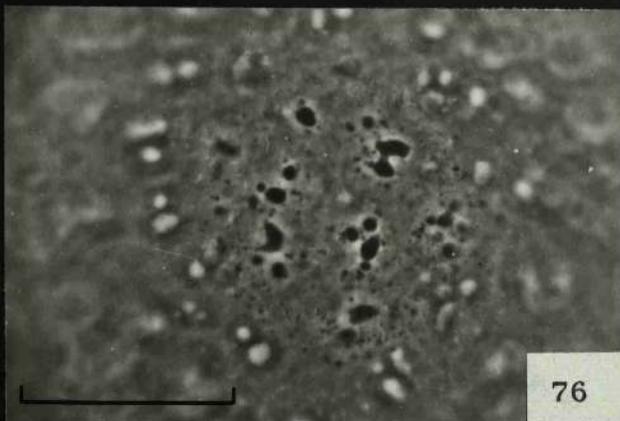
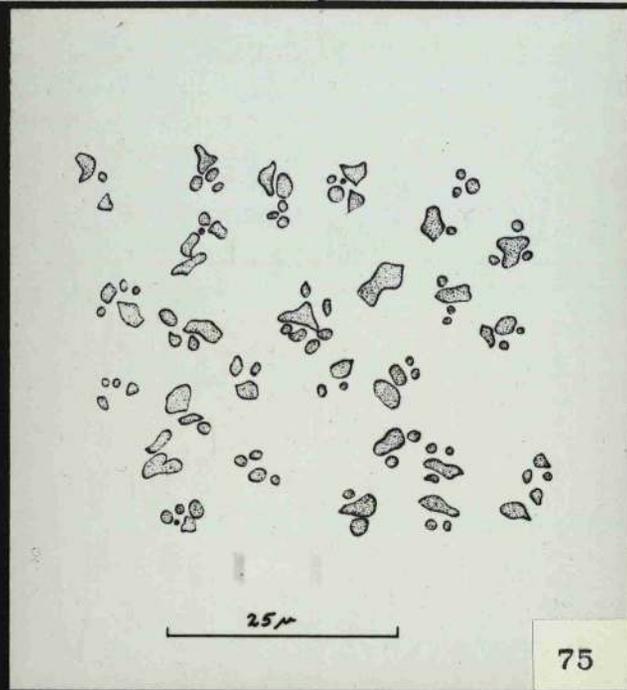
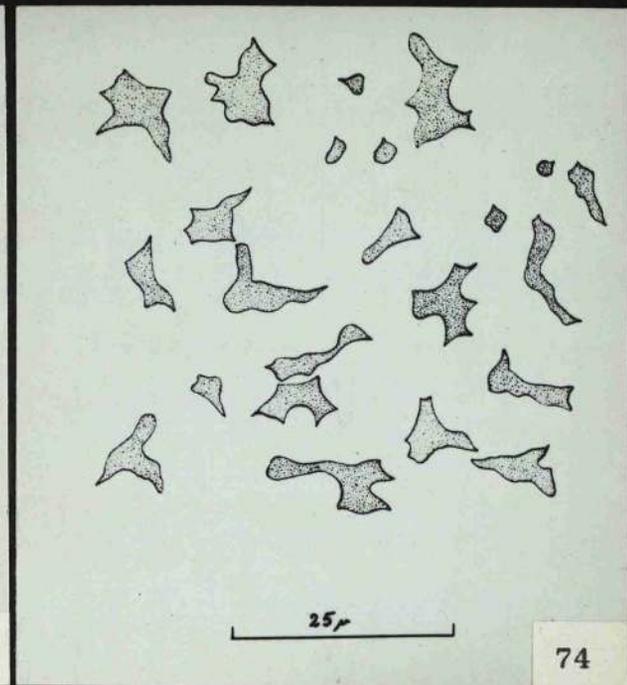
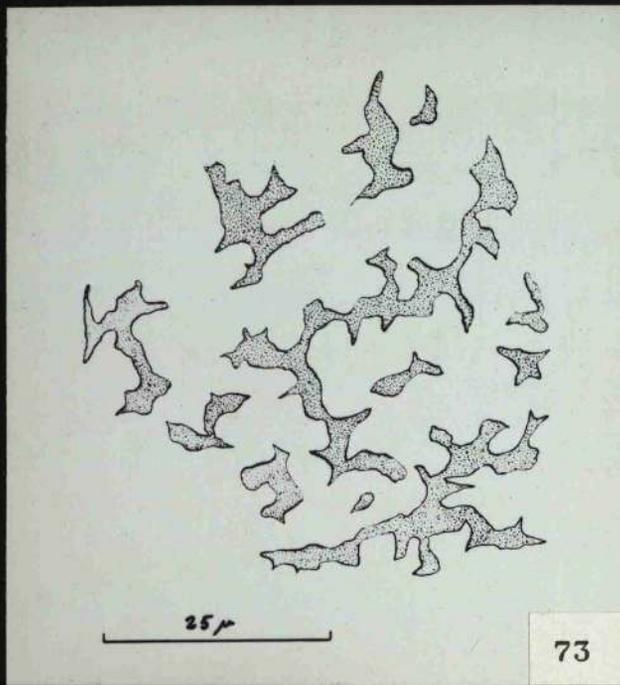
Oocyte diameter = 0.14mm  
Nuclear diameter = 95 $\mu$

Scale = 25 $\mu$

- (77) carnifex 63, early secondary "nucleoli".

Oocyte diameter = 0.21mm  
Nuclear diameter = 138 $\mu$

Scale = 25 $\mu$



(78) carnifex 63, secondary "nucleoli"

Oocyte diameter = 0.46mm

Nuclear diameter = 320 $\mu$

Scale = 25 $\mu$

(79) carnifex 63, secondary "nucleoli" and free "nucleoli".

All "nucleoli" in this picture were nearly round. Some were attached to the nuclear membrane, others were free and fell into the field as the nuclear sap hydrated.

Oocyte diameter = 0.7mm

Nuclear diameter = 365 $\mu$

Scale = 25 $\mu$

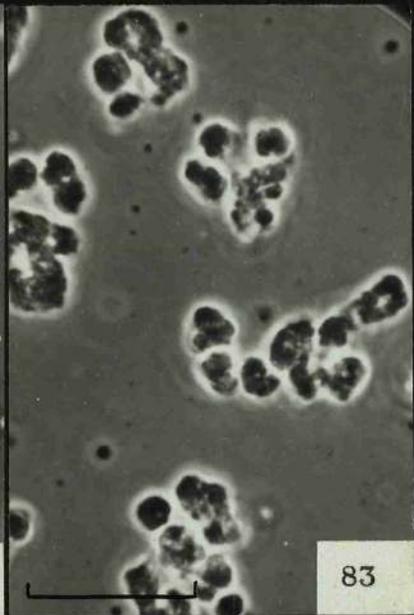
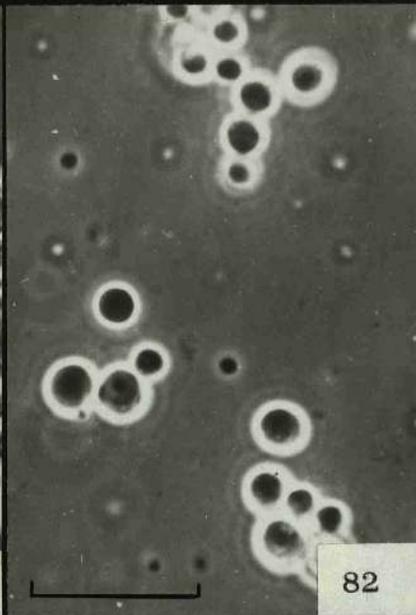
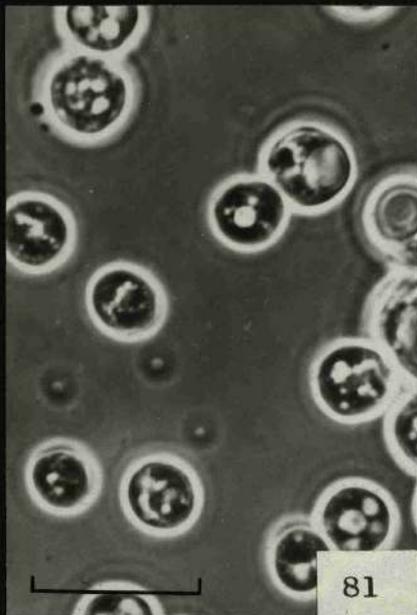
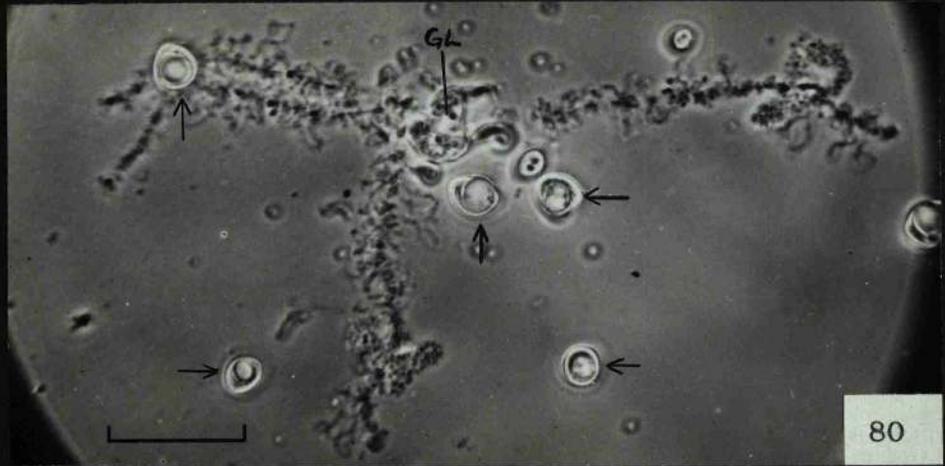
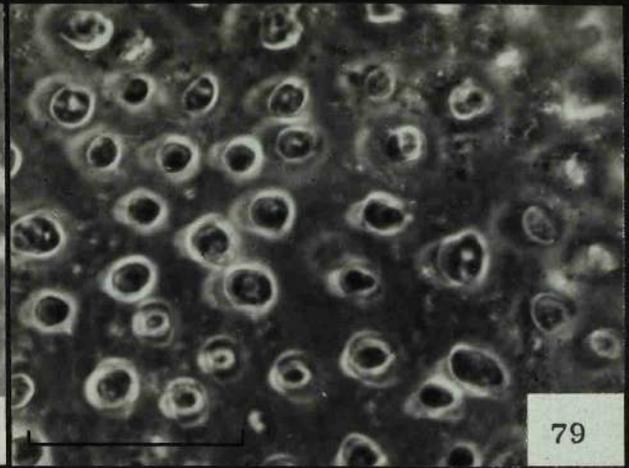
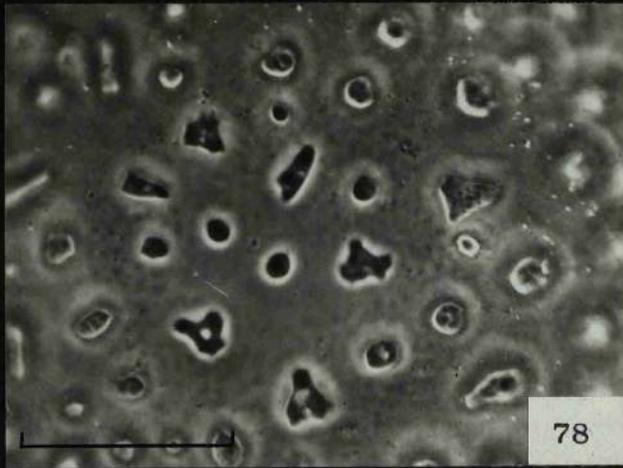
(80) Photographed with the IPCM, x 40 objective and a xenon flash light source. Bivalent XII (cristatus). The large bright bodies (arrowed) are thought to be shed products of the giant loops (GL).

Scale = 40 $\mu$

Figures 81 to 83.

Photographed with the IPCM and x 95 objective. Free "nucleoli" from standard-size oocytes of 3 anonymous carnifex females.

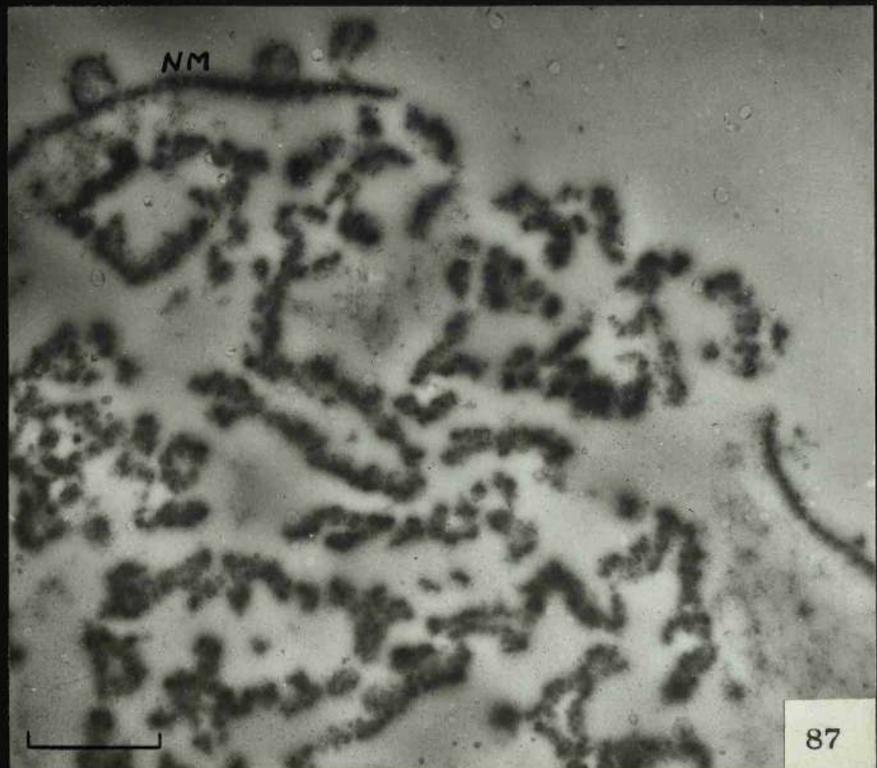
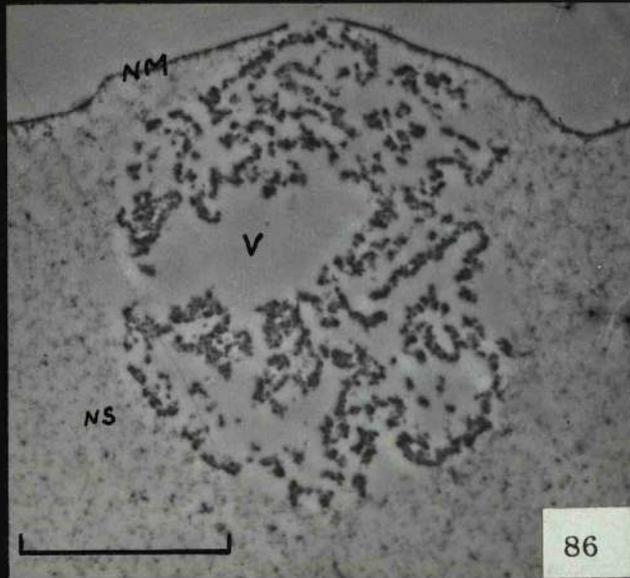
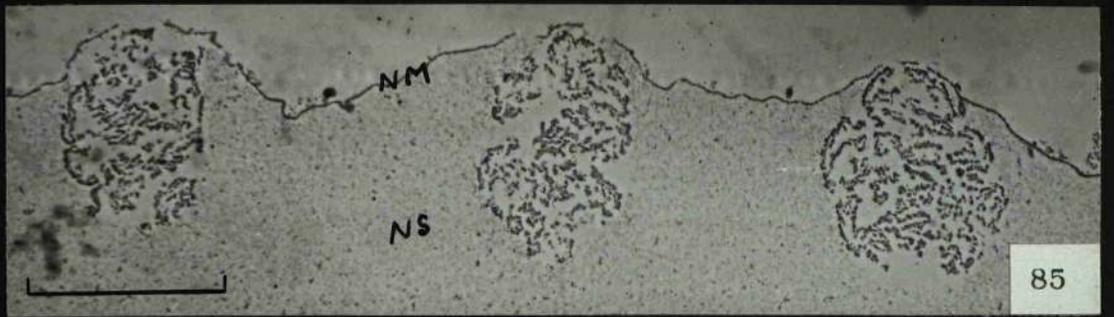
Scale = 20 $\mu$



Figures 85, 86, and 87.

Thin sections through the nuclei of carnifex oocytes. Nuclei from oocytes of 0.5 to 0.6mm diameter were isolated in 0.1 M 5:1 K/NaCl, fixed in OsO<sub>4</sub>, and embedded in methacrylate.

- (85) Three secondary "nucleoli" closely applied to the nuclear membrane (NM). Note the gaps in the nuclear membrane beside each "nucleolus". (NS) nuclear sap. Instrument magnification 1000 x.  
scale = 10 $\mu$
- (86) A secondary "nucleolus" with a large vacuole (V) which appears to be empty. Instrument magnification 2000 x. Scale = 5 $\mu$
- (87) The region of contact between a "nucleolus" and the nuclear membrane. Note the similar electron absorbing properties of the nuclear membrane and the "nucleolar" material. The poor preservation of the ultrastructure of the nuclear membrane is apparent here. Instrument magnification 8000 x. Scale = 1 $\mu$



Figures 88 to 92.

Thin sections of free "nucleoli" and a giant loop from standard-size oocytes of carnifex. Chromosomes and "nucleoli" were isolated in C medium, fixed in formalin vapour and in  $OsO_4$ , stained in uranyl acetate, and embedded in Vestopal W.

- (88) Part of a giant loop on bivalent XII.  
Instrument magnification 2000 x. Scale =  $5\mu$
- (89) A free "nucleolus" with a conspicuous electron-dense shell. The material in the lower half of the picture is an unidentified part of a lampbrush chromosome.  
Instrument magnification 2000 x. Scale =  $5\mu$
- (90) A free "nucleolus" with a well defined shell and a single vacuole. The latter appears empty and is smooth walled.  
Instrument magnification 10,000 x. Scale =  $1\mu$
- (91) Part of the giant loops of bivalent XII. The matrix of the loop appears structureless and the vacuoles are rough walled.  
Instrument magnification 10,000 x  
Scale =  $1\mu$
- (92) Part of a free "nucleolus". The "nucleolar" material including the shell appears finely granular, the granules being of the order of  $100\text{\AA}$  in diameter.  
Instrument magnification 10,000.  
Scale =  $1\mu$

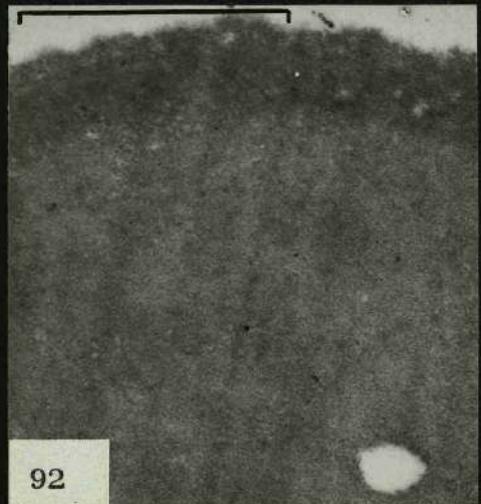
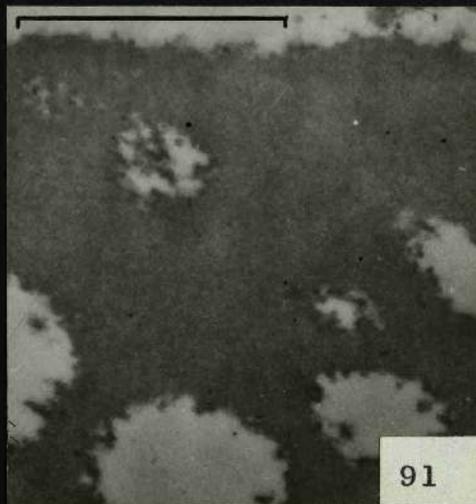
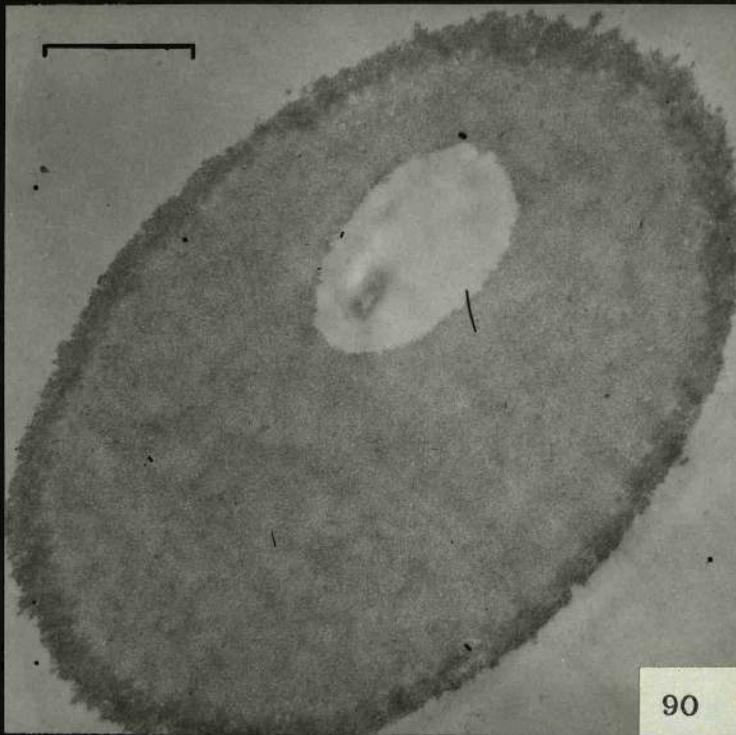
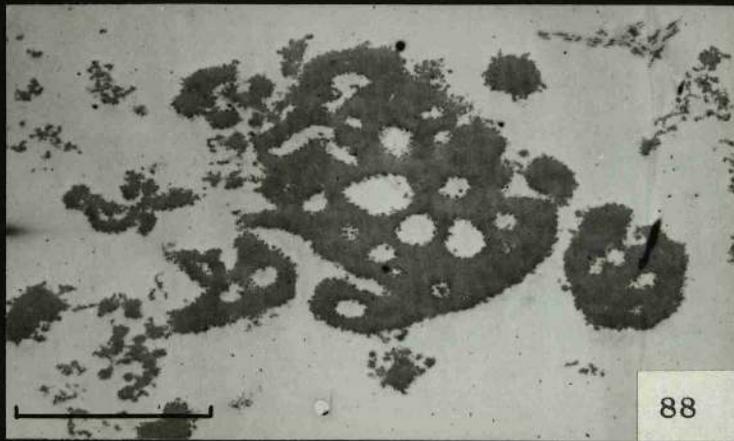
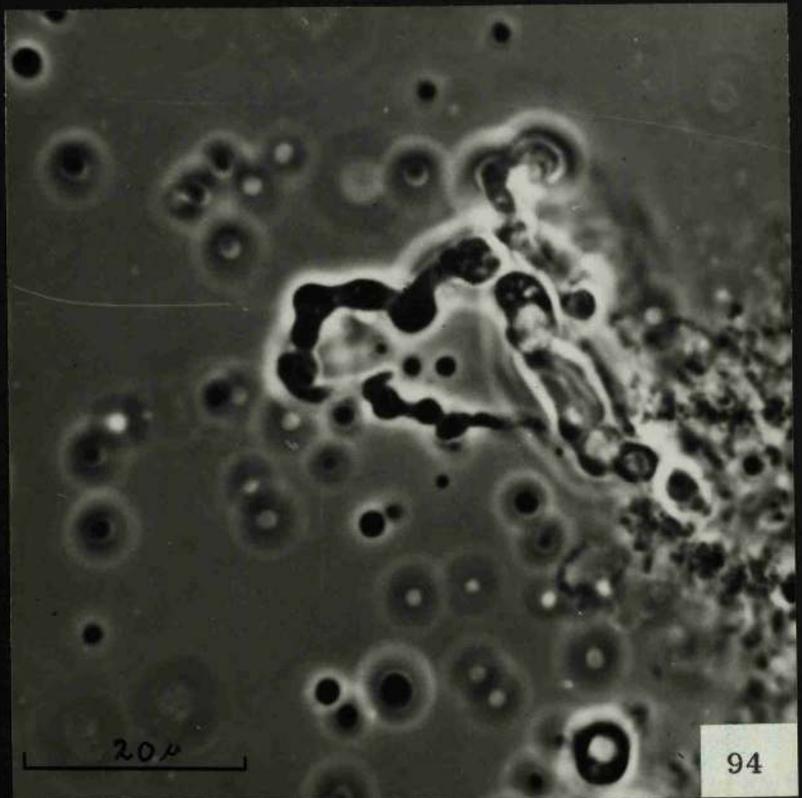
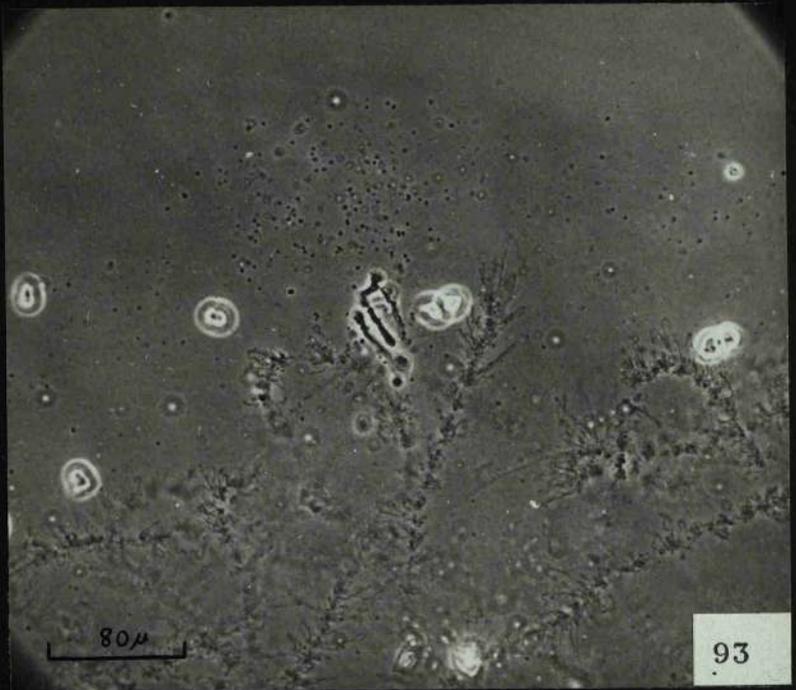


Figure 93.

Photographed with the IPCM and x 20 objective.  
carnifex 60 after treatment with 400 I.U. CG + PMS.  
A pair of giant loops on the edge of a partly dispersed  
chromosome group. There are large numbers of free  
granules beside the giant loops but not elsewhere.  
Figure 94 shows a similar situation (photographed with  
the IPCM and x 95 objective) in another preparation  
from the same newt. Note the resemblance between  
the free granules and some parts of the giant loop.



- APPENDIX I -

- ILLUSTRATIONS -

- THE ACTIONS OF ENZYMES ON LAMPBRUSH CHROMOSOMES -

shown in Figures 3 and 4 were taken with phase contrast at one or other of two objective magnifications: x 40, referred to throughout as "low power" and x 65 referred to as "high power".

Text-figure 1, Callan and Lloyd's diagram of the working map of the lampbrush chromosomes, is not shown. Figure 1 in the main part of this book

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St. Salvator's College

University of St. Andrews.

## APPENDIX I

### INTRODUCTORY REMARKS

Negatives of all photographs except those shown in Figures 3 and 4 were taken with phase contrast at one or other of two objective magnifications : x 40, referred to throughout as "low power", and x 95 referred to as "high power".

Text-figure 1, Callan and Lloyd's (1960b) working map of the lampbrush chromosomes of carnifex is not included here: it has already been shown as Figure 1 in the main part of this thesis.

---

## APPENDIX I

### Text figure 2.

Camera lucida drawing to show the increase in length of the axial bars of karelinii as a result of pan-protease digestion at pH 7.8. On the left is a drawing of the middle region of bivalent XII. The axial bars are enclosed in square brackets to show their length in the undigested chromosome. The left arms of the two chromosomes are above the axial bars in this drawing. On the right is a drawing of the same chromosome region after it had been treated for 1 hour with pan-protease at pH 7.8. The axial bar material is enclosed in square brackets. A dense centromere granule lies midway along each axial bar.

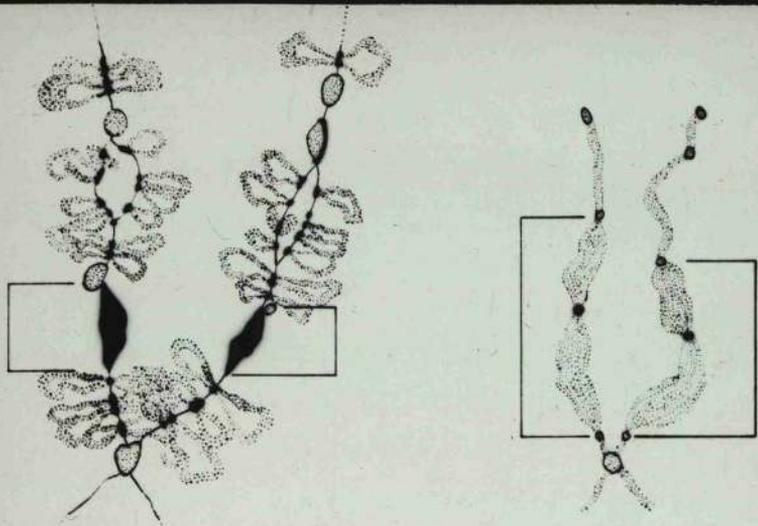
### Text figure 3.

Camera lucida drawing of the middle region of bivalent VI (karelinii) after it had been digested with pepsin at low pH for 6 hours. The left arms of both chromosomes point to the left, the right arms to the right. Arrows indicate places where the interchromomeric thread was invisible, but where its continuity could be inferred by the side-to-side movements of an unattached chromomere lying between two chromomeres which were firmly attached to the glass surface, (see insert below camera lucida drawing).

This drawing also illustrates the effect of peptic digestion on the axial bars of karelinii. Here, centromeres were fused and pepsin caused the constriction (C) between adjacent fused sets of axial bars to widen.

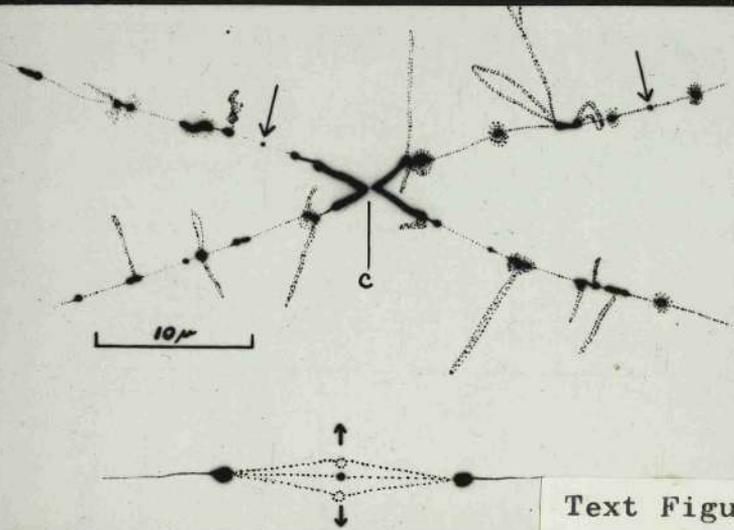
### Text figure 4.

Camera lucida drawings of the middle region of bivalent VIII (carnifex) to show the different types of fusion which may occur between axial granules and between chromosomes' axes at axial granule loci. Arrows indicate centromere positions.

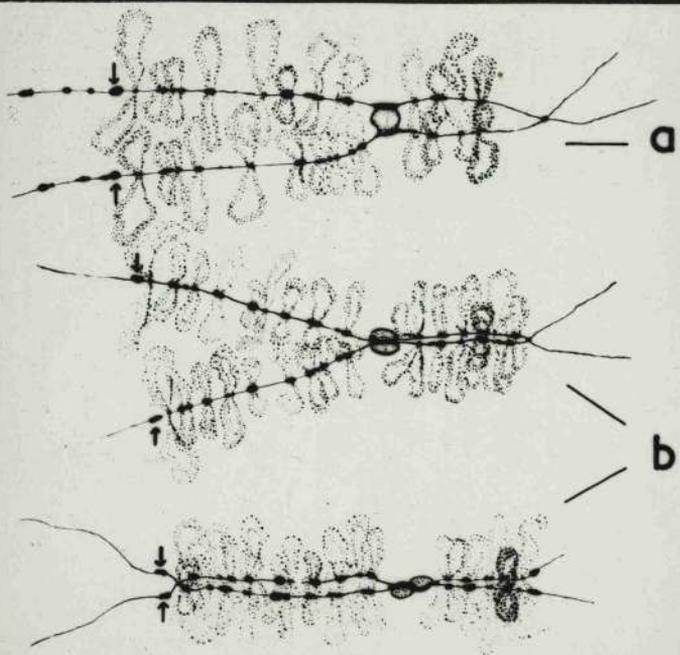


10 $\mu$

Text Figure 2



Text Figure 3



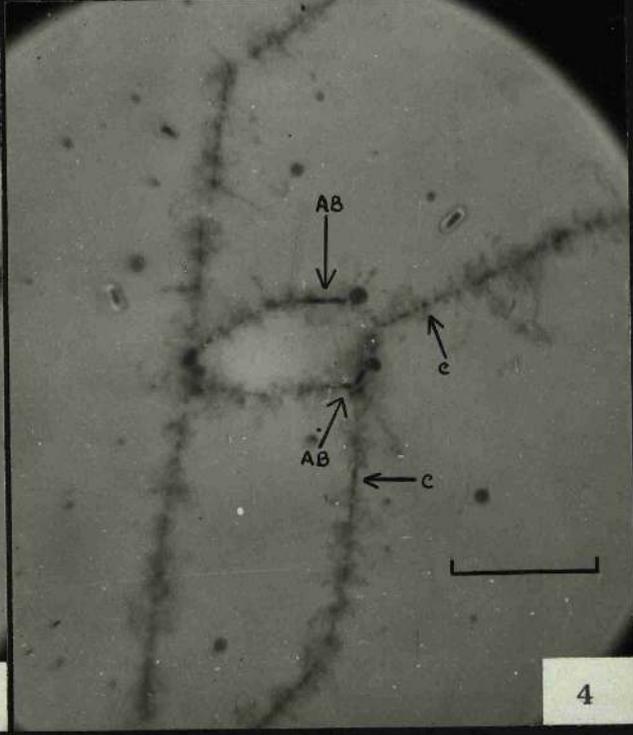
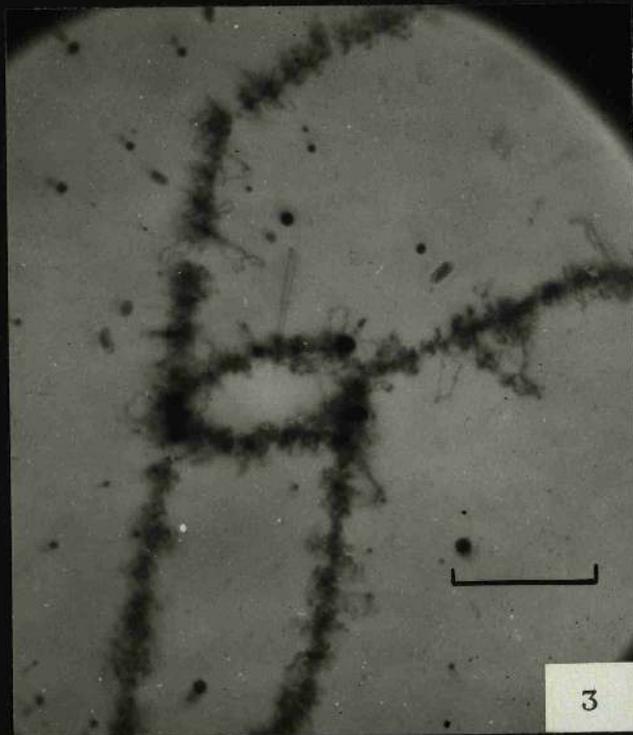
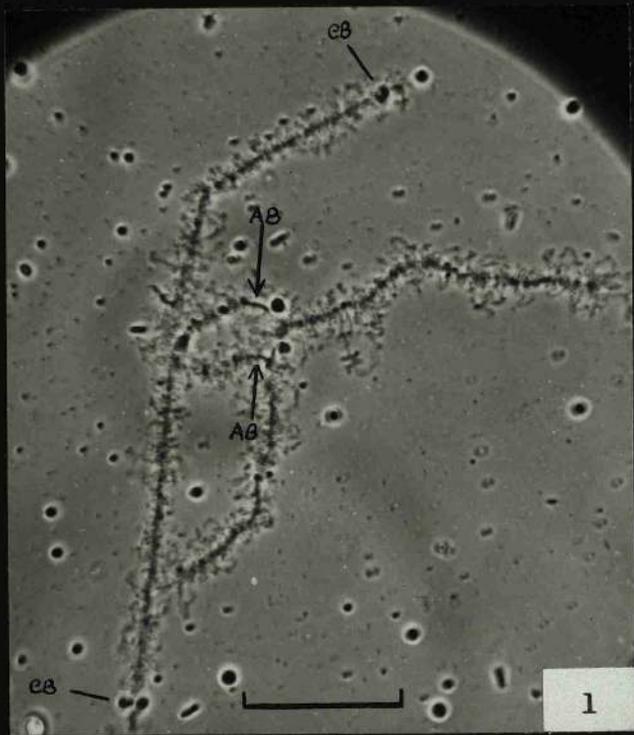
Text Figure 4

## APPENDIX I

### Figures 1 to 4.

#### Fixation and Feulgen/light green staining of lampbrush chromosomes.

- (1) Low power, karelinii. Bivalent XI entire, unfixed. C medium. Arrows "AB" point to axial bars. "CB" indicates the currant buns which lie at 2 units on the left arms of each chromosome.  
Scale = 50 $\mu$
- (2) Same as (1) after the preparation had been exposed to formalin vapour for 30 minutes. The chromosomes in this picture are attached to the glass surface upon which they lie and are fixed in the histological sense.  
Scale = 50 $\mu$
- (3) Middle region of the bivalent shown in Figures (1) and (2) stained with Feulgen and light green, and photographed using the inverted microscope fitted with a short working distance condenser and an ordinary x 80 objective. A cell containing Feulgen's reagent developed with formalin was used as a light filter to show the distribution of light green staining material.  
Scale = 25 $\mu$
- (4) Same as (3) but photographed using a cell containing light green in 70% alcohol as a filter to show the distribution of Feulgen staining material. Arrows "AB" point to Feulgen-positive axial bars. Arrows "C" point to stretches of chromosome axis in which Feulgen positive chromomeres are particularly evident.  
Scale = 25 $\mu$



## APPENDIX I

### Figures 5, 6, and 7.

#### The effects of increasing pH.

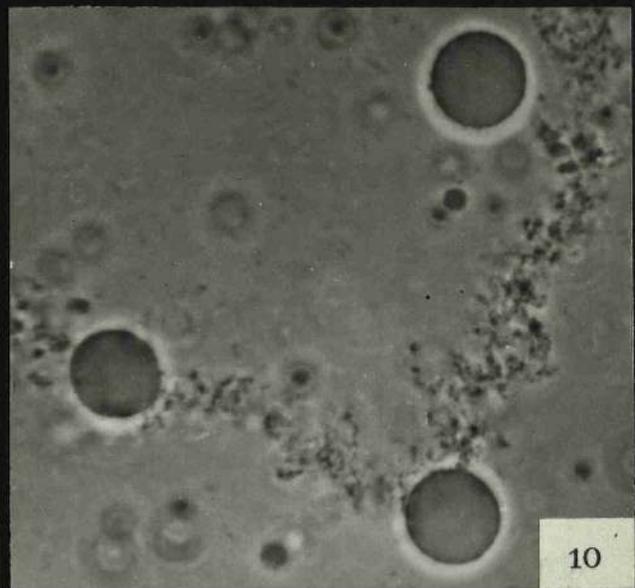
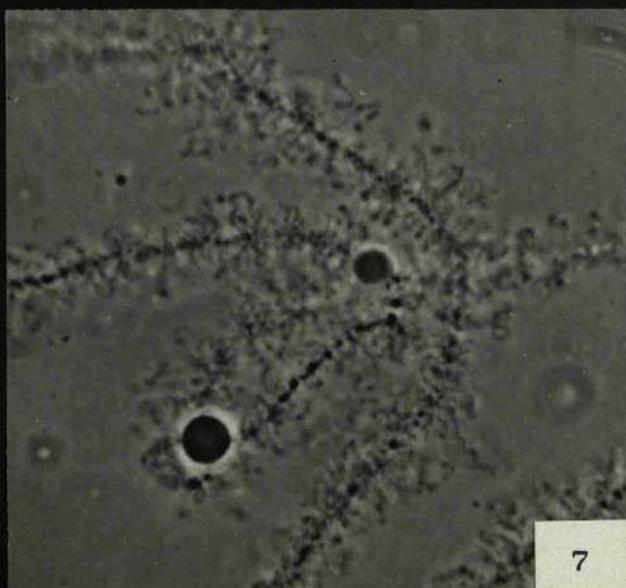
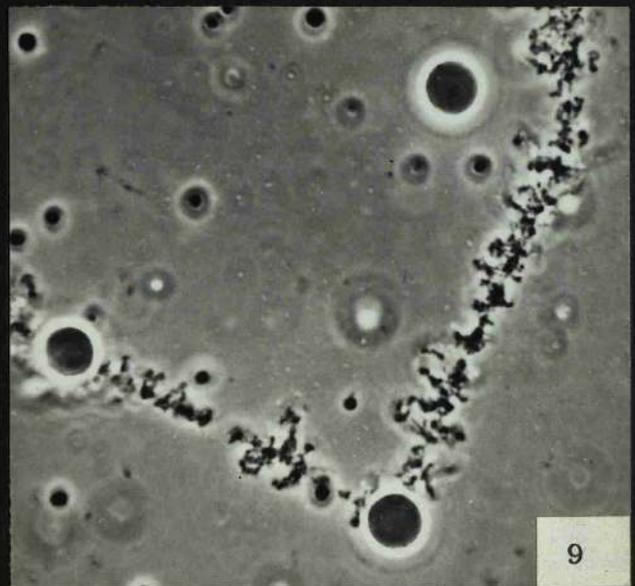
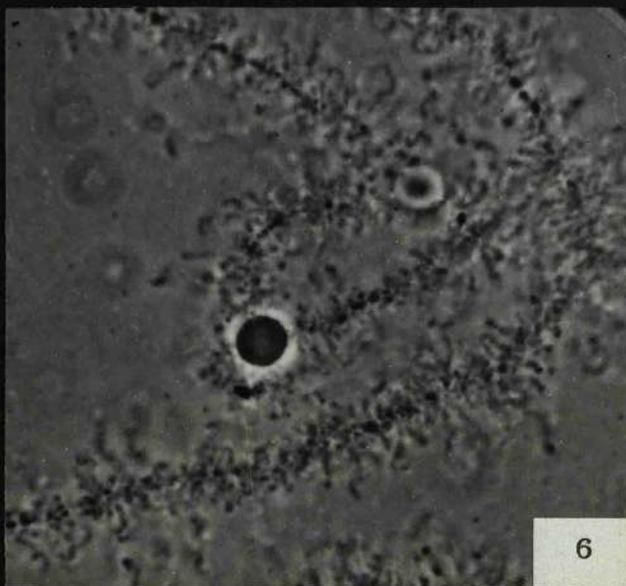
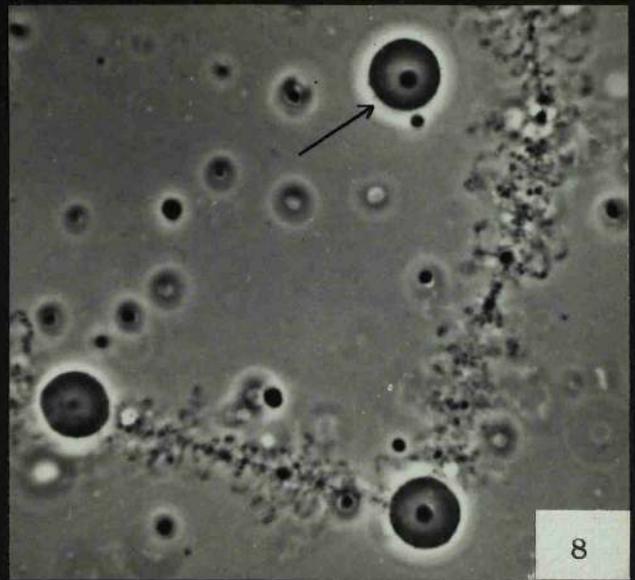
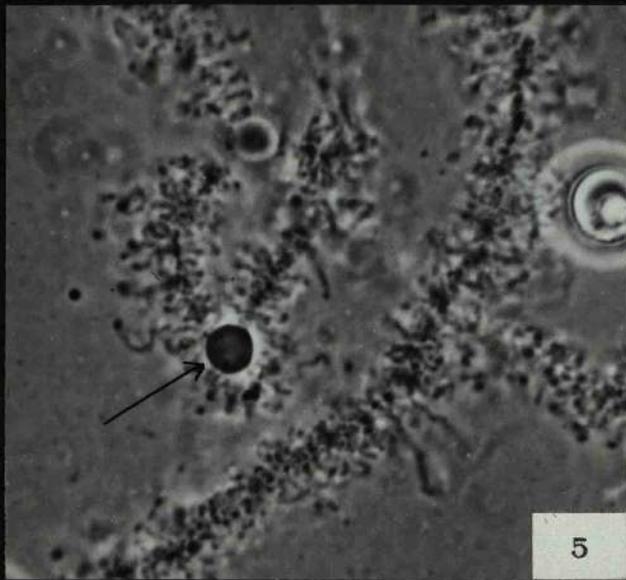
- (5) High power, carnifex. Ends of the left arms of bivalent V showing a single fused telomere granule and single fused spheres at 2 (arrowed) and 11 units. C medium, pH 6.2.
- (6) Same as (5) but pH increased to 7.
- (7) Same as (5) but pH increased to 8.
- 

### Figures 8, 9, and 10.

#### The effects of lowering pH.

- (8) High power, carnifex. End of the left arm of chromosome V showing spheres at 2 and 11 units, also one free sphere (arrowed). C medium, pH 6.2.
- (9) Same as (8) but pH lowered to 5. This picture illustrates the fixed condition described on Pages 26 and 35 (of Appendix 1).
- (10) Same as (8) but pH lowered to 2. This picture illustrates the "relaxed" condition described on Pages 26 and 35 (of Appendix 1).

Scale = 30 $\mu$



[ ]

## APPENDIX I

### Figures 11 and 12.

#### The action of TRYPSIN at pH 6.2.

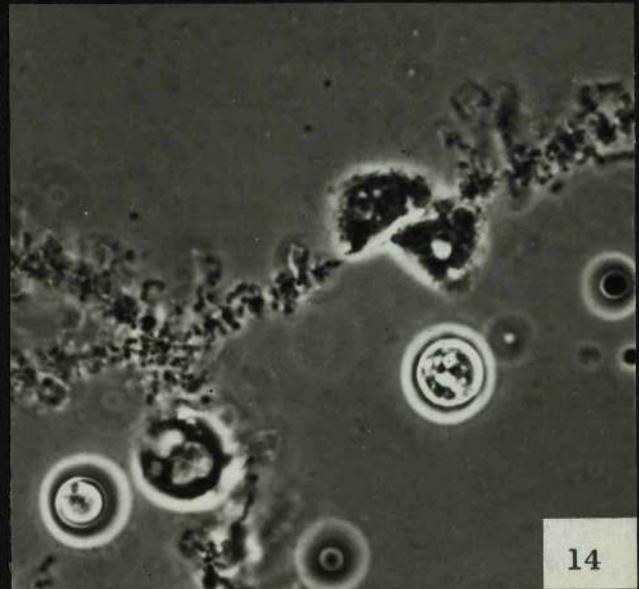
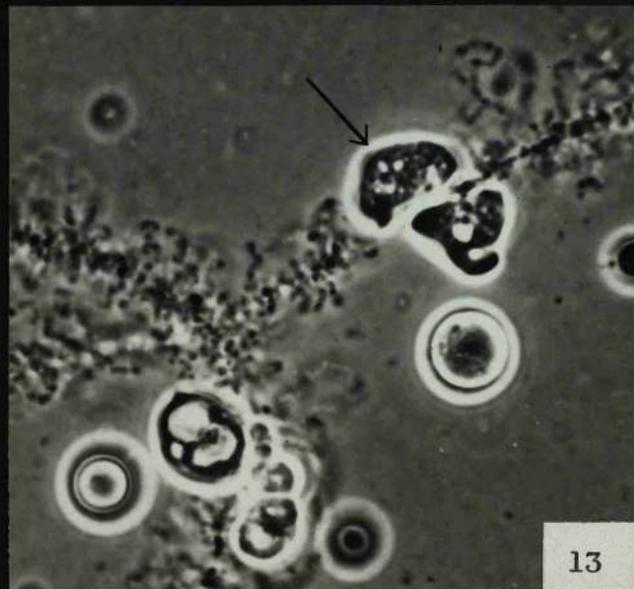
- (11) High power, carnifex. Parts of the left arms of bivalent XI showing the giant loops at 24 units. C medium, pH 6.2.
- (12) Same as (11) 45 minutes after application of the enzyme solution. Arrows indicate the remaining giant loop material.

### Figures 13, 14, 15, and 16.

#### Rapid destruction of giant loops by TRYPSIN at pH 6.2.

- (13) High power, carnifex. Parts of the left arms of bivalent XI showing the giant loops at 24 units; Arrow points to one pair of giant loops which have formed a double bridge. C medium, pH 6.2.
- (14) Same as (13) 3 minutes after application of the enzyme solution.
- (15) Same as (13)  $3\frac{1}{2}$  minutes after application of the enzyme solution.
- (16) Same as (13) 5 minutes after application of the enzyme solution. Arrow indicates double bridge formed by unbroken axes of the giant loops.

Scale = 30 $\mu$



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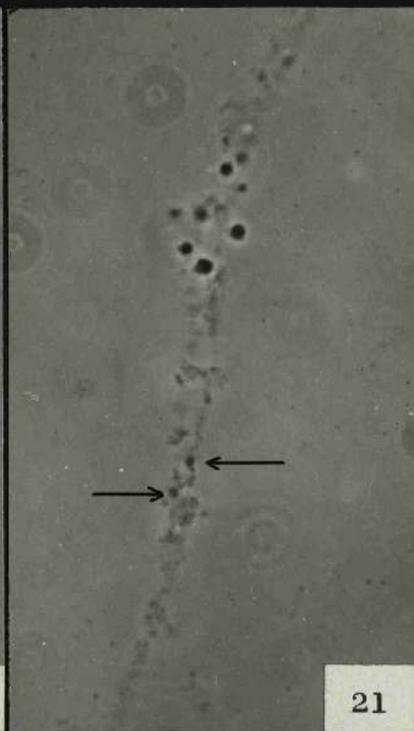
APPENDIX I

Figures 17 to 22.

The action of TRYPSIN at pH 7.8

- (17) High power, carnifex. Middle region of bivalent II from about 58 to 69 units showing a series of lumpy objects towards the top of the picture. Arrows indicate centromere granules. C medium pH 6.2.
- (18) Same as (17) 1 minute after application of the enzyme solution.
- (19) Same as (17) 2 minutes after application of the enzyme solution.
- (20) Same as (17) 15 minutes after application of the enzyme solution.
- (21) Same as (17) 55 minutes after application of the enzyme solution. Arrows indicate centromere granules.
- (22) Same as (17) 65 minutes after application of the enzyme solution. Fixed by adding 10% formalin to the preparation.

Scale = 30 $\mu$



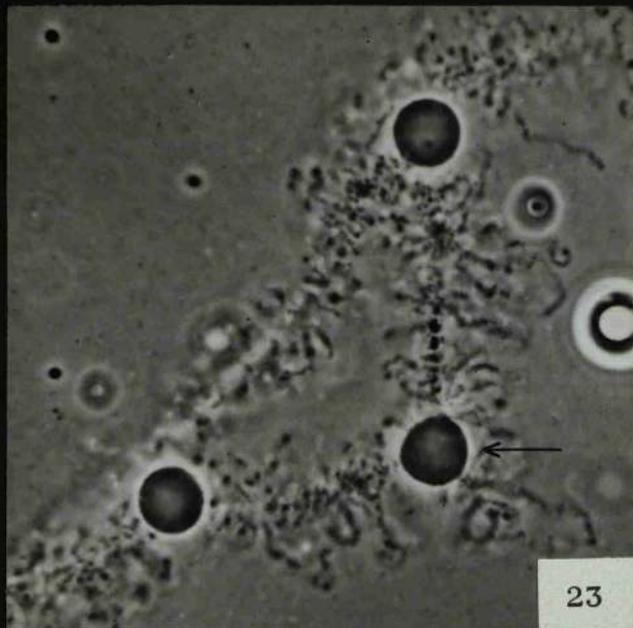
APPENDIX I

Figures 23 to 26.

The action of TRYPSIN at pH 7.8.

- (23) High power, carnifex. Ends of the left arms of bivalent V showing a single fused sphere at 2 units (arrowed), and a single sphere at 11 units on each chromosome. C medium, pH 6.2.
- (24) Same as (23) 5 minutes after application of the enzyme solution.
- (25) Same as (23) 15 minutes after application of the enzyme solution.
- (26) Same as (23) 1 hour after application of the enzyme solution. Arrow indicates what remains of the fused sub-terminal sphere.

Scale = 30 $\mu$



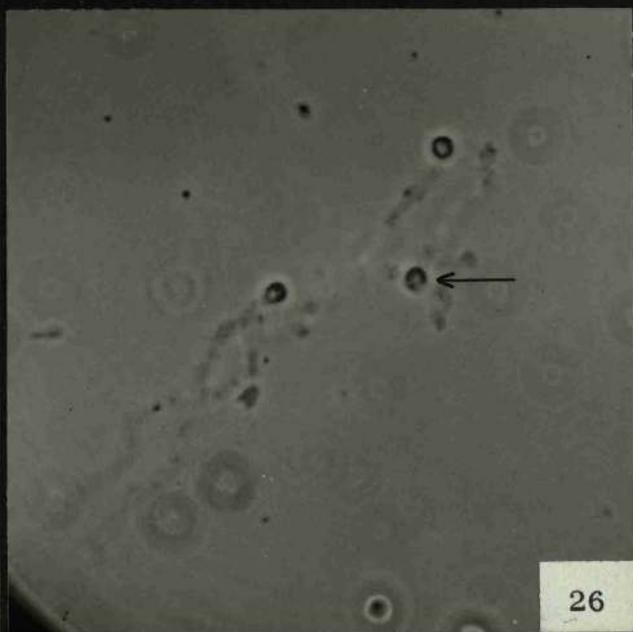
23



24



25



26

## APPENDIX I

### Figures 27 to 32.

#### The action of PAN-PROTEASE at pH 7.8 on the centromere granule and axial bars of karelinii.

- (27) High power, karelinii. Part of bivalent XII showing the two sets of axial bars. The set in the lower half of the picture is out of focus. Arrow indicates the position of the centromere granule on one chromosome. C medium, pH 6.2.
- (28) Same as (27) 10 minutes after application of the enzyme solution.
- (29) Same as (27) 15 minutes after application of the enzyme solution.
- (30) Same as (27) 30 minutes after application of the enzyme solution. Arrow points to the centromere granule.
- (31) Same as (27) 40 minutes after application of the enzyme solution. Arrow points to the centromere granule.
- (32) Same as (27) 45 minutes after application of the enzyme solution. Fixed by adding 10% formalin to the preparation.

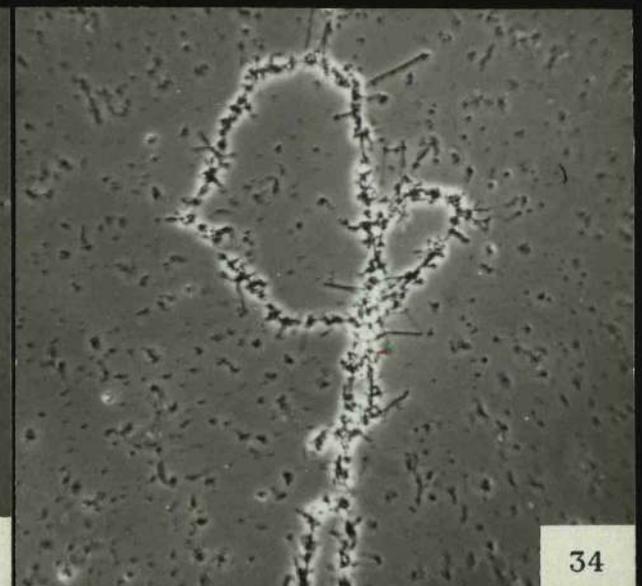
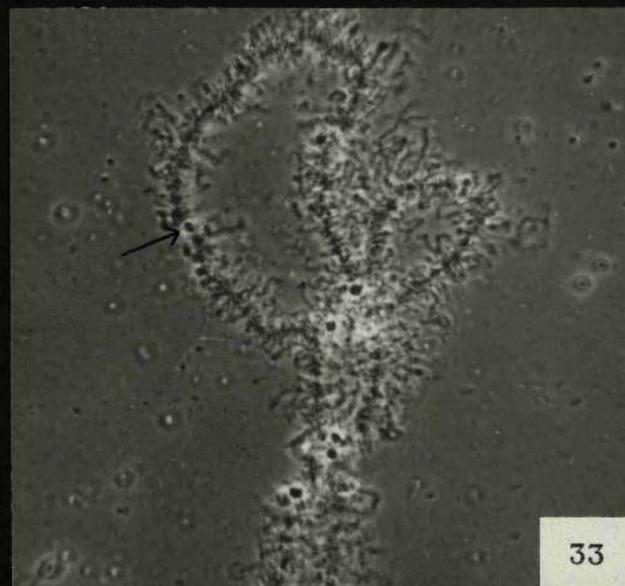
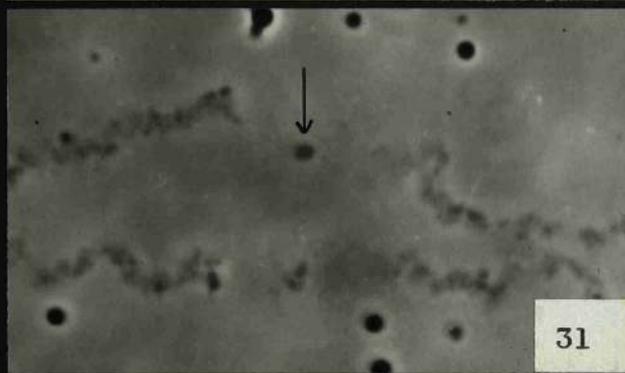
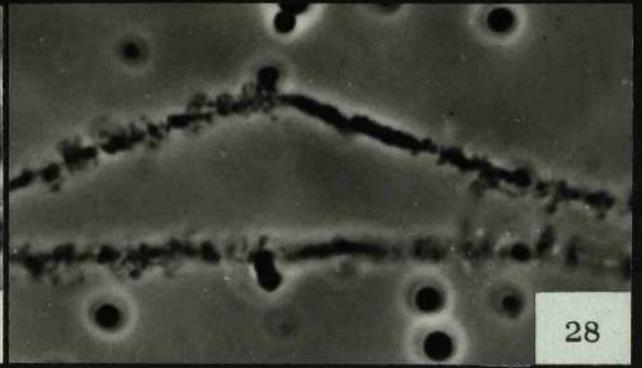
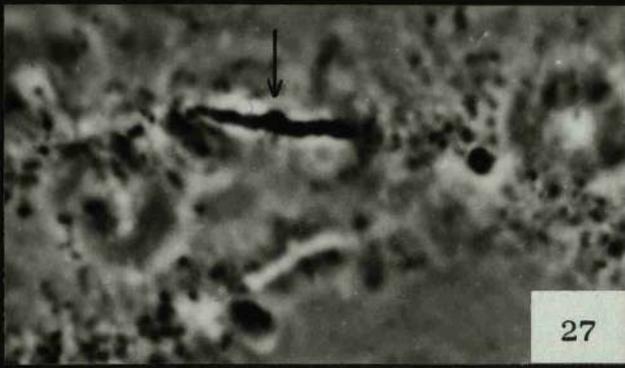
Scale = 20 $\mu$

### Figures 33 and 34.

#### The effect of applying a solution of PEPSIN at pH 1.8

- (33) Low power, carnifex. Left arms of bivalent VII. Arrow indicates the position of a telomere fusion. C medium, pH 6.2.
- (34) Same as (33) 10 minutes after application of the enzyme solution. Particles scattered over the field are of precipitated nuclear sap.

Scale = 50 $\mu$



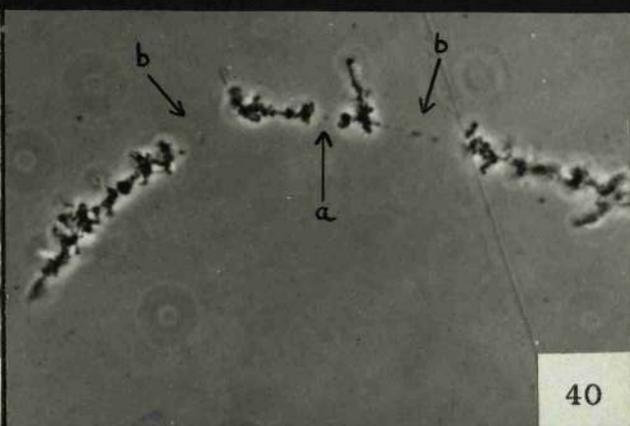
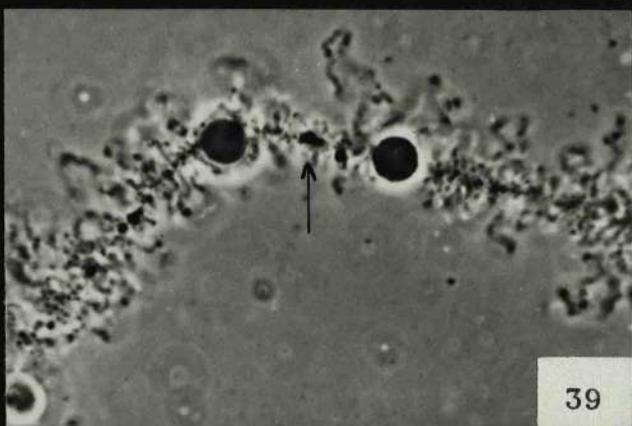
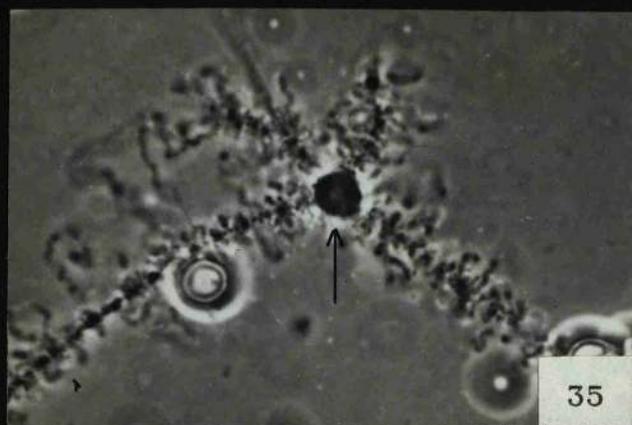
APPENDIX I

Figures 35 to 40

The action of PEPSIN at pH 6.2 and the effect of lowering pH after peptic digestion has begun.

- (35) High power, carnifex. Ends of the left arms of bivalent VIII showing telomere granules and a single fused sub-terminal sphere (arrowed). C medium, pH 6.2.
- (36) Same as (35) 10 minutes after application of a solution of pepsin in ordinary C medium.
- (37) Same as (35) 10 $\frac{1}{2}$  minutes after the application of the enzyme solution, and immediately after pH had been lowered to 1.8 by adding a modified C medium. The sphere has dissolved and the two chromosomes have drifted apart where they were originally joined. Like Figure (10), the picture illustrates the relaxed condition of the chromosomes which follows a drop in pH to below 2.
- (38) Same as (35) 20 minutes after application of the enzyme solution, and 10 minutes after pH had been lowered to 1.8.
- 
- (39) High power, carnifex. Ends of the left arms of bivalent V. Arrow indicates telomere fusion on either side of which lie the sub-terminal spheres. C medium pH 6.2.
- (40) Same as (39) 20 minutes after application of a solution of PEPSIN in ordinary C medium, and 10 minutes after pH had been lowered to 1.8 by adding a modified C medium. Arrow (a) indicates a tiny thickening - the remains of the telomere material - midway along the thread which joins the terminal chromomeres. Arrows (b) point to places where the spheres were originally located.

Scale = 30 $\mu$



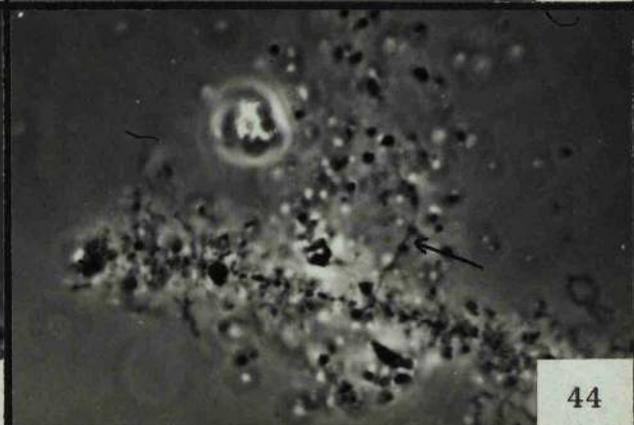
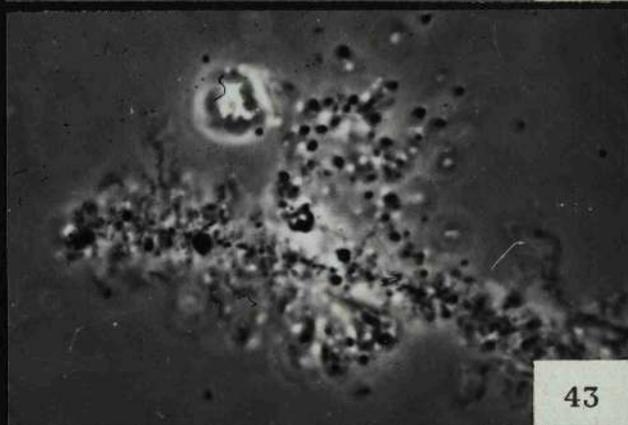
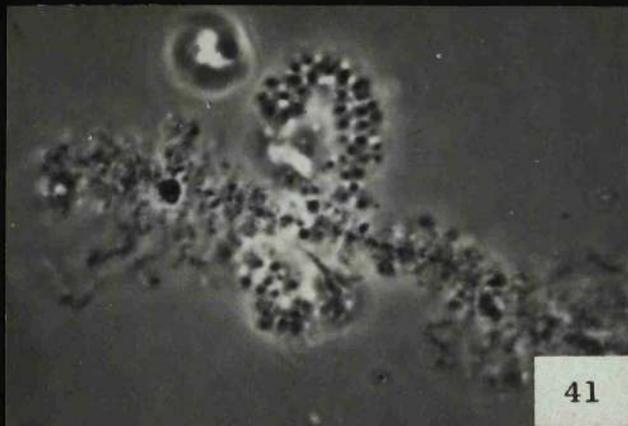
APPENDIX I

Figures 41 to 46.

The action of RIBONUCLEASE at pH 6.2.

- (41) High power, cristatus. End of the left arm of chromosome XII showing a pair of giant granular loops. C medium, pH 6.2.
- (42) Same as (41) 3 minutes after application of the enzyme solution. Arrow indicates two granules which have come adrift from the giant granular loop as a result of the breakdown of loop matrix.
- (43) Same as (41) 5 minutes after application of the enzyme solution.
- (44) Same as (41) 7 minutes after application of the enzyme solution. Arrow points to the loop axis which has been stripped of its granules and matrix.
- 
- (45) High power, carnifex. Part of bivalent XI showing two pairs of giant loops. The matrices of sister loops are fused. C medium, pH 6.2.
- (46) Same as (45) 30 minutes after application of the enzyme solution. Compare with Figure (12).

Scale = 30 $\mu$

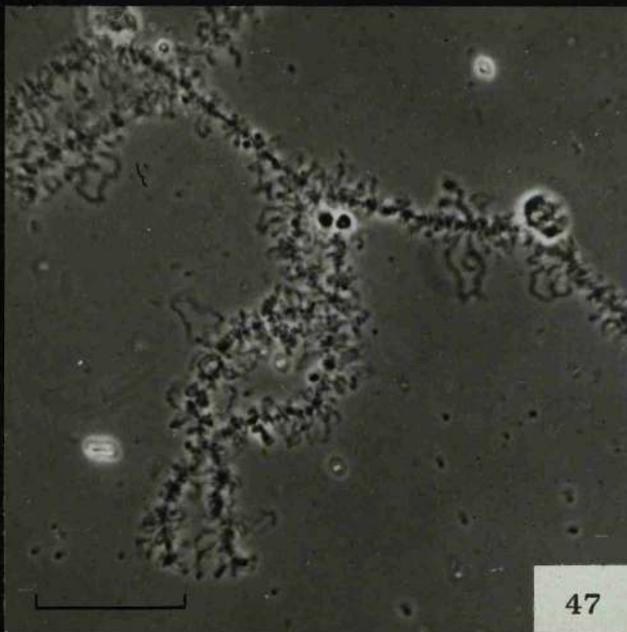


APPENDIX I

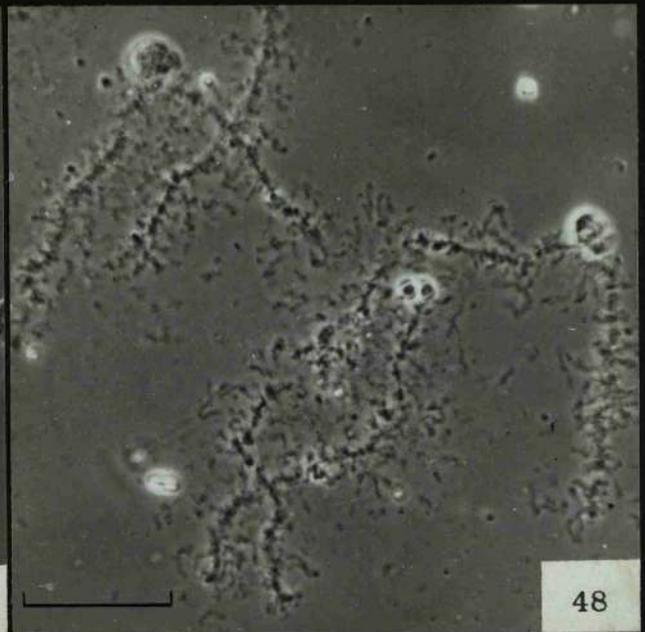
Figures 47 to 51.

The action of DN'ASE at pH 6.2

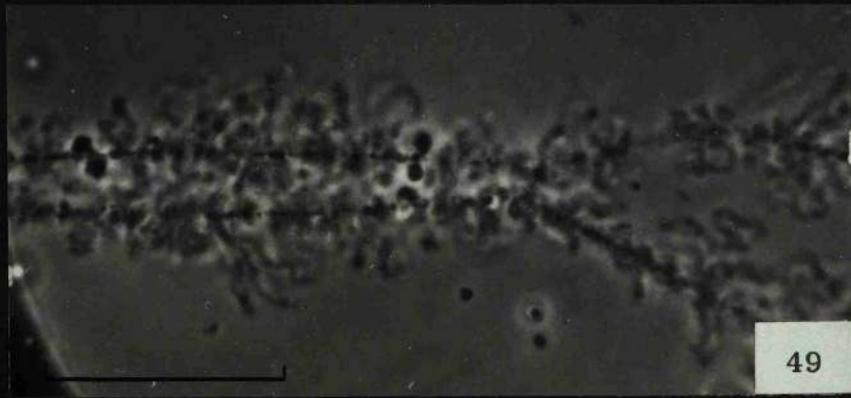
- (47) Low power, carnifex. Bivalent X homozygous for the giant loops at the "G" locus. C medium, pH 6.2.  
Scale = 50 $\mu$
- (48) Same as (47) 5 minutes after application of the enzyme solution. Axial breaks and fragmented lateral loops are visible.  
Scale = 50 $\mu$
- 
- (49) High power, carnifex. Middle region of bivalent VII.  
C medium, pH 6.2.  
Scale = 30 $\mu$
- (50) Same as (49) 5 minutes after application of the enzyme solution. Arrow points to the first axial break which occurred in this region.  
Scale = 30 $\mu$
- (51) Same as (49) 8 minutes after application of the enzyme solution. A second axial break can be seen on the extreme right of the picture. Arrows point to loop fragments.  
Scale = 30 $\mu$



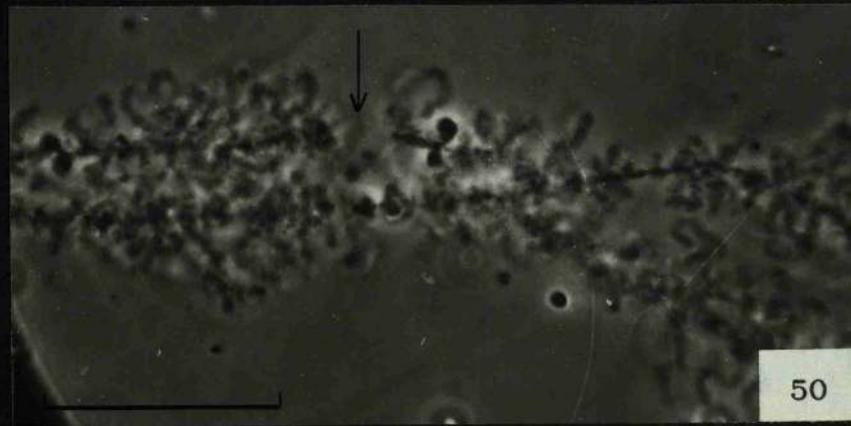
47



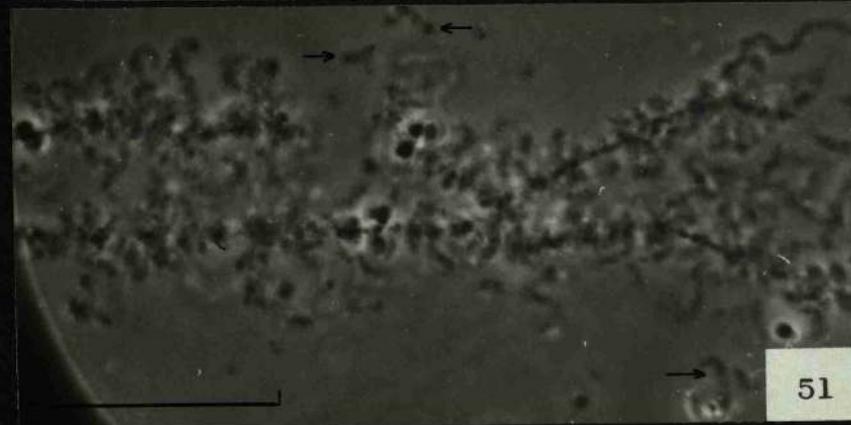
48



49



50



51

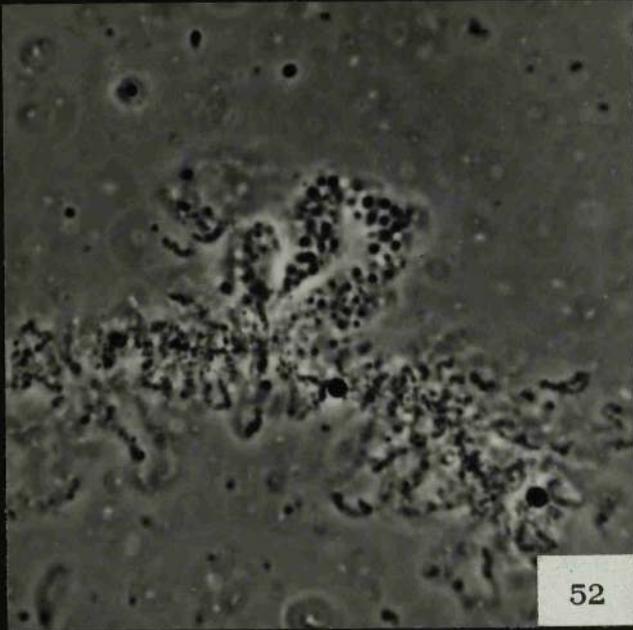
APPENDIX I

Figures 52 to 55.

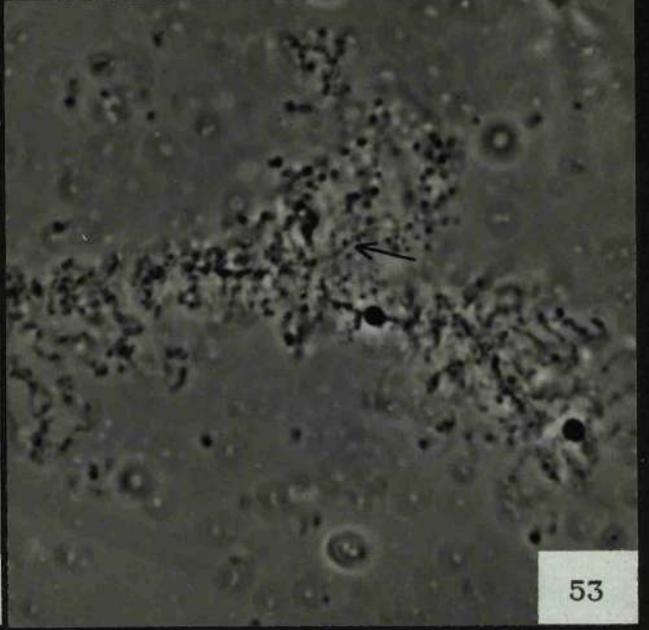
The action of DN<sup>A</sup>SE at pH 6.2.

- (52) High power, cristatus. End of the left arm of chromosome XII showing a pair of giant granular loops. C medium, pH 6.2.
- (53) Same as (52) 5 minutes after application of the enzyme solution. Arrow indicates the axis of one of the giant granular loops.
- (54) Same as (52) 5½ minutes after application of the enzyme solution. Giant granular loops are almost completely stripped of matrix and granules.
- (55) Same as (52) 10 minutes after application of the enzyme solution. Only the dense thin ends of the giant granular loops remain to mark the sites of the original structures. No axial breaks are visible although many occurred in other parts of this preparation.

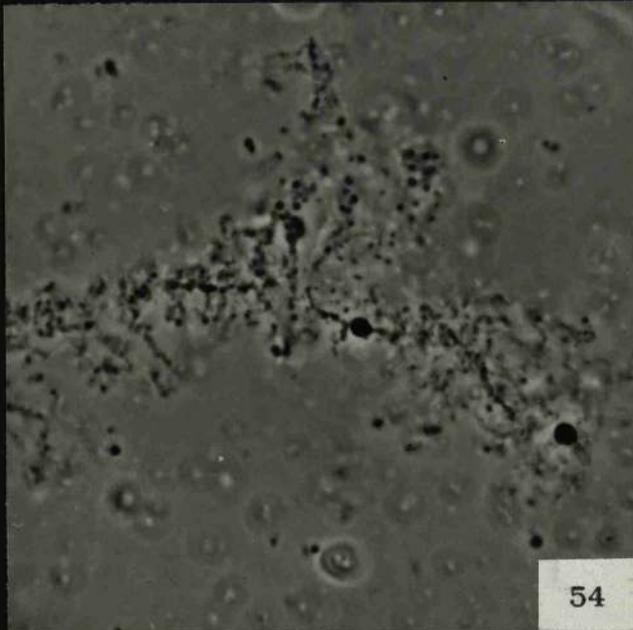
Scale = 30μ



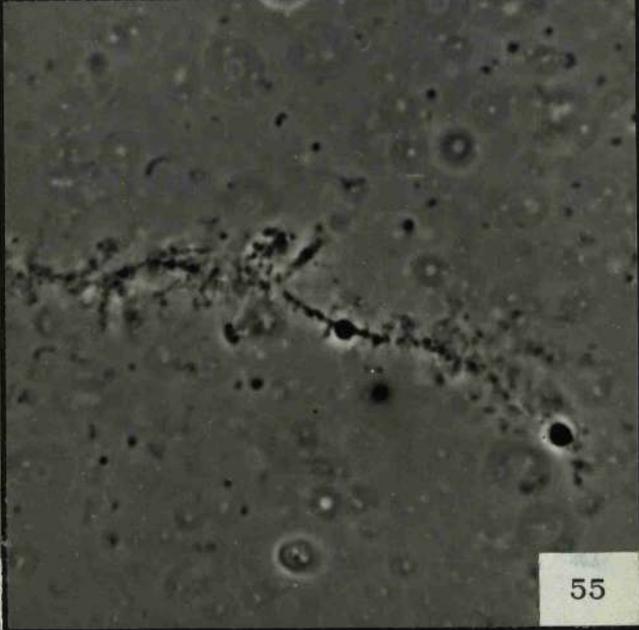
52



53



54



55



- APPENDIX II -

- ILLUSTRATIONS -

- THE BEHAVIOUR OF ISOLATED NUCLEI -

by

H. C. Macgregor

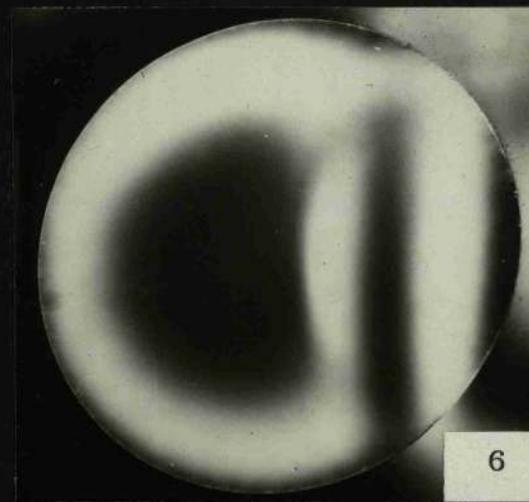
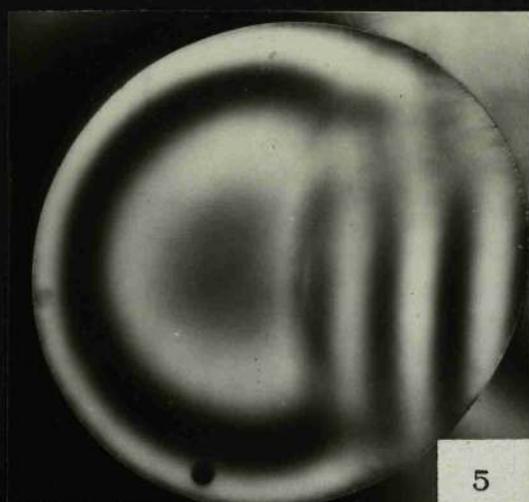
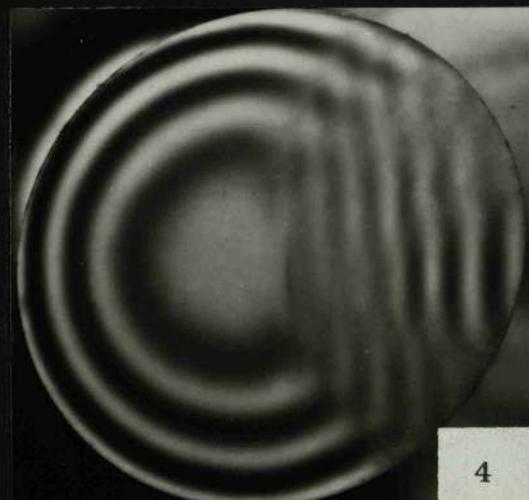
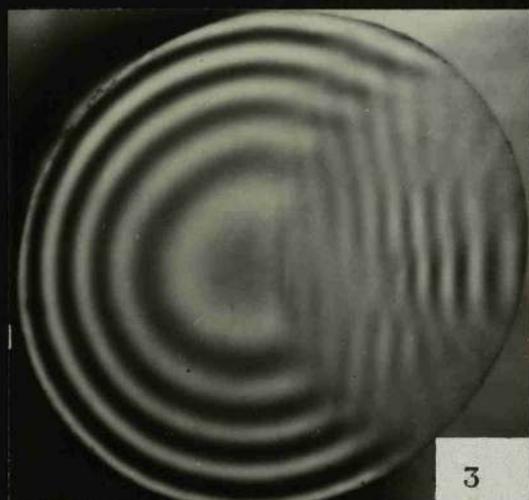
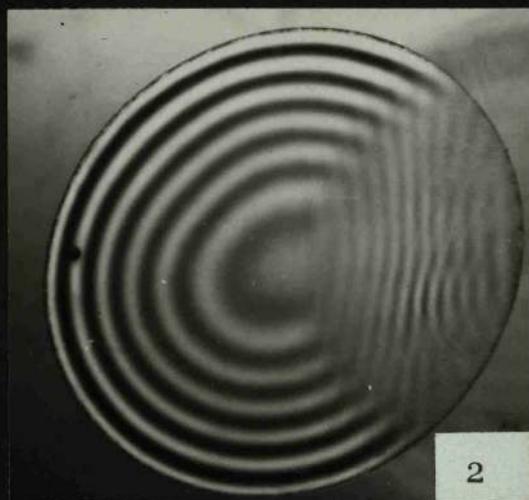
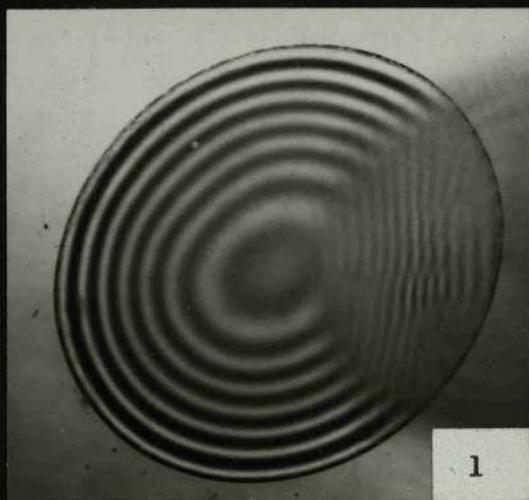
## APPENDIX II

Figures 1 to 6 show the changes in volume and interference properties of a carnifex nucleus isolated in 0.3M 5:1 K/NaCl. The nucleus was photographed with a Baker interference microscope fitted with a x 10 shearing objective and a single exposure camera loaded with Ilford Micro-Neg Pan film. A conventional light source was used.

- (1) 40 seconds after isolation: 7 dark fringes.
- (2) 2 minutes after isolation: 6 dark fringes.
- (3) 5 minutes after isolation: 4 dark fringes, the 5th disappearing in the centre of the nucleus.
- (4) 8 minutes after isolation: 3 dark fringes. Note the bright area immediately outside the nucleus at the top left hand corner of the picture; this indicates leading of nuclear sap.
- (5) 15 minutes after isolation: 1 dark fringe, the second disappearing in the centre of the nucleus.
- (6) 30 minutes after isolation. Optical retardation is now between 1 and  $1\frac{1}{2}$  wavelengths.

Swelling is evident in Figures 1, 2, and 3, deflation in Figures 4, 5, and 6.

Scale = 0.5mm

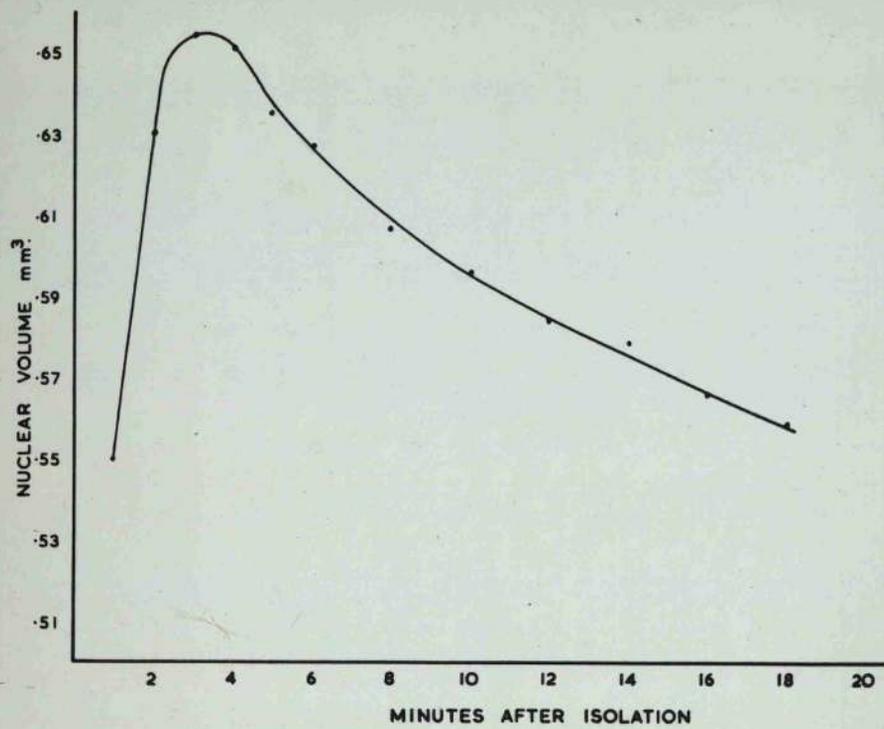


## APPENDIX II

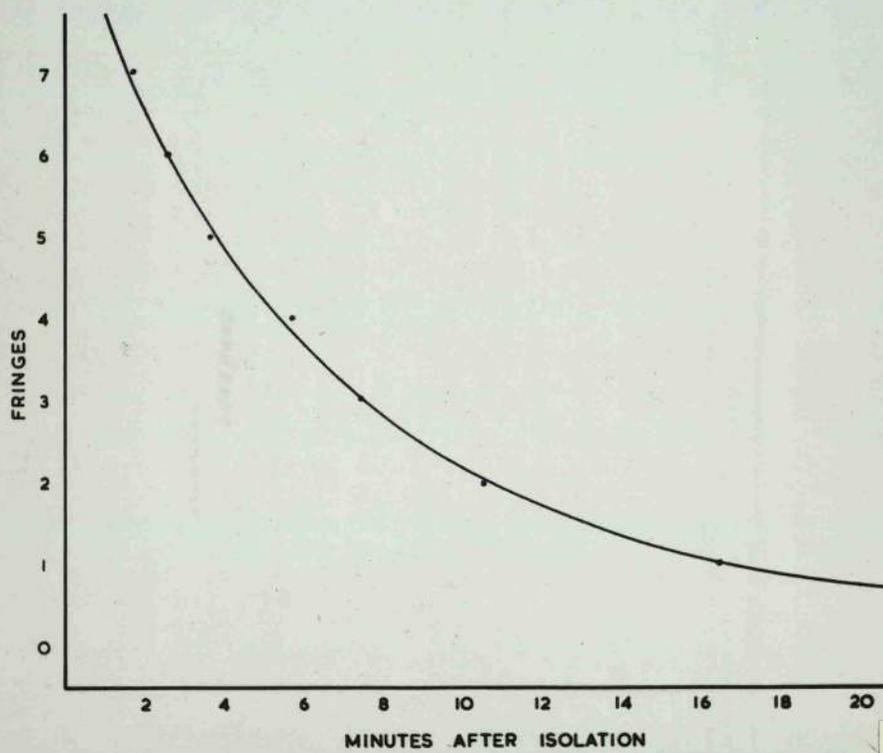
### Figures 7 to 10.

Each point in all of these figures represents the mean of comparable values obtained from 5 like-sized oocyte nuclei.

- (7) Volume changes with respect to time for nuclei isolated in 0.3M 5:1 K/NaCl
- (8) Optical retardation, measured in integral wavelengths (fringes), with respect to time for nuclei isolated in 0.3M 5:1 K/NaCl.



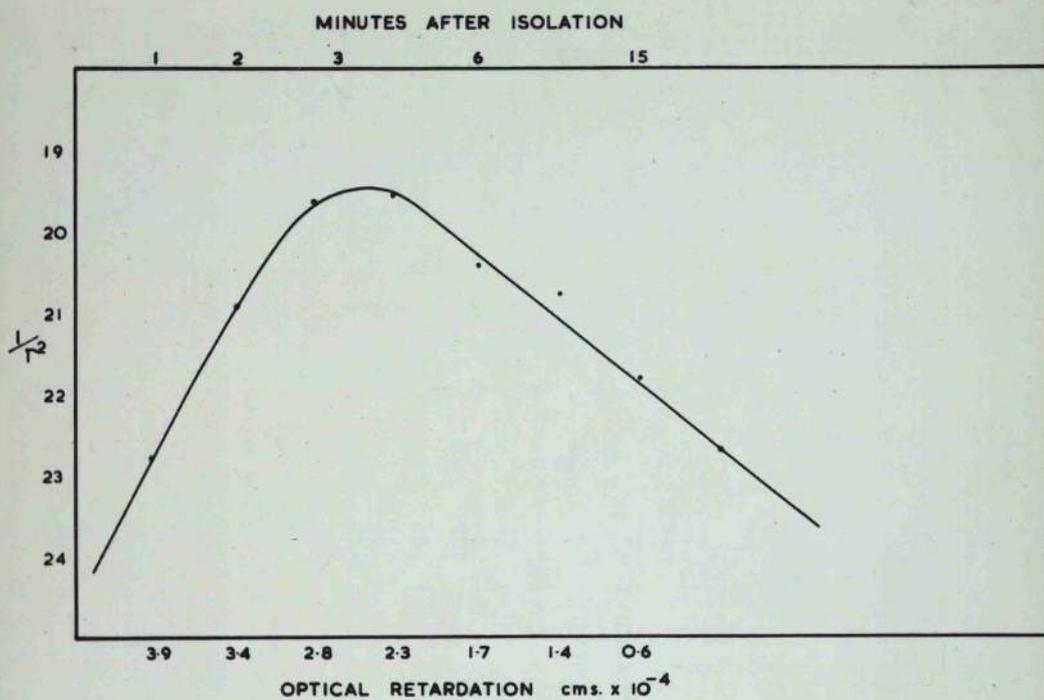
7



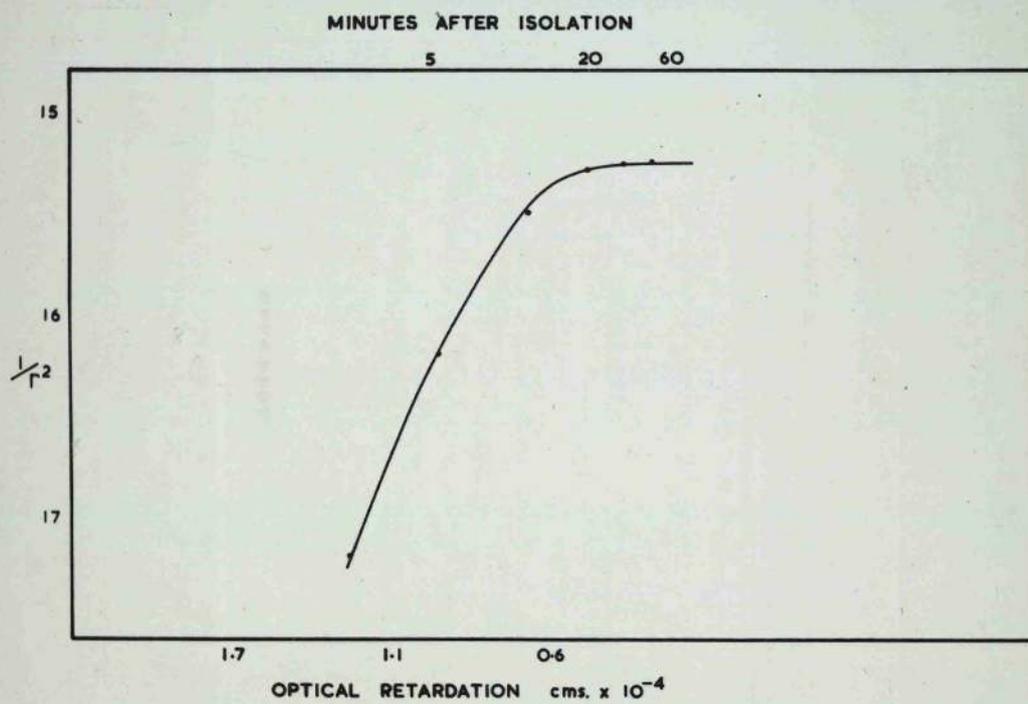
8

APPENDIX II

- (9)  $\frac{1}{r^2}$  plotted against optical retardation (f) for nuclei isolated in 0.3M 5:1 K/NaCl. The upper horizontal scale ("minutes after isolation") is included for comparison with Figures 7, 8, and 10: it played no part in determining the positions of the points on the graph, and the spacing of the numbers is incidental.
- (10)  $\frac{1}{r^2}$  plotted against optical retardation (f) for nuclei isolated in a 3.5% solution of BSA in 0.2M 5:1 K/NaCl. The upper horizontal scale is included for comparison only.



9



10