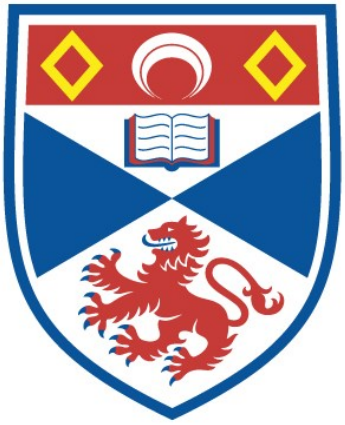
THE STRUCTURAL ORGANIZATION OF NEWT MITOTIC CHROMOSOMES

Edwina-Anne Rudak

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1976

THE STRUCTURAL ORGANIZATION OF NEWT MITOTIC CHROMOSOMES

by

EDWINA-ANNE RUDAK

Department of Zoology

University of St. Andrews

A thesis submitted for the Degree of Doctor of Philosophy

October, 1976

DECLARATION

I hereby declare that this thesis is my own composition, and that the experimental work was performed by me alone, except for some of the data included in Chapter I which has been published jointly with Professor H.G. Callan in Chromosoma 56, 349-362 (1976).

None of the material in this thesis has been submitted for any other degree.

8.10.76 EDWINA-ANNE RUDAK.

CERTIFICATE

I certify that Ms Edwina-Anne Rudak has spent 12 terms at research work on the structural organization of newt mitotic chromosomes, that she has fulfilled conditions of Ordinance No. 16 (St. Andrews) and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

12.10.76. H.G. CALLAN.

UNIVERSITY CAREER

I began my University career in the School of Biological Sciences at the University of Leicester in October 1970, and graduated with an upper second class Honours B.Sc. in June 1973. I entered the Zoology Department of the University of St. Andrews in October 1973, and spent three years doing post-graduate research into the structure and organization of the mitotic chromosomes of newts of the genus Triturus. The results of my work are presented here for the degree of Doctor of Philosophy.

CONTENTS

C H A P T E R III.

C H A P T E R IV.

GENERAL INTRODUCTION

A study of the basic organization of the DNA-protein complex which forms each of the eukaryote chromosomes is fundamental to an understanding of gene action, regulation and inheritance. Such studies can be undertaken from two aspects, (i) a biochemical investigation involving the isolation of DNA *in vitro* and performing analyses of its renaturation kinetics under specific conditions, from which the complexity, abundance and degree of interspersion of the different classes of DNA in the genome can be inferred, and (ii) a predominantly morphological study involving a microscopical analysis of some features which chromatin fibres show when they are maximally condensed at the metaphase stage of mitosis. The latter type of study relies on the specificity with which different regions of the chromosomes will bind to certain stains and radioactively labelled DNA or RNA molecules under identical conditions. My thesis is an account of one of the latter types of investigation.

Any study of chromosome morphology is greatly facilitated if the animals to be used have a fairly large amount of DNA per cell which is packed into comparatively few chromosomes. The animals whose chromosomes I have looked at belong to the amphibian Class Urodela, genus *Triturus*, more commonly referred to as newts. Newts of the genus *Triturus* have a high C-value (*T. cristatus* = 29 pg DNA) and few chromosomes (2n = 24) compared to, for example, human cells C = 3.65, 2n = 46). Thus newt tissues are excellent material to use for studies of chromosome morphology.

My thesis is divided into four chapters. The first chapter contains an account of the differential staining properties of newt mitotic chromosomes, the way in which the chromatin fibres are packed into the metaphase

chromosomes, and how this is affected by culture of the cells at low temperatures before fixation. During the course of this work, it became apparent that it would be advantageous if I could establish a newt cell line because this would provide a ready supply of mitotic chromosomes. The methods by which I have established a fibroblast culture of T.c. carnifex cells are described in Chapter II. Chapter III describes some experiments I have done to locate the positions of ribosomal (5S, 18 + 28S) genes on the mitotic chromosomes of T.c. carnifex and T. vulgaris by the technique of molecular in situ hybridization. The last chapter is an account of the frequency at which sister chromatid exchanges occur during mitosis in BUdR-treated cultures of T.c. carnifex.

CHAPTER I.

Part 1.

Differential staining and chromatin packing of the mitotic chromosomes of the newt *Triturus cristatus*.

Most of the work which I would like to present in this chapter has been published (Rudak & Callan, 1976). To avoid much repetition, I have enclosed a copy of this publication (overleaf) which should be regarded as the major part of Chapter I. Following this in Part 2, are some additional illustrations which were not included in Rudak & Callan (1976) and others which were but are presented again to demonstrate some of the staining features with greater clarity. The illustrations in Part 2 are preceded by a short description of the main features they show.

Part 2.

1. Differential staining of the mitotic chromosomes of T.c. carnifex, T.c. cristatus and T. vulgaris.

Rudak & Callan (1976) describe the patterns of Geimsa banding and cold-induced secondary constrictions which are shown by the mitotic chromosomes of Triturus cristatus and suggest that the degree of chromatin packing might be one of the factors responsible for the production of differentially stained regions of the chromosomes. The predominantly pericentric location of most of those chromosome regions which stain intensely with Giemsa has been described for all species of newt that have been studied to date: Triturus vulgaris meridionalis, T. italicus (Nardi et al., 1973; Ragghianti et al., 1973; Schmid & Krone, 1976) T. marmoratus (Mancino et al., 1973; Ragghianti et al., 1973; Schmid & Krone, 1976); T. alpestris apuanus (Ragghianti et al., 1973), T. alpestris (Schmid & Krone, 1976), T. cristatus (Mancino et al., 1973; Ragghianti et al., 1973; Rudak & Callan, 1976; Schmid & Krone, 1976), Pleurodeles waltlii (Bailly et al., 1973), Notophthalmus (= Triturus) viridescens (Hutchison & Pardue, 1975), and Cynops pyrrhogaster (Schmid & Krone, 1976). I therefore decided to extend my study of differential staining patterns of the mitotic chromosomes of T. cristatus and look at these patterns in T. vulgaris, to see whether this species too has percentrically-located chromosome regions which stain intensely with Giemsa.

(a) T.c. carnifex

Fig. I - 1a (fig. 1 in Rudak & Callan, 1976) shows the C-banding pattern of T.c. carnifex. In each chromosome, the centromere is apparent as a deeply staining spot. Pericentrically located intensely staining regions are also visible. Figs. I - 2a (Fig. 3 in Rudak & Callan, 1976), I - 3a and 3b (fig. 4 in Rudak & Callan, 1976) show the regions of the

mitotic chromosomes which stain intensely with Giemsa after cold treatment. In these preparations the centromeres are never stained. Fig. 1 - 4a and 4b (figs 9 and 10 in Rudak & Callan, 1976) are electron micrographs showing regions of metaphase chromosomes from the tail-fin of a cold- and colchicine-treated T.c. carnifex larva. Both figures include regions of densely packed chromatin thought to correspond to the constricted and intensely stained regions visible in the light microscope.

(b) T.c. cristatus

Figs I - 5 to I - 7 are Giemsa-stained mitotic chromosomes of brain cells of cold-treated T.c. cristatus. The diagrams of cut-out chromosomes from each set illustrate the similarity of the pattern of intensely staining regions to that of T.c. carnifex (fig. I - 2b). The mitotic metaphase sets of this larva regularly showed three secondary constrictions which did not stain intensely with Giemsa. These constrictions are located near the end of the long arm of both chromosomes X, and halfway down one arm of the larger chromosome, probably chromosome IV. If these constrictions mark the locations of the nucleolar organizers in T.c. cristatus, they are in positions different from those of T.c. carnifex (see Chapter III for further details). Fig. I - 8a and 8b show the intensely staining granules seen in interphase nuclei which have been stained with Giemsa under conditions which produce intensely-staining regions on the chromosomes.

(c) T. vulgaris

Pericentrically-located deeply-staining regions are also seen in the chromosomes of T. vulgaris (fig. I - 9a). These chromosomes are from the brain cell of a T. vulgaris larva. The larval newt spent 4 days at 4°C before fixation. The asterisks on chromosomes II and X denote the positions of some of the ribosomal (28 + 18S) genes (see Chapter III).

In the newts then, it would appear that during the course of evolution, the positions of the heterochromatic chromosomal regions have remained more or less constant. Speciation has occurred with only slight rearrangement of the pericentrically located heterochromatic areas and variation in the arm lengths of each of the 24 chromosomes.

2. The production of secondary constrictions in the mitotic chromosomes of T.c. carnifex after culture in medium containing BUdR.

The production of secondary constrictions in the mitotic chromosomes of some eukaryote species by culture at low temperatures (for references see Rudak & Callan, 1976) is also known to occur when cells are grown in medium containing the thymidine analogue, 5-bromodeoxyuridine (BUdR). Mammalian cells grown in medium containing BUdR have elongated secondary constrictions (Hsu et al., 1961; Kaback et al., 1964) which are a result of differential spiralization of those regions of the chromosomes where the DNA is replicated in the latter part of the S-phase. I have found that secondary constrictions can be induced to form in mitotic chromosomes of T.c. carnifex tissue culture cells after one round of DNA replication in medium containing 1 µg/ml. BUdR (Fig. I - 9b). These constrictions are in positions identical to those induced by low temperature treatment (fig. I - 9a).

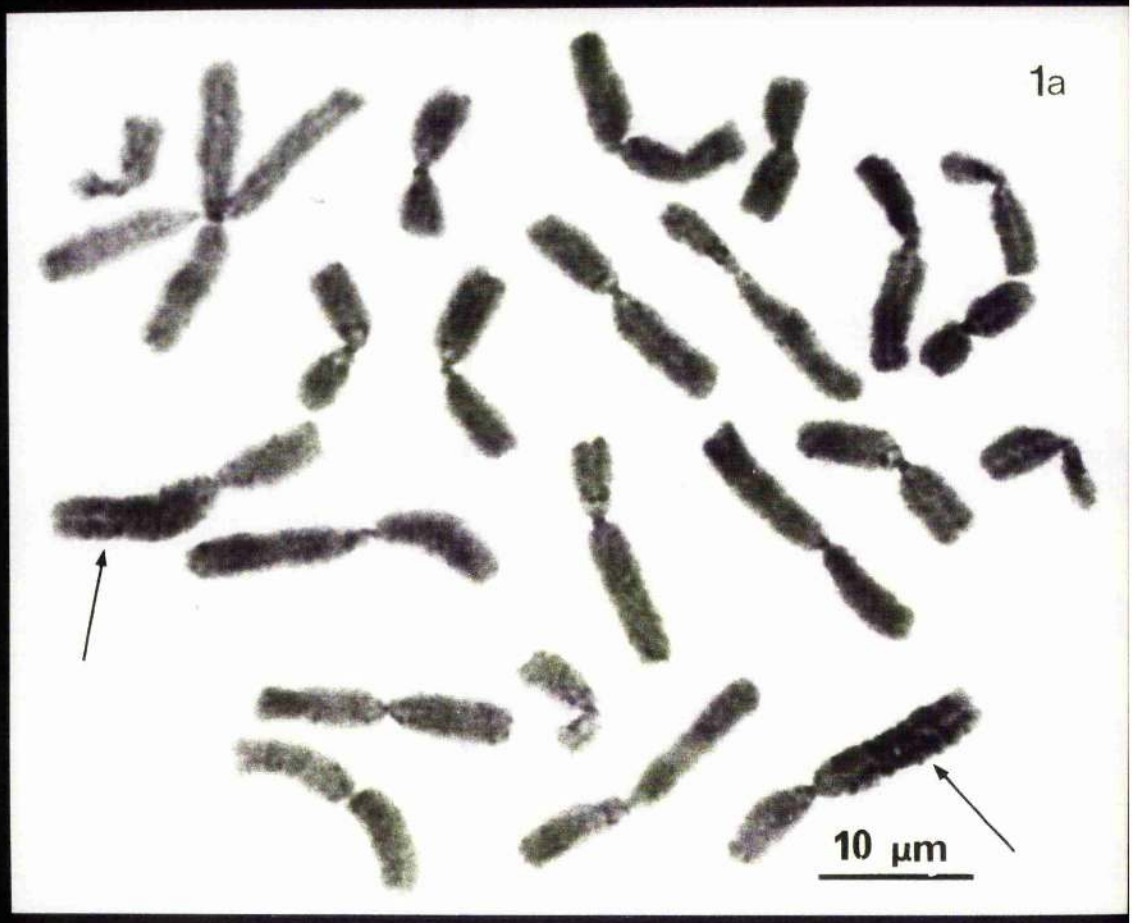
3. Differential spiralization of the mitotic chromosomes of T.c. carnifex after treatment with hypotonic saline.

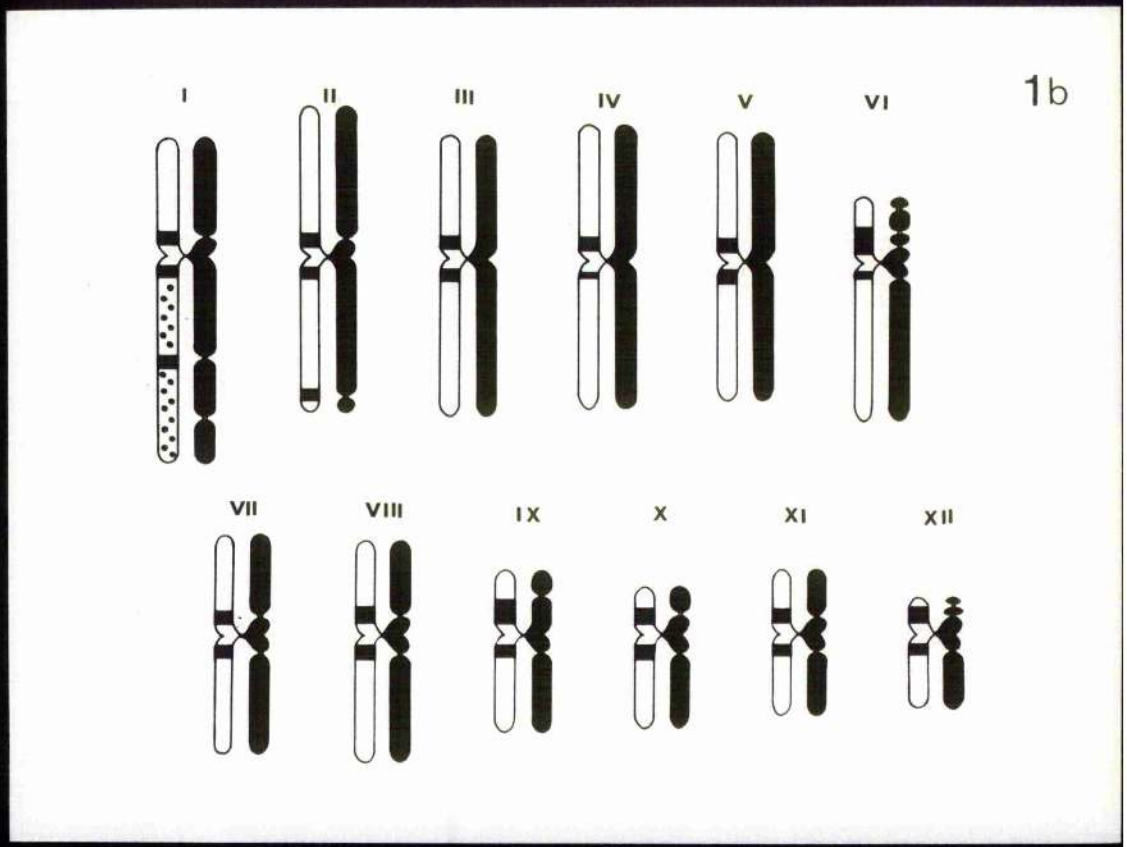
Ohnuki (1968) has demonstrated that human mitotic metaphase chromosomes are extensively despiralized after treatment with a hypotonic saline solution (a 4 : 1 : 1 : 2 mixture of 0.055 M KCl, 0.062 M KSCN, 0.055 M $NaNO_3$ and 0.055 M CH_3COONa) which allows the coiling pattern of each chromatid to be seen. I have observed a similar type of despiralization in the mitotic chromosomes of spermatogonial mitoses in cold- and colchicine-treated adult T.c. carnifex after a brief hypotonic treatment of testis fragments with distilled water before fixation (Rudak & Callan, 1976) (fig. I - 10a). I have also found that chromosome despiralization can be induced in T.c. carnifex tissue culture cells by hypotonic treatment with 0.07 M KCl for 50 - 60 mins before fixation (fig. I - 10b). It would appear that hypotonic treatment in some way releases the chromosome spirals from the factors which usually ensure normal condensation.

Figure Legends

I - 1a. Mitotic chromosomes of a spermatogonium of *Triturus cristatus carnifex*. The newt spent 4 days at 2°C, colchicine being injected 16 hrs before fixation. The preparation was stained according to the C-banding technique. The longer arms of chromosomes I are indicated by arrows.

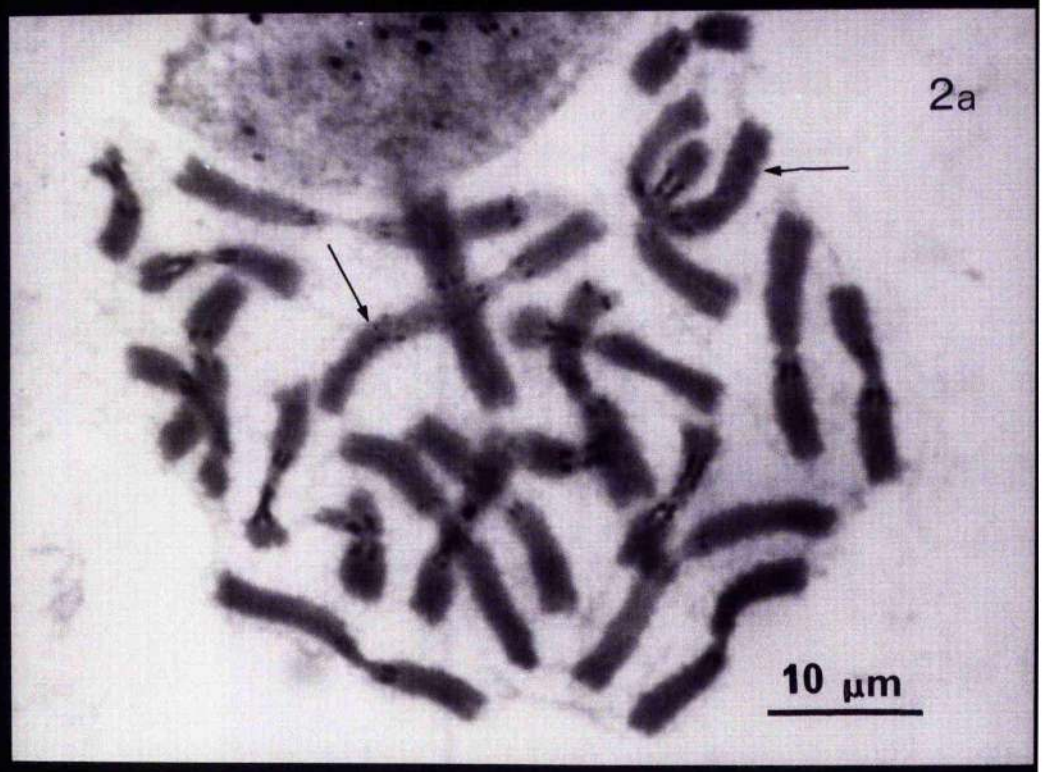
I - 1b. A diagram of the haploid chromosome complement of cold-treated *T.c. carnifex*. In each chromosome the chromatid to the left is drawn so as to indicate the regions which stain intensely with Giemsa; the chromatid to the right is drawn so as to indicate the positions of secondary constrictions.

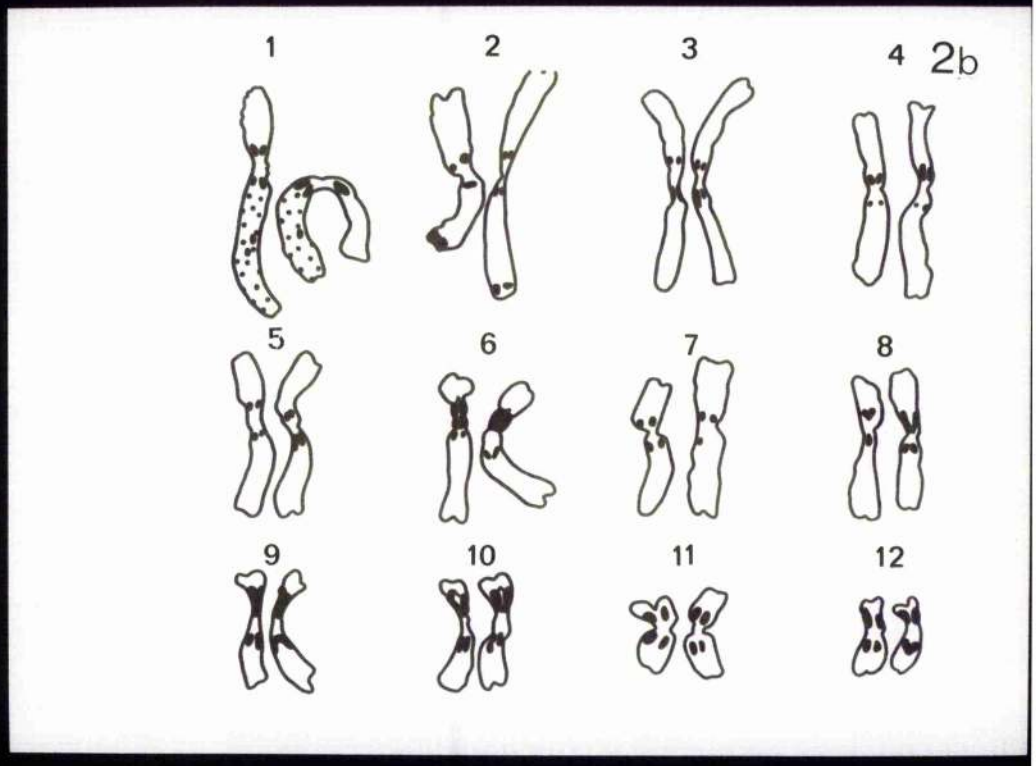

1a
10 µm


1b
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

I - 2a. A Giemsa-stained mitotic chromosome complement of a brain cell of T.c. carnifex. The larval newt spent 4 days at 2°C, and was in colchicine for the last 18 hrs before fixation. These chromosomes show deeply stained constrictions. The longer arms of chromosomes I are indicated by arrows.

I - 2b. A diagrammatic representation of the chromosomes in fig. I - 2a to show the pattern of deeply stained constrictions on homologous chromosomes.


2a
10 µm


1
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3
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2b
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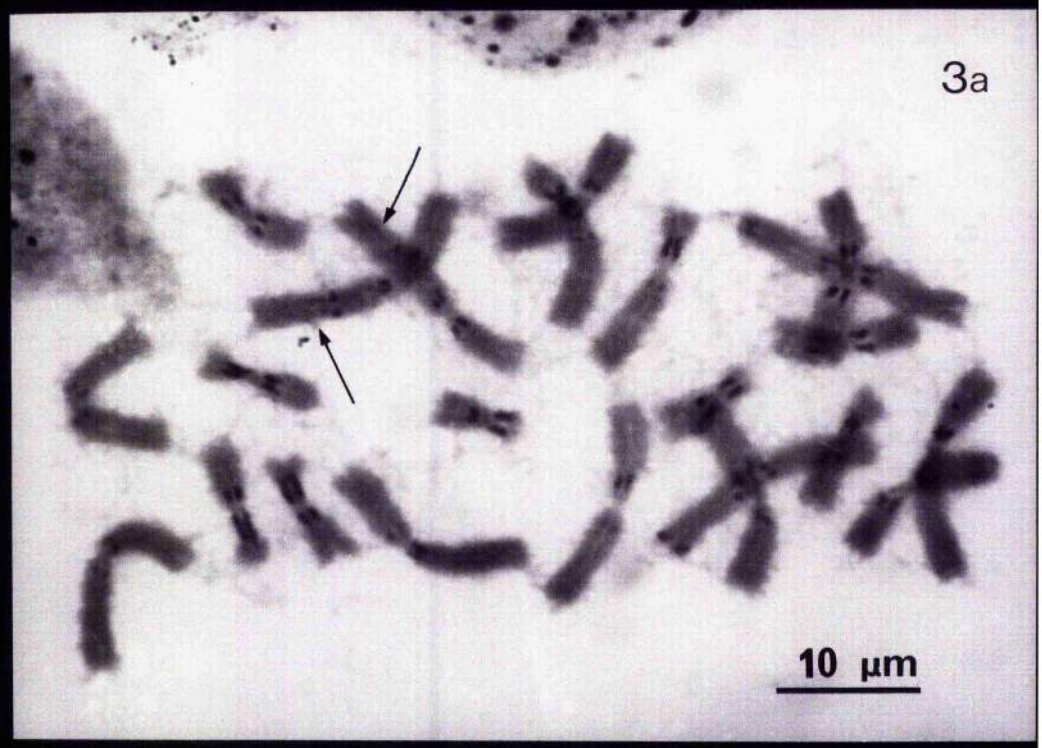
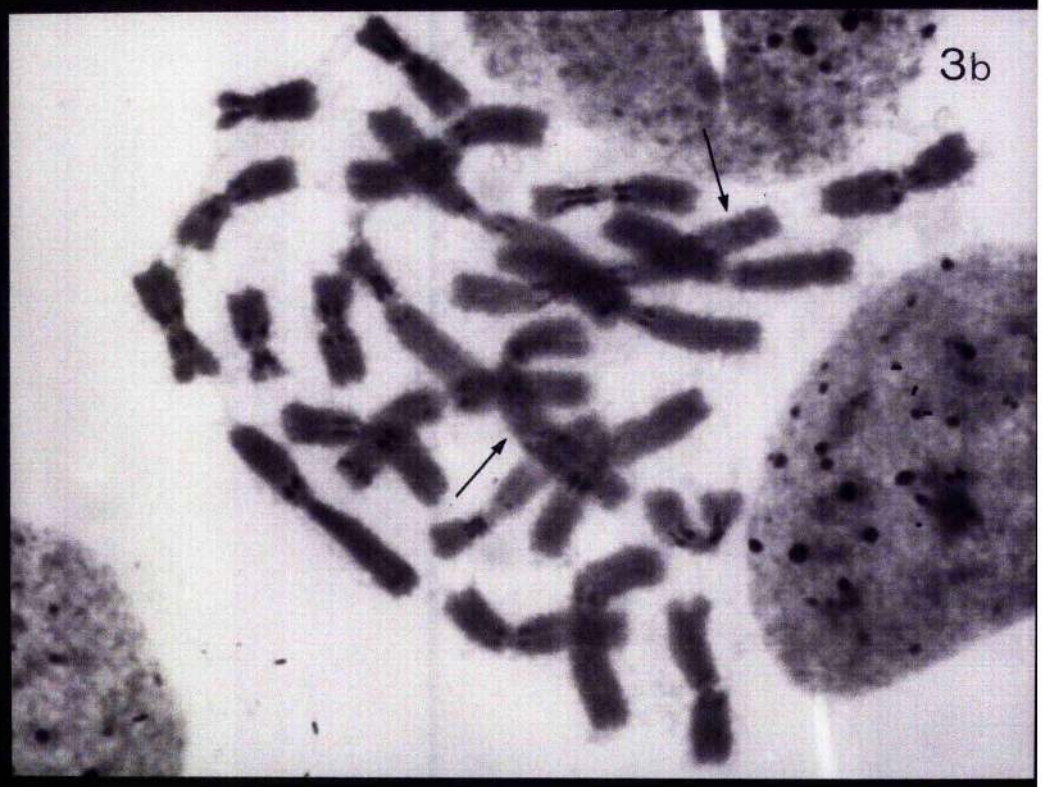
12

I - 3a. As fig. I - 2a.

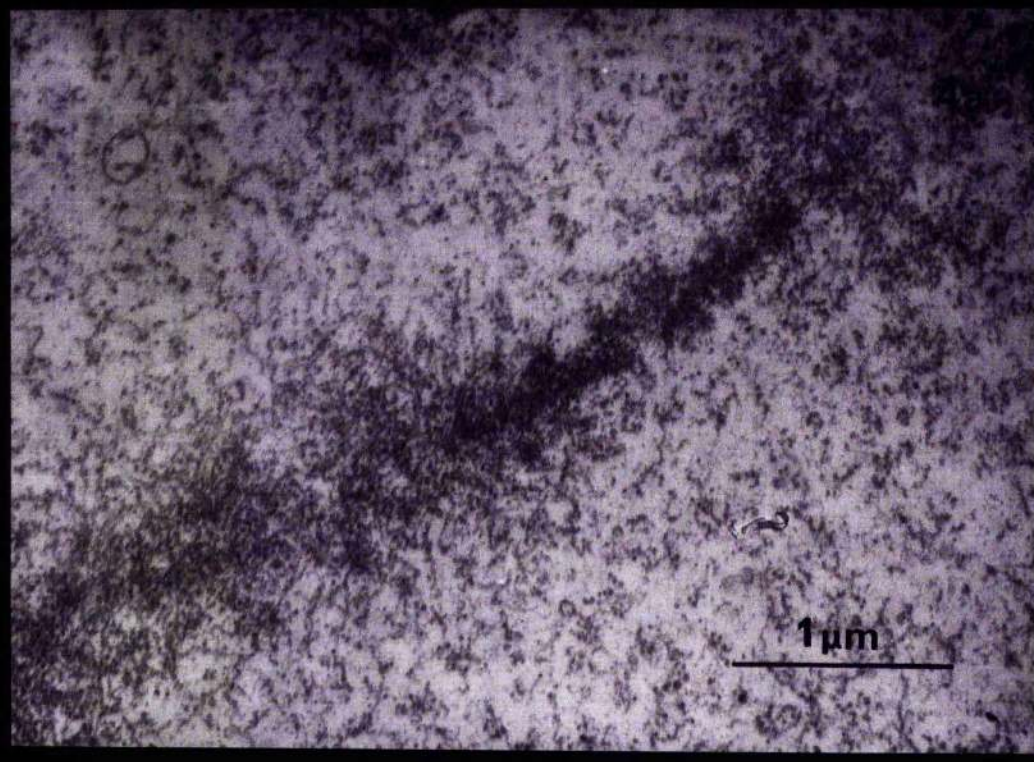
I - 3b. As fig. I - 2a.

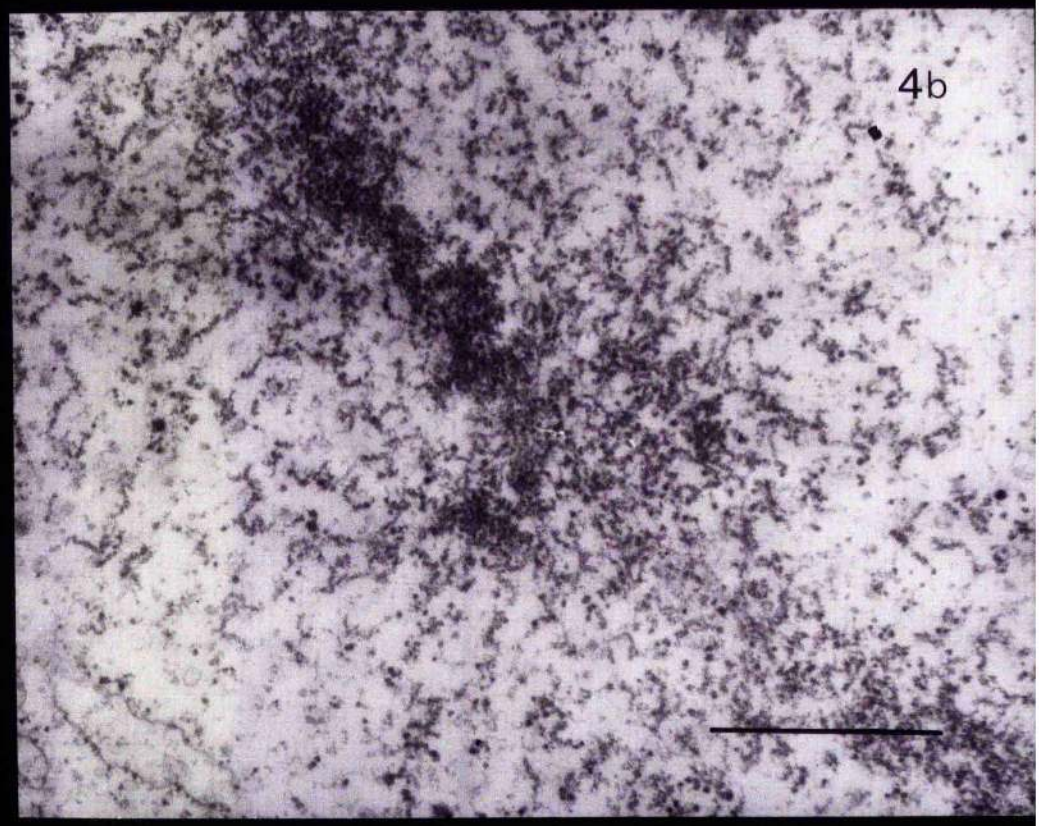

3a
10 µm


3b

I - 4a. Ultrathin sections of parts of mitotic chromosomes of T.c. carnifex in epithelial cells of a larval tail fin. The sections were cut in the plane of the tail fin. The larva spent 4 days at 2^{o} C prior to fixation, the last 18 hrs being in the presence of colchicine. The figure includes a constricted region, where the chromatin fibres are more densely packed then elsewhere.

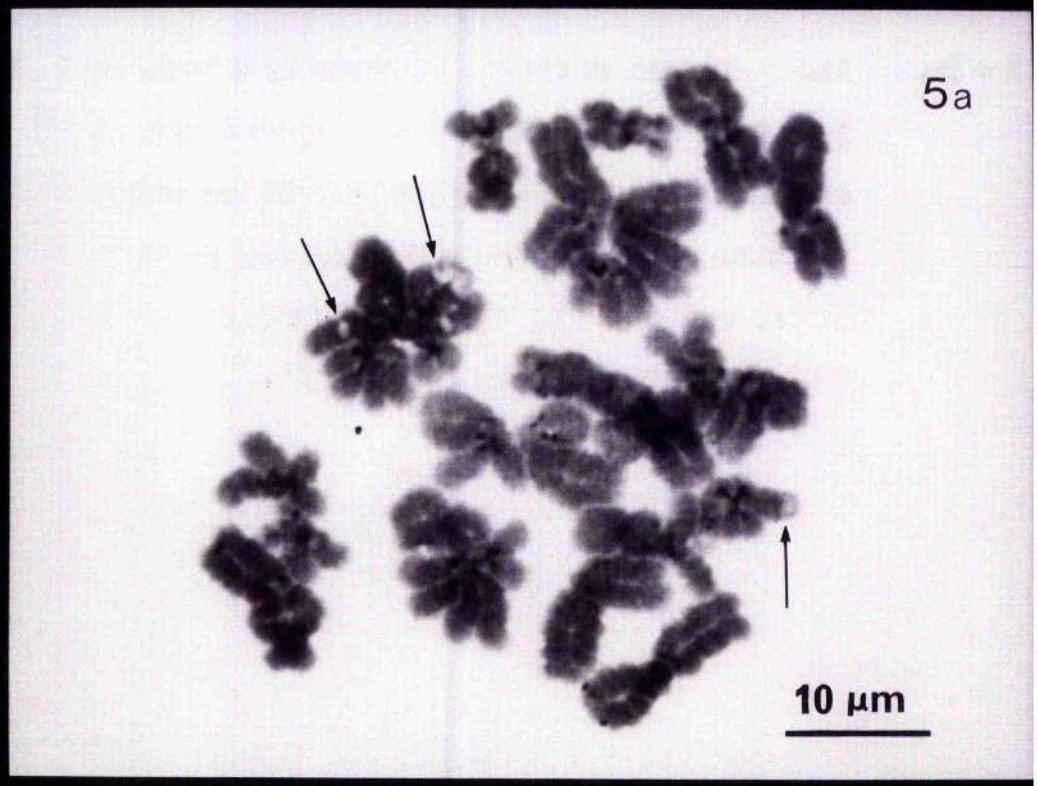
I - 4b. As fig. I - 4a.

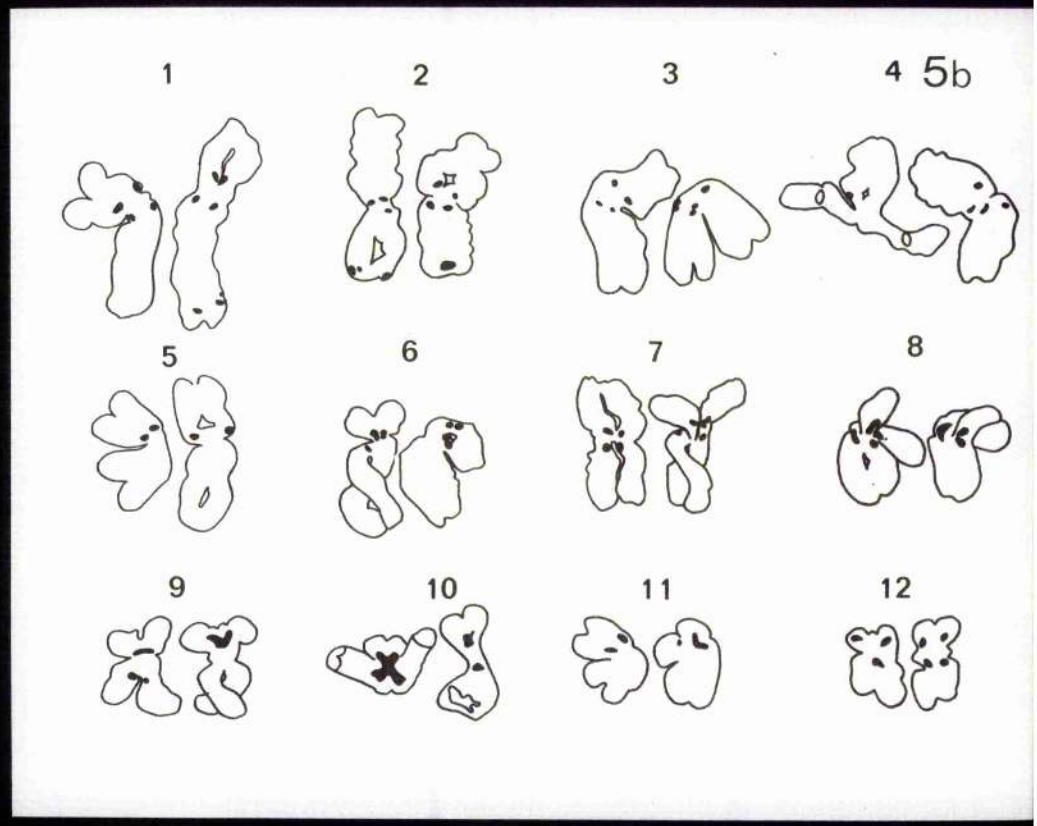

1µm


4b

I - 5a. Giemsa-stained mitotic chromosomes of a brain cell of T.c. cristatus. The larval newt spent 4 days at 4°C and was in colchicine for the last 18 hrs before fixation. The preparation was digested by trypsin before staining. 3 presumed nucleolar organizer constrictions are indicated by arrows.

I - 5b. A diagrammatic representation of the chromosomes in fig. I - 5a. The presumed nucleolar organizer constrictions of this animal lie near the end of the long arms of both chromosomes X and halfway down one arm of one larger chromosome, probably IV.


5a
10 µm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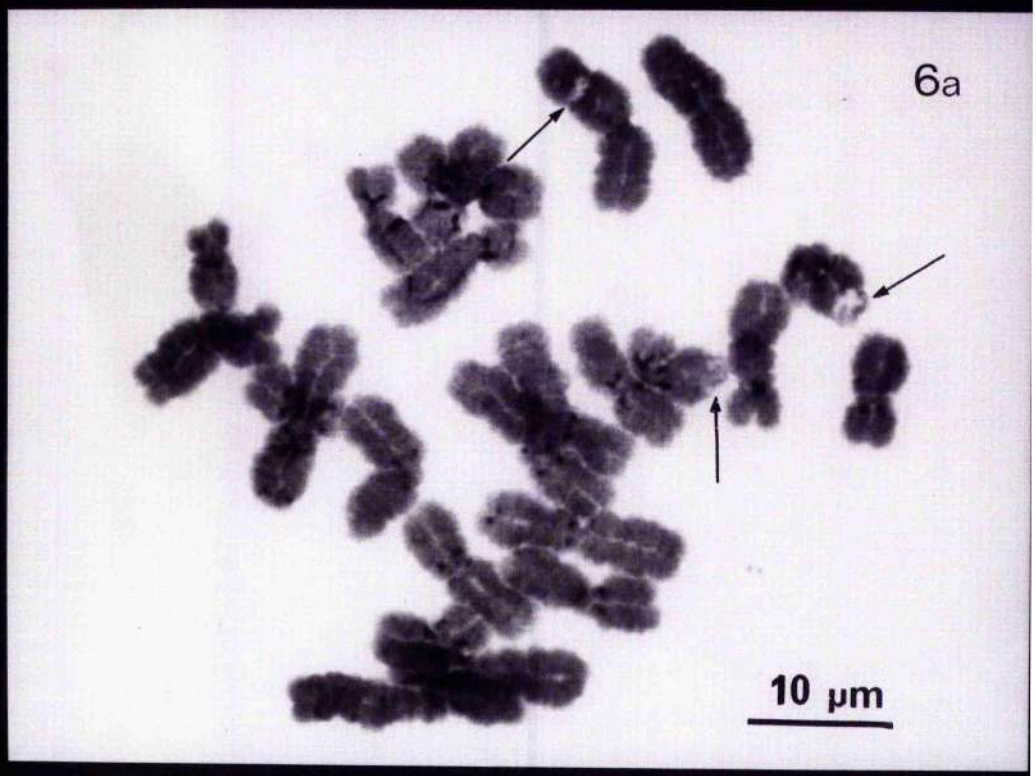
12

I. - 6a. As Fig. I - 5a.

I - 6b. As fig. I - 5b.


6a
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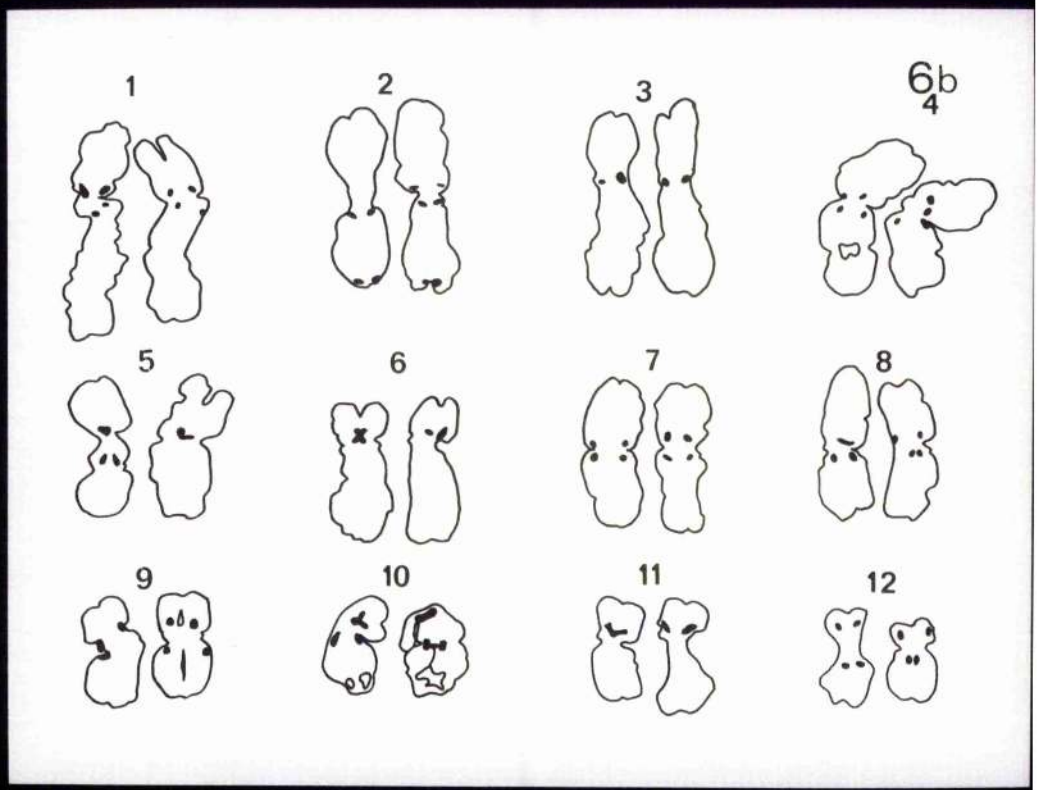
6b
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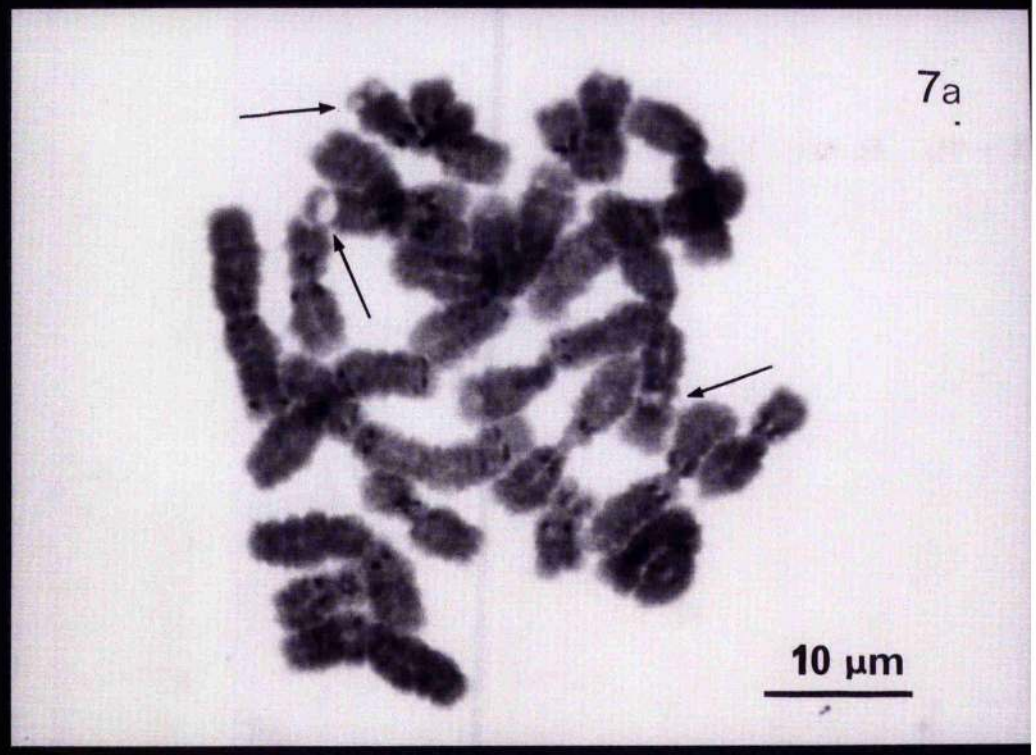
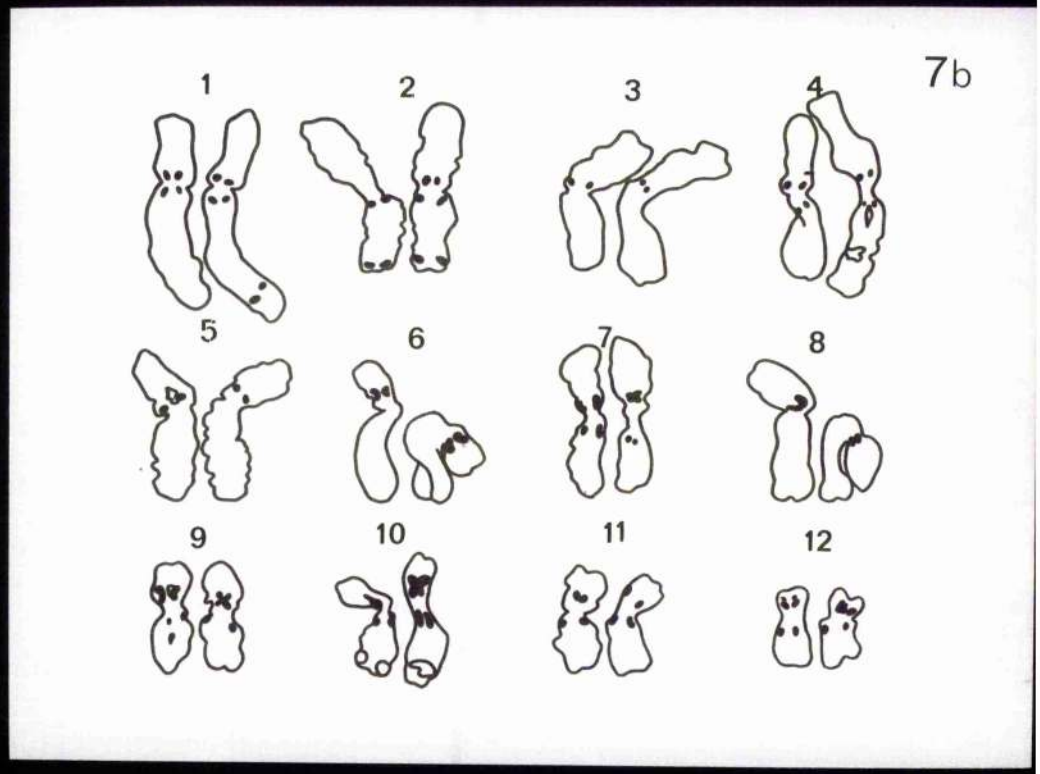
I - 7a. As fig. I - 5a.

I - 7b. As fig. I - 5b.


7a
10 µm


7b
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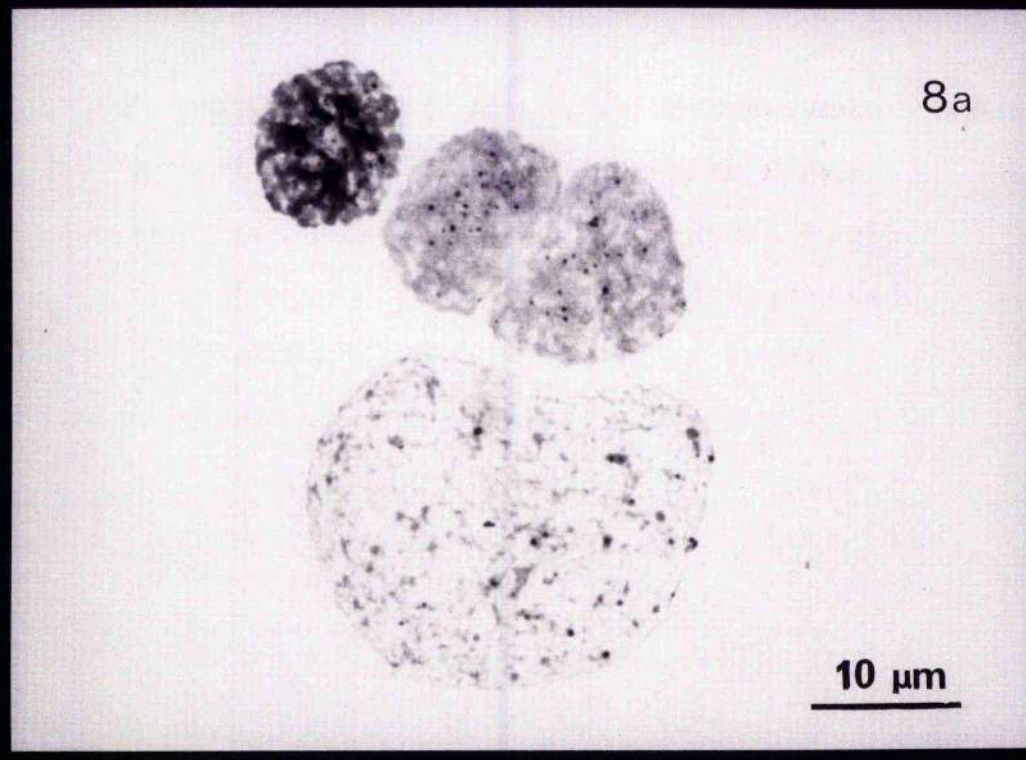
I - 8a. Giemsa stained brain cells of T.c. cristatus. The larval newt spent 4 days at 4°C and was in colchicine for the last 18 hrs before fixation. The preparation was digested with trypsin (0.025% Difco Bacto-Trypsin in Earles' balanced salt solution lacking Ca and Mg) for 6 mins before staining. Several deeply-staining "spots" can be seen in each nucleus.

I - 8b. As fig. I - 8a.


8a
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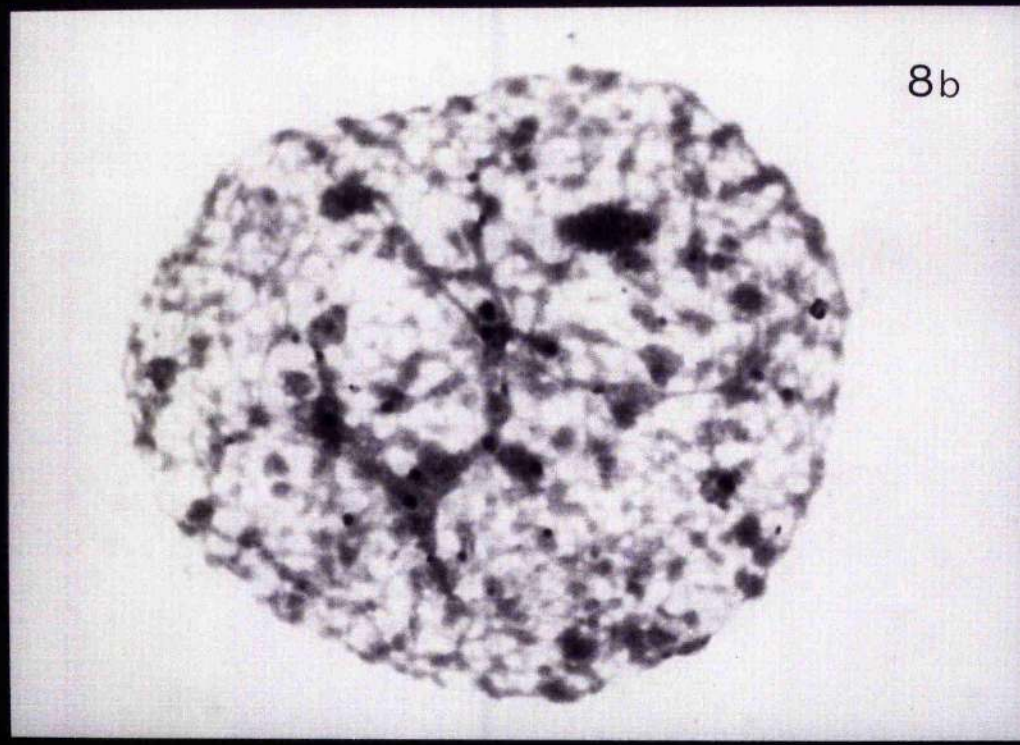
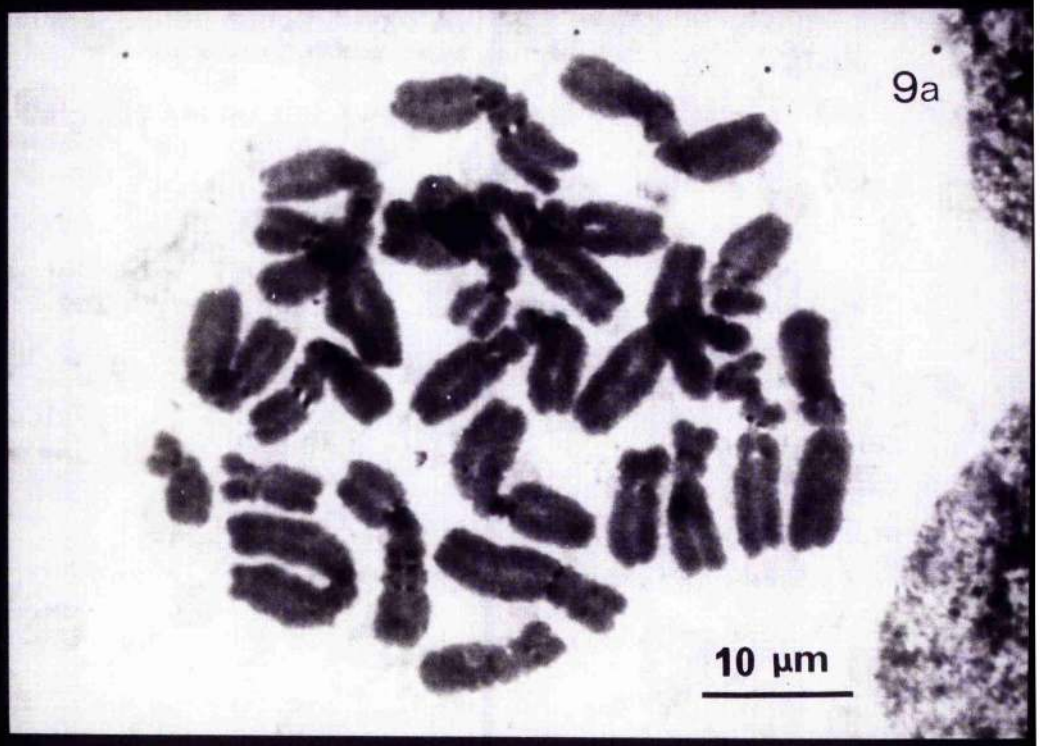

8b

I - 9a. Giemsa-stained mitotic chromosomes of a brain cell of *T. vulgaris*. The larval newt spent 4 days at 4°C and was in colchicine for the last 18 hrs before fixation.

I - 9b. A diagram of the haploid chromosome complement of *T. vulgaris* to show the distribution of those regions of the chromosomes which stain intensely with Giemsa after cold treatment. Chromosome XII is represented by two chromosomes of differing arm lengths. This variability in chromosome XII of *T. vulgaris* was reported by Callan (1942). I have never seen the subtelocentric chromosome XII in any of my preparations. The asterisks on chromosomes II and X indicate the positions of some of the sites of ribosomal DNA as identified by *in situ* hybridisation with ^{125}I-labelled ribosomal RNA. (See Chapter III.)


9a
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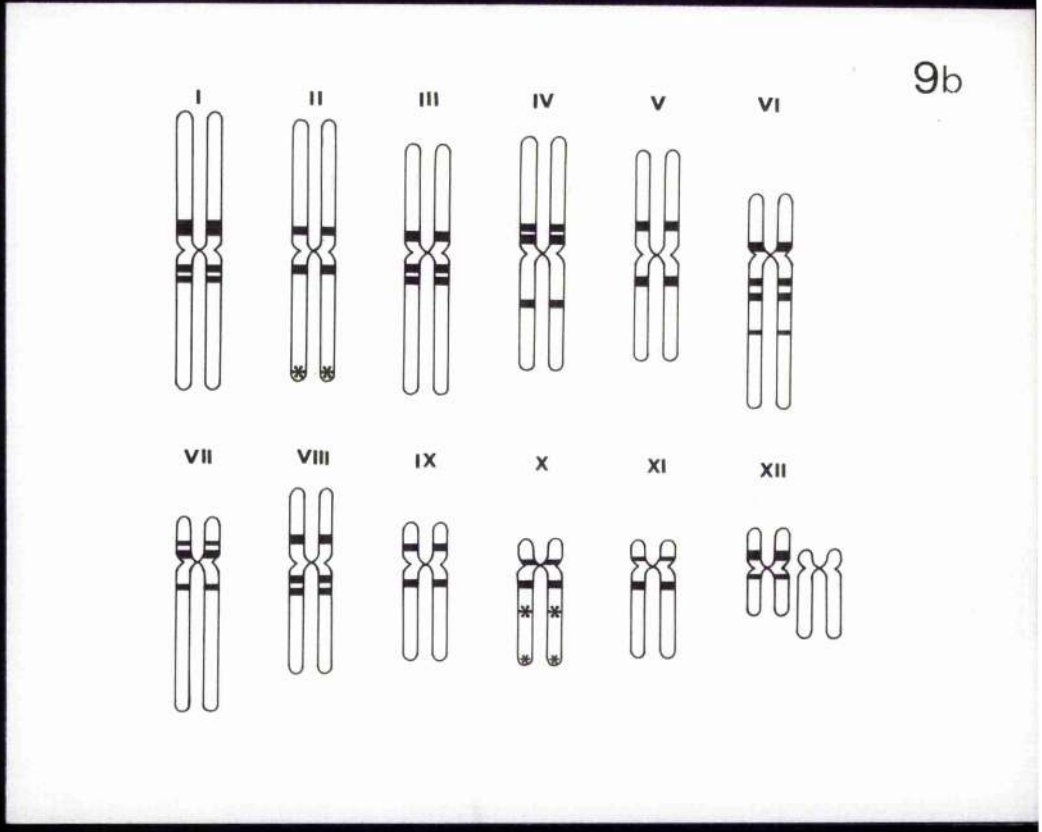
9b
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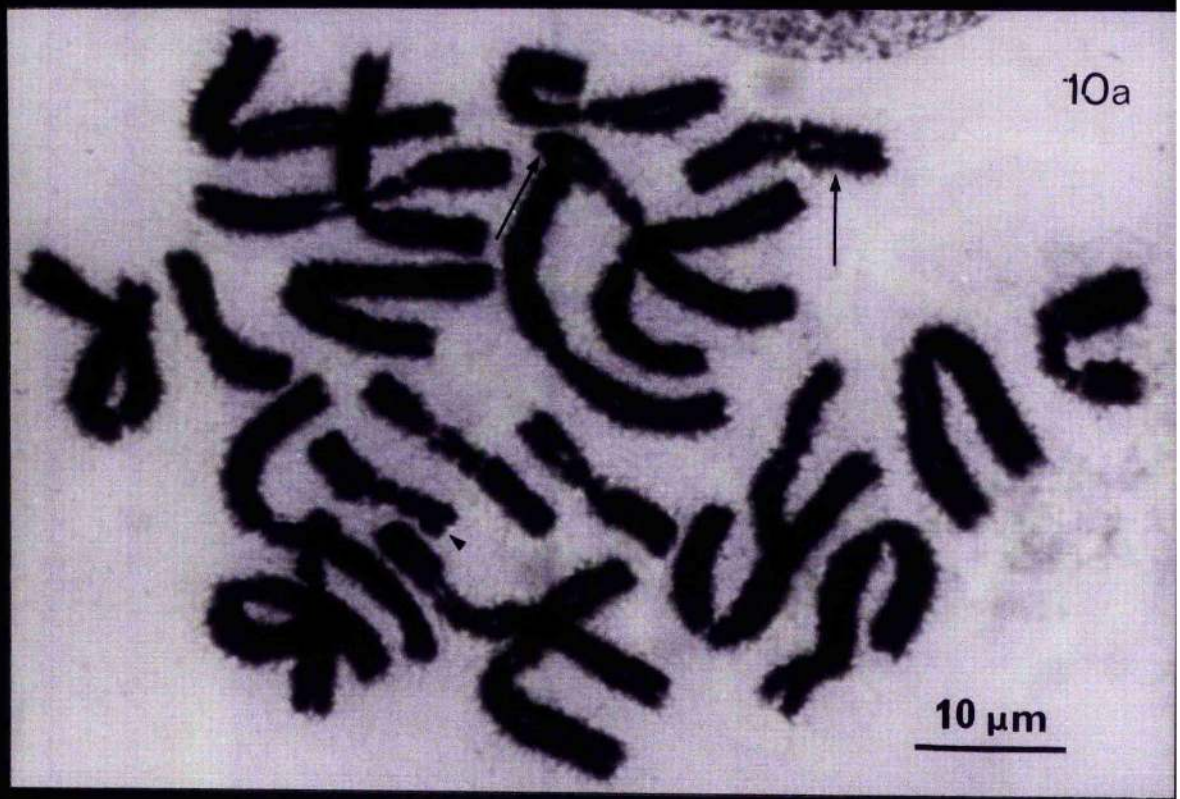
I - 10a. Giemsa-stained mitotic chromosomes of a gut cell of T.c. carnifex. The larval newt spent 4 days at 2°C, and was in colchicine solution for the last 18 hrs before fixation. The presumed nucleolar organizer constrictions are visible on one each of chromosomes VI and IX, and these are indicated by arrows.

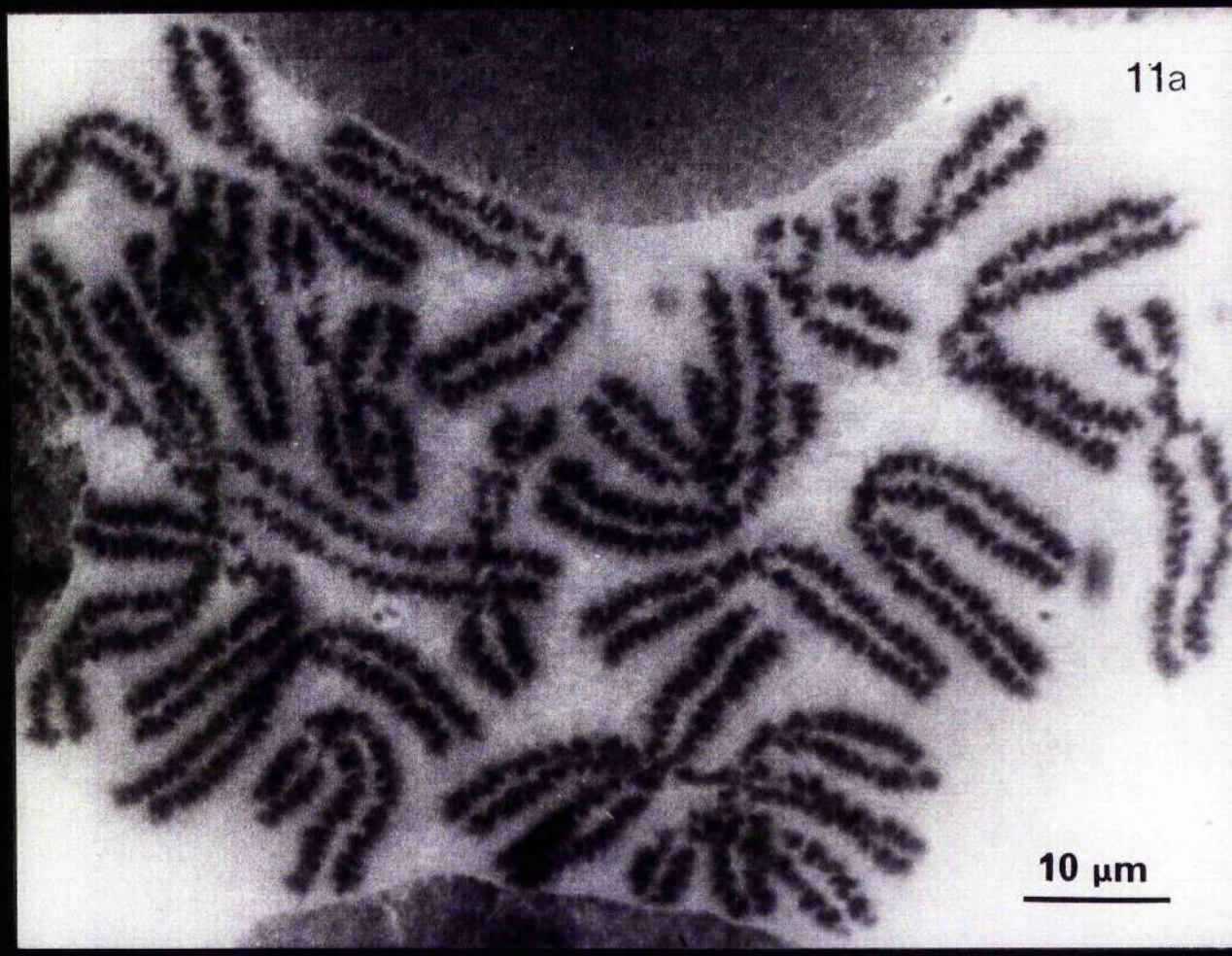
I - 10b. Geimsa-stained mitotic chromosomes of a cell from the T.c. carnifex tissue culture. The cells were cultured at 25°C in medium containing bromodeoxyuridine (1 µg/ml.) for 8 days, the last 18 hrs of this period being spent in medium also containing colchicine (1 µg/ml.). The secondary constrictions are in identical positions to those induced by culture at low temperature (fig. I - 10a). One of chromosomes XII is labelled in fig. I - 10a and I - 10b to illustrate the identical constriction pattern shown by both.

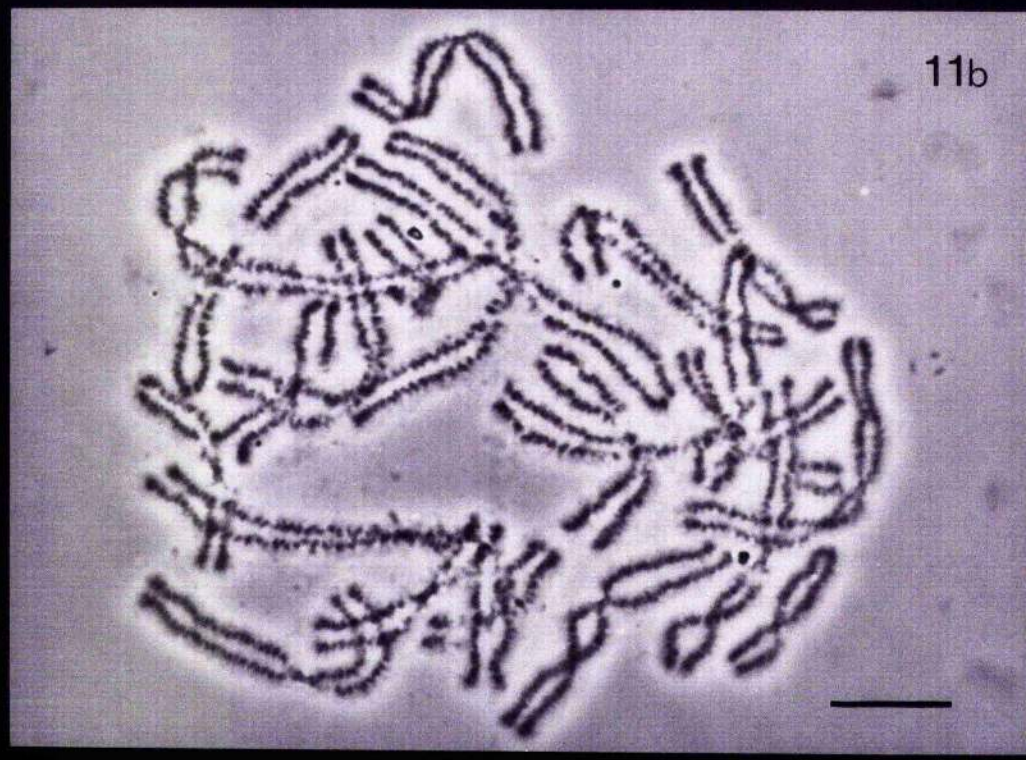

10a
10 µm


10b

I - 11a. An orcein-stained mitotic chromosome complement of a spermatogonium of T.c. carnifex showing spiral structure. The adult newt spent 4 days at 3°C, colchicine being injected 14 hrs before fixation. Immediately prior to fixation testis fragments were placed in distilled water for 15 min.

I - 11b. A preparation of mitotic metaphase chromosomes from the T.c. carnifex tissue culture. Colchicine (1 µg/ml. final concentration) was added to the cells for the last 18 hrs of culture. The cells were hypotonically treated with 0.075 M KCl for 60 mins before fixation in 3:1 absolute ethanol:glacial acetic acid. Cells were squashed in 45% acetic acid and photographed in phase contrast. All chromatids show spiral structure.


11a
10 µm


11b

CHAPTER II.

Method for obtaining and maintaing a fibroblast line of *Triturus cristatus carnifex* (female) cells in culture.

1. INTRODUCTION

During the early months of my research, while experiencing difficulties in obtaining somatic mitoses from both regenerating liver cells and spermatogonial cells of *T.c. carnifex*, it became apparent that there were many advantages to be gained if I could successfully establish a cell line of this animal in culture. For some of the studies on the mitotic chromosomes of *T.c. carnifex* which I wanted to carry out, particularly those involving the incorporation of specific metabolites, notably 5-bromodeoxyuridine, into the DNA (Chapter IV), a cell culture was essential.

The methods by which amphibian tissues can be cultured are identical to those applicable to mammalian cells, that is, either by culture of phytohaemoagglutinin-stimulated leukocytes obtained from a blood sample, or by growing cells from pieces of explanted tissue in monolayer culture. Leukocyte cultures have been successfully obtained from several species of amphibians, including *Necturus maculosus* (Seto *et al.*, 1964), *Pleurodeles waltlii* (Jaylet, 1965), *Rana pipiens* (Volpe *et al.*, 1966), *Rana nigromaculata* (Seto & Rounds, 1969), and *Bufo pardalis*, *B. calamita* and *B. marinus*, (Griffin *et al.*, 1970). Leukocyte culture is useful for performing a karyotype analysis of the animal studied and is comparatively simple to perform. However, leukocyte cultures can only be temporary since the cells will not continue to divide for more than a few generations, which makes long term studies impossible. Long-term studies are made possible by establishment of a monolayer culture of cells from portions

of tissues aseptically removed from the live animal. Under optimal culture conditions, these cells will continue to grow and divide, and occasionally, the culture may fortuitiously become a permanent cell line, the latter transformation often involving the loss of some chromosomes from the diploid set to give an aneuploid line. Only a few amphibian cell lines have been grown in monolayer culture, and these have been derived mostly from anuran tissues; Rana pipiens kidney (Auclair, 1961; Seto, 1964) haploid and diploid R. pipiens embryos (Freed, 1962, 1969) Taricha torosa, Necturus maculosus and Triturus pyrrhogaster lung, (Seto, 1964), R. catesbeiana tongue (Wolf & Quimby, 1964), "Bullfrog" lenses (Rothstein et al., 1965), R. pipiens kidney tumour (Freed & Rosenfeld, 1965), R. sylvatica and Xenopus laevis kidney, X. laevis liver, R. pipiens, R. clamitans, Hyla caerulea and R. sylvatica whole hatching embryos, (Rafferty, 1969), R. nigromaculata kidney (Seto & Rounds, 1969) and T. pyrrhogaster iris (Eguchi et al., 1974).

Several attempts to set up permanent cultures of Triturus cells have previously been made in this laboratory by Dr. J.H. Priest, who used explants of kidney, heart, lung and spleen. However, those cultures which proliferated initially were rather slow-growing and none of the cultures have survived. To the best of my knowledge, the only permanent amphibian cell cultures still surviving and regularly used in several laboratories are an aneuploid cell line initiated from tongue tissue of an adult, female bullfrog, Rana catesbeiana (Wolf & Quimby, 1964), and a culture of aneuploid Xenopus laevis kidney cells, line A-6, which was established by Rafferty in 1965 (Rafferty, 1969). Reese established a culture of iris cells from Triturus viridescens in 1967, which was reported to be in phase III culture stage in 1971 (personal communication from Dr. J.H. Priest to Prof. H.G. Callan). Phase III cultures have an increased generation time and reduced mitotic activity prior to total degeneration of the culture.

Using an amalgamation with modifications of the techniques previously described by other workers for establishing monolayer cultures of amphibian cells, I have established a line of fibroblast-like cells from T.c. carnifex. The methods for establishing and maintaining the cell culture are described below.

2. MATERIALS AND METHODS

(i) Sterilisation Procedures

(a) Tissue culture room

All work concerned with the culture of cells under sterile conditions took place in a room used for tissue culture only. The room and its contents can be irradiated with UV light from 2 wall-mounted bactericidal lamps (Hanovia model 13A) and these were usually switched on overnight, but for at least 2 hours before use.

Sterile procedures involving setting-up cultures, subculturing or filter sterilisation of solutions took place in the TC room under a sterile hood (Bassaire) fitted with a fan and light. The working surface, sides and top of the hood were initially sterilised by swabbing with 70% ethanol on a cotton wool pad, and were repeatedly swabbed during use. When the fan was turned on, a bunsen burner with a small strong flame was placed inside the hood and used to flame-sterilise instruments, necks of bottles, etc.

(b) Instruments

The stainless steel instruments used to dissect animals whose tissues were to be removed for culturing were first thoroughly cleaned with 70% ethanol and a paper tissue, then placed with scissor and forcep points downmost in a beaker of 96% ethanol under the sterile hood. The surface

of the ethanol in the beaker came to within 2 cm. of the end of the instruments so that they could be picked out and flamed easily without the risk of burning fingers. The instruments were flamed immediately before use, then propped up against an ethanol-swabbed glass rod whilst the ignited ethanol burnt away. After use, any large pieces of debris were cleaned from the instruments using an ethanol-soaked swab, and the instruments were returned to the beaker of 96% ethanol.

(c) Glassware

Glassware to be sterilized was first soaked in a 2% solution of RBS 25 detergent in hot tap water overnight. It was then washed thoroughly, and rinsed in running tap water for 2 hours, or in 5 consecutive rinses of running tap water by hand. It was given two final rinses in distilled water and dried in a heated drying cabinet. The mouths and necks of all flasks, bottles etc. were securely covered with a double-layer cap of aluminium foil. Pasteur pipettes were lightly plugged with cotton wool and placed, tips downwards, in 20.5 x 3.0 cm. boiling tubes. The mouths of the tubes were capped with a double layer of foil. The glassware was sterilized by dry heat in an oven at 150°C for 2 - 4 hours.

(d) Bottle caps.

All caps were washed in the manner described for glassware. Large aluminium bottle caps with rubber liners were placed in bags made from aluminium foil and the bags were sealed. The smaller types of plastic cap were placed in glass petri dishes which were then sealed with autoclave tape. The caps were sterilized in a pressure cooker (Prestige) by autoclaving at 15 lb. pressure for 20 - 30 mins.

The sterilized bottles were carefully capped with sterile caps, this operation being done under the sterile hood next to the bunsen flame. The foil caps from the bottles were placed over the plastic or aluminium caps on the assembled bottles for added protection from contamination.

(e) Solutions

Triple distilled water was sterilized by autoclaving 100 ml. aliquots in sterile glass bottles with the caps loosened (to allow equilization of the pressure inside and outside the bottle when heated) at 15 lb. pressure for 20 - 30 mins. After cooling, the caps were screwed down tightly.

(ii) Solutions required for tissue culture

(a) Amphibian wash solution (AWS) lacking Ca and Mg

per litre:-	NaCl	6.100 gm.
	KCl	0.580 gm.
	N_aHPO_4	1.065 gm.
	α-D glucose	0.400 gm.
	Bovine serum albumin (30%)*	10.0 ml.
	phenol red (0.5% w/v)**	2.0 ml.
	3 x distilled water	to 1000 mls.

* Armour Pharmaceutical Company Ltd., Eastbourne, England; 25 ml. vials (sterile)

** Gibco: 100 ml. bottles (sterile) Cat. No. 614.

To sterilize:- Millipore filter.

To store:- 100 ml. aliquots in sterile glass bottles Refrigerated at + 4°C.

To use:- 1.0 ml. penicillin/streptomycin (pen/strep) (see below) was added per 100 mls AWS before use.

(b) Penicillin/Streptomycin ("pen/strep")*

Obtained sterile in desiccated form in vials, each containing 100,000 units penicillin G and 100,000 µg streptomycin sulphate. Each vial is rehydrated with 10.0 mls of sterile distilled water from a sterile 10 ml. disposable syringe fitted with a sterile 21G2 (Yale) needle.

To store:- 1.0 ml. aliquots in sterile plastic tubes (Falcon). Frozen at -20°C. Un-reconstituted vials were refrigerated at +4°C.

To use:- 1.0 ml. pen/strep was added per 100 - 110 ml. volume of AWS or tissue culture medium before use.

* Difco: boxes of 6 vials, Code No. 5854.

(c) Trypsin*

Obtained sterile in desiccated form in vials. Each vial is rehydrated with 10.0 mls of sterile distilled water from a sterile disposable syringe fitted with a sterile 21G2 (Yale) needle. This gives 10.0 mls of a 5% solution of trypsin.

To store:- 1.0 ml. aliquots in sterile plastic tubes (Falcon). Frozen at -20°C. Un-reconstituted vials were refrigerated at +4°C.

To use:- Diluted with AWS to desired concentration.

For subculturing procedures, 0.1% trypsin in AWS was used (1.0 ml. 5% trypsin + 49.0 ml. AWS).

* Difco Bacto-trypsin: boxes of 6 vials; Code No. 0153.

(d) Phytohaemagglutinin (PHA)*

Obtained sterile in desiccated form in vials. Each vial was rehydrated with 10.0 mls of sterile distilled water from a sterile disposable syringe fitted with a sterile 21G2 (Yale) needle.

To store:- Refrigerated in vial at +4°C for up to 6 weeks.

To use:- At a final concentration of 3 - 6% (v/v) when added to the culture. Usually, 0.2 ml. PHA was added to a flask containing 5.0 mls of medium (4% PHA).

* Gibco: PHA (M form); 10.0 ml. vials; Cat. No. R15-0576.

(e) Tissue culture medium*

Stock: Eagles MEM, purchased sterile in 100 ml. or 500 ml. bottles.

To store:- Refrigerated at +4°C.

To use:- Cells are cultured in a medium containing

100 mls Eagles MEM*
10 mls Foetal Calf Serum**
1 ml. glutamine***
1 ml. pen/strep

} referred to as "tissue culture medium".

* Gibco: Eagles MEM - Minimal Essential Medium (1x) with Earle's salts, without L-glutamine. 100 ml. and 500 ml. bottles. Cat. No. 109. Flow: Minimal Essential Medium (MEM Modified) with Earles' Balanced Salt Solution (EBSS). Liquid. With 0.85 g/l. sodium bicarbonate but without L-glutamine. 100 ml. bottles Cat. No. 1-065 D.
500 ml. bottles Cat. No. 1-065 M.

** Gibco: sterile; in 100 ml. bottles; Cat. No. 614. Stored in 10.0 ml. aliquots in sterile plastic tubes (Falcon). Frozen at -20°C.

*** Flow: 200 mM L-glutamine; sterile; in 50 ml. bottles; Cat. No. 6-134 C. Stored in 1.0 ml. aliquots in sterile plastic tubes (Falcon). Frozen at -20°C.

Cells in 25 cm^2 growth area flasks (see 'Hardware') are cultured in 4 - 5 mls of the above medium. Cells in 75 cm^2 growth area flasks are cultured in 12 mls of medium. Stored refrigerated at +4°C. Used within 6 weeks of adding serum etc.

(f) Colchicine*

This was used on the cultures at a final concentration of approx. 1 µg/ml. A stock solution of 100 µg/ml. colchicine was made up by dissolving 1.0 mg. colchicine in 10.0 ml. distilled water.

To sterilize:- Millipore filter.

To store:- Refrigerate non-sterile powder at +4°C.

Colchicine solutions were not stored because they soon lose their activity.

* BDH: non-sterile powder; Cat. No. 27805.

To use:- 0.12 ml. of 100 µg/ml. colchicine solution was added to a flask containing 12.0 mls of culture medium. Final colchicine concentration is 1 µg/ml.

(g) Hypotonic saline (not required sterile)

0.075 M KCl = 0.558 gm. KCl in 100 mls distilled water.

To store:- Refrigerated at +4°C.

(iii) 'Hardware'.

Glassware

(a) Graduated 100 ml. glass bottles, initially obtained from 'Flow' or 'Gibco' containing medium, serum, glutamine, etc.

(b) Pyrex 15.0 ml. graduated conical centrifuge tubes, made by Jobling Laboratory Glassware, obtained from Macfarlane Robson, Ltd., Burnfield Avenue, Thornliebank, Glasgow G46 7TP.

Sterile, Disposable Plastic-ware		Cat. No.
(a) Tissue culture flasks, 25 cm^2,	20 per pack, 500 per case	3013
75 cm^2,	5 per pack, 100 per case	3024
(b) Petri dishes, 60 x 15 mm,	20 per pack, 500 per case.	3002
(c) Pipettes, 1.0 ml. serological.	Individually wrapped 200/shelf pack	7521
5.0 ml. "	25 per pack	7529
10.0 ml. "	25 per pack	7530
(d) Tubes with caps, 17 x 100 ml. (15 ml.)		2001

All obtained through 'Gibco' or 'Flow'.

(e) Syringes (Luer-lok), 250 x 1.0 ml.	00012-11
40 x 20.0 ml.	00011-0-11
50 x 50.0 ml.	05607---11

by Beckton, Dickinson and Co., Ltd.

Order from Macfarlane Robson, Ltd., Glasgow.

90 x 2.0 ml.
40 x 10.0 ml.

by Grahams' Medical Products,

Order from Macfarlane Robson, Ltd., Glasgow.

Cat. No.

(f) Needles, Yale microlance 21G2 (green) 05129-0-11
26G 3/8 (brown) 05110-0-11

by Beckton, Dickinson and Co., Ltd.,
Order from Macfarlane Robson, Ltd., Glasgow.

(iv) Culture Methods

(a) Procedure used for setting-up a culture from the tissues of T.c. carnifex

A female newt was anesthetized in a 0.1% solution of MS222 (Sandoz) for several minutes, then placed in a 10% solution of "Savlon" antiseptic for 5 mins. The body surface of the newt was swabbed thoroughly with a pad of cotton wool soaked in 70% ethanol, then the body cavity was opened along the dorsal surface just left of centre using ethanol- and flame-sterilized scissors and forceps. Portions of several tissues (spleen, liver, brain and abdominal skin) were removed, and placed separately in sterile plastic tubes (Falcon) each containing 5 mls of tissue culture medium. The tubes were capped and gently inverted a few times to wash the tissues. The medium was decanted off and the tissues washed again in 5 mls fresh medium. The medium was decanted off, and the tissue fragments were each placed in a sterile, 60 x 15 mm, plastic petri dish (Falcon) containing 5 mls medium. The tissues were torn into fragments using two pairs of sterile No. 4 watchmakers' forceps and iridectomy scissors. The minced tissue fragments and medium were placed in sterile culture flasks (Falcon, 25 cm^2) which were tightly capped. The cells were cultured at 25°C in a water-cooled incubator. After 2 weeks, fragments of spleen and brain were adhering to the culture flask, and observation through an inverted microscope showed that there was a halo of proliferating cells around these fragments. Thereafter, the medium was changed weekly, with 5.0 mls fresh medium. These cells continued to grow

for 4 months but eventually died. The flask containing portions of abdominal skin remained undisturbed for 9 weeks. After this time, there was extensive cell growth around all skin fragments, forming an almost confluent layer of cells. The medium in this flask was changed weekly for the next 4 weeks. The cells were subcultured in order to obtain a cell monolayer without large tissue fragments. The flasks were agitated manually until the large tissue fragments had detached from the flask, then the fragments were tipped off with the medium. 5.0 mls of trypsin (Difco Bacto-Trypsin) 0.1% in amphibian wash solution (AWS) was added. At room temperature the cells detach from the flask in 3 - 6 mins. The cell suspension was centrifuged in 10 ml. sterile plastic tubes for 5 mins at 900 rpm. The trypsin solution was poured off and the cell pellet was resuspended in 5.0 mls of fresh culture medium and put into another 25 cm^2 culture flask. The cells had reattached to the flask in 24 hours and formed a near-confluent monolayer. One week later, the cells were trypsinized as described above, and split to 2 x 25 cm^2 culture flasks, each containing 5.0 mls of culture medium. To one flask, 0.2 ml of phytohaemagglutinin (M form) (Gibco) was added. Thereafter, the cells were split 1:2 every 3 weeks for the next 12 weeks (the flasks of cells from the original culture inoculated with PHA did not show any peculiar growth features and were therefore "pooled" with the non-PHA-treated cells after 6 weeks). The cells continued to proliferate at a slightly increased rate, and were then split 1:3 or 1:4 every $2\frac{1}{2}$ - 4 weeks for the next 32 weeks. The cells are still growing well after a total period of 12 months in culture. A 1:3 split can also be done by transfering the cell pellet obtained by trypsinizing one 25 cm^2 flask, to 12 mls of medium in one 75 cm^2 flask.

(b) Routine subculturing

The old medium is poured off the cells and 2 mls (to a 25 cm^2 flask) or 6 mls (to a 75 cm^2 flask) of 0.1% trypsin is added. The flask is rocked gently to promote detachment of the cells. The cells detach in about 5 mins. If the cells are in contact with the trypsin solution for longer than 5 mins, a small volume (about 2 - 3 mls) of medium is added to the cell suspension before centrifugation to suppress the action of the trypsin. The cells are pelleted by centrifugation at 900 rpm for 10 mins, and the trypsin solution poured off. The cell pellet is resuspended in an appropriate volume of fresh culture medium and 'seeded' into fresh flasks.

(v) Preparation of Slides

In order to look at metaphase chromosomes, the cultures were treated with colchicine (final concentration 1 µg/ml) for the last 16 - 22 hours of culture. The cells were trypsinized by the method described for routine subculturing, and the cell pellet obtained was given hypotonic treatment by resuspending the cells in a siliconized conical Pyrex centrifuge tube in about 5.0 mls of 0.075 M KCl solution at room temperature, by careful agitation with a pasteur pipette, then left to swell for 50 - 60 mins. The cells were pelleted by centrifugation at 900 rpm for 10 mins, and the hypotonic saline decanted off. The cell pellet was disrupted by sharply flicking the end of the tube. The cells were fixed by adding about 5.0 mls of a 3:1 mixture of absolute methanol and glacial acetic acid dropwise from a pasteur pipette, while the tube and its contents were simultaneously agitated by a 'Whirlimixer' (Fisons). The cells were left to fix at +4°C for 1 - 2 hours, then pelleted by centrifugation and refixed 2 - 3 times in the manner described above.

(a) Squash preparations

The fixative was decanted off the fixed cell pellet, and the cells were resuspended in about 0.5 - 2.0 mls of 45% acetic acid, using a siliconised pasteur pipette. A small drop of the cell suspension was placed in the centre of a siliconised 22 mm^2 coverslip (Chance, No. 1) and the cell suspension was squashed onto a subbed slide, then made permanent in the manner described in Chapter I.

(b) Air dried preparations

The fixative was decanted off the fixed cell pellet and the cells were resuspended in an appropriate volume of fresh fixative. The amount of fixative required to give a cell concentration of suitable density for air-drying varies according to the number of cells in the pellet, and the length of time they received treatment with hypotonic saline. As a rough guideline, the cells obtained after splitting 1 x 75 mm^2 flask to 3 x 75 mm^2 flasks, letting them grow for 8 days, with colchicine-treatment for the last 20 hours of culture, trypsinising 2 of the flasks, hypotonically treating the cells for 60 mins, the final fixed cell pellet should be resuspended in about 1.0 ml. of fresh fixative. Fixed cells were stored in fixative in 1.5 ml. capped plastic tubes (Eppendorf) at +4°C.

A $6\frac{1}{2}$" diameter x $3\frac{1}{2}$" deep Pyrex glass crystallising dish was filled to within $\frac{1}{2}$" of the top with crushed ice, then also filled to this level with distilled water. Several pre-washed, scrupulously clean slides were stood on end in the iced water, with the ground glass ends uppermost, and the slides were left to cool for about 5 minutes. One slide was removed from the dish, held at the ground glass end between index finger and thumb of the left hand, and shaken once to remove excess water. If the slides are clean the water will form a thin film over both surfaces.

1 - 2 drops of the fixed cell suspension from a siliconised pasteur pipette was placed near the ground glass end of the slide, at the top edge, and the slide simultaneously tilted at an angle of 45° to the long axis downwards. The bottom end of the slide was placed on a pad of paper tissues to help the fixative drain down the slide. When the film of cells in fixative was 2/3 of the way down the slide, the slide was tilted at 45° to its short edge and drained off on the pad of tissues. This operation gives a spread of cells over most of the surface area of the slide. The preparations were air dried at room temperature or with the aid of a cold-air fan. Dried preparations were stored in cardboard trays in dustproof black cardboard boxes at room temperature.

(vi) Photography

Living cells on the surface of 25 mm^2 culture flasks were photographed on Ilford Pan F film using a Zeiss Plankton Microscope fitted with a x20 Neofluar (phase) objective.

Squash preparations of fixed cells in 45% acetic acid were photographed on Ilford Pan F film using a Zeiss microscope fitted with a x100 (oil) Neofluar objective.

3. Results

The initial cell layer formed after inoculation of a flask consists of cells bearing many granule-containing cytoplasmic extensions. Fig. II - 1 shows some of these cells. In a few days, as the cell sheet becomes more dense, individual cells become compressed by their neighbours and take on the spindle-shaped appearance of the typical fibroblast-like cell.

After one year in culture, the cells are still diploid, although there are some larger-than-normal cells which are obviously polyploid. Some metaphase sets frequently contain double the diploid number of chromosomes, and counts made of the number of nucleoli per cell (Chapter III) go up to double (9) the mean number (4) seen in the majority of cells.

Fig. II - 2 shows a set of metaphase chromosomes from a squash preparation. In general, the chromosomes tend to be longer and less condensed than those chromosomes obtained from squashes of larval tissues or adult testis or liver. Although the cells in Fig. II - 2 were grown in the presence of BUdR, their general morphology, disregarding the constricted regions, is much the same as normally cultured cells.

4. DISCUSSION

During the course of my research it became apparent that there would be many advantages in establishing a cell line of T.c. carnifex, both for my own use and for that of other members of the department and close associates to this laboratory. In the absence of a cell culture, it is quite time-consuming to obtain good preparations of newt mitotic chromosomes. For the latter, I have used 3 techniques which are described in detail in Chapter I. Apart from the difficulties encountered in keeping the animals in good conditions following partial hepatectomy or at the relatively high temperature of 25°C, used to stimulate spermatogonial divisions, there are many problems which are encountered when pieces of tissue thought to contain mitoses, are squashed in 45% acetic acid. Unless the slides are properly 'subbed' and the coverslips well siliconised, the cells may be literally torn apart when the coverslip is flicked off, with fragments remaining attached to both coverslip and slide. This can still

happen even when the coverslips and slides have been correctly prepared. for unknown reasons. Squashing pieces of fixed tissue in 45% acetic acid, while usually ensuring good flattening of the chromosomes, does not influence spreading of individual members, and preparations are frequently obtained where the metaphase chromosomes are so tangled as to make karyotype analysis impossible. The degree to which chromosome sets will spread is predominantly determined by the hypotonic treatment given to the cells before fixation, and this is impossible to regulate when working with pieces of tissue many cells thick. The same difficulties related to chromosome spreading are found when squashing larval tissues, with the additional problem that larval mitoses can only be obtained for 4 months of the year from March to June, and even then only after overcoming difficulties related to obtaining fertilised eggs, then keeping them in good condition while the embryos are developing. Newt eggs have a tendency to contract mould growth very quickly unless the mouldy eggs are immediately removed from the tanks.

The method used to obtain air dried preparations of metaphase chromosomes from tissue culture cells has proved very successful and the chromosome morphology is excellent. It is a technique which is usually applied to human lymphocytes.

The methods usually used when setting up a culture from explanted tissues involve trypsinization of the tissue to dissociate the cells. Amphibian tissues appear to be unusually sensitive to trypsinization at this early stage in the establishment of a culture, and it is frequently not successful, probably due to the large cell size. I have attempted several times to set up cultures of newt liver cells obtained from slow, tryptic digestion at room temperature, and although a reasonable number of cells are dissociated from the liver fragments, only very few attach to

the culture flask even after 3 days, and these eventually round up and die after a few weeks. As I have described previously, the method used to initiate this culture merely involved mincing the tissues into small fragments using iridectomy scissors, and without the aid of enzymatic digestion. I have also found this method to work well with tissues taken from Rana temporaria and Amphiuma means which I have recently established as primary cell lines in culture.

The fibroblast-like nature of the cells is also a characteristic shown by mammalian and avian cell lines that have been derived as mass cultures from dissociated organs. In fact, in mammalian cell cultures, all diploid lines are fibroblastic and epithelial lines are heteroploid. Rafferty (1969) on the other hand, found that the anuran cell lines he established were either fibroblastic or epitheloid, yet either type of cell line could be diploid or heteroploid.

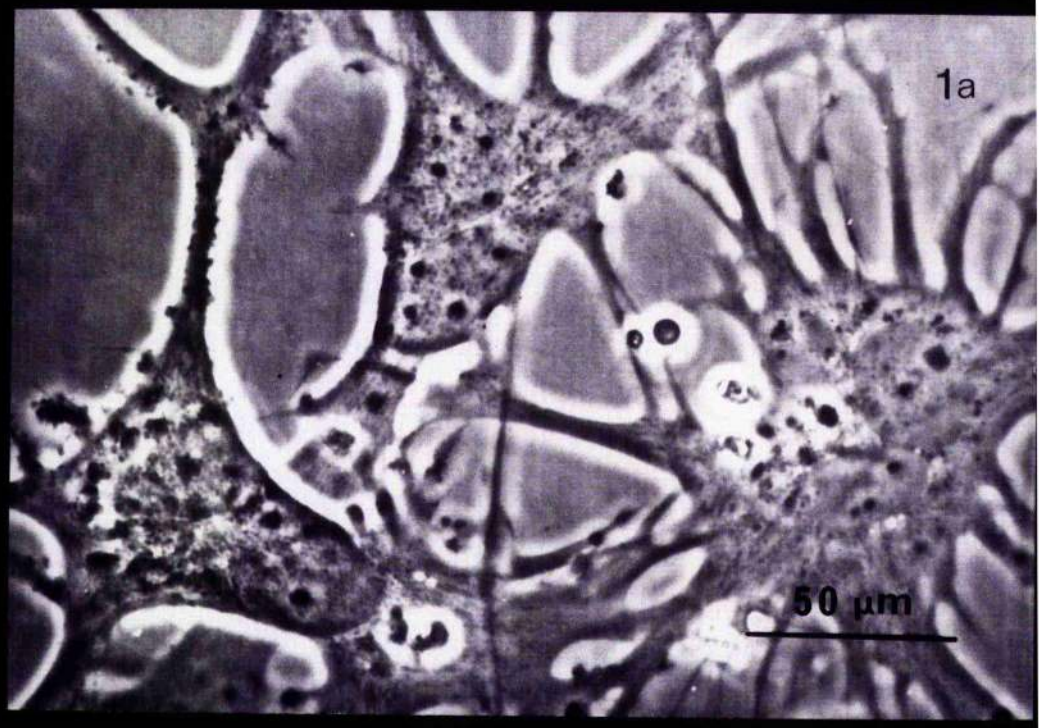
The culture of T.c. carnifex spleen cells which I established was epitheloid in morphology until its death, while the heart cells in culture were fibroblast-like. The T.c. carnifex culture is basically very easy to handle and maintain, and has no special requirements apart from a culture temperature of 25°C, at which the cells grow optimally. It is unusual, however, that it will grow successfully in medium originally formulated for mammalian cells (Eagle, 1959). Other workers (e.g. Wolf & Quimby, 1964; Seto & Rounds, 1969) have found that in order to grow amphibian cells in culture, the osmotic pressure of the medium must be reduced by dilution with distilled water. It appears that the T.c. carnifex cells in culture will tolerate the relatively high osmotic pressure of undiluted medium. The 'hardiness' of the cells is also shown by the fact that a healthy culture can be obtained by splitting flasks of cells which had been left to grow to a high density for 2 - 3 weeks longer than usual.

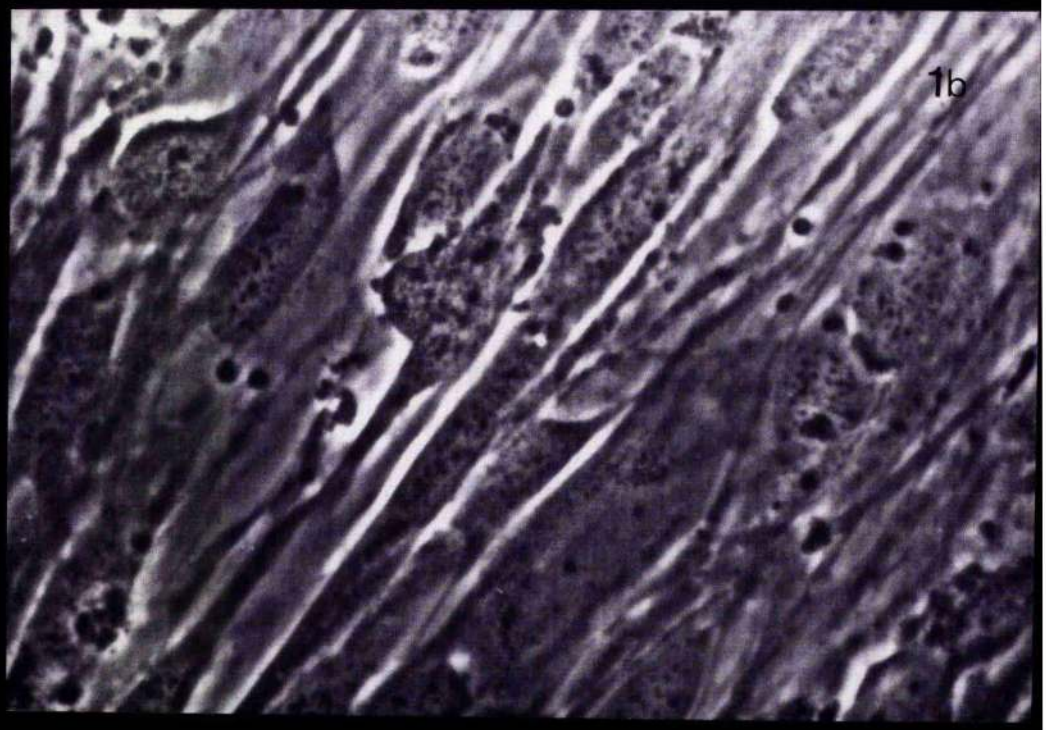
After this time the cell layer becomes so dense that the regions around the periphery begin to slough and the medium is yellow (acid) in colour. However, when these cells are trypsinized and seeded into fresh cultures, the resulting monolayers are healthy and normal.

It is speculative whether this cell line will remain predominantly diploid, or whether it will become aneuploid in order to persist. Hayflick (1965) found that the culture lifetime of diploid fibroblasts from human foetal lung is limited to 100 cell generations or less, therefore he proposed that cells which do not become aneuploid are limited in the number of possible _in vitro_ cell generations by intrinsic factors related to the longevity of the donor. However, Rafferty's A-1 line derived from _Rana sylvatica_ survived in diploid form for 90 generations, then in euploid form for 90 generations before being lost (due to contamination) without any apparent decline in vigour. At the present time, the _T.c. carnifex_ cell culture is the only diploid, urodele culture maintained in any laboratory.

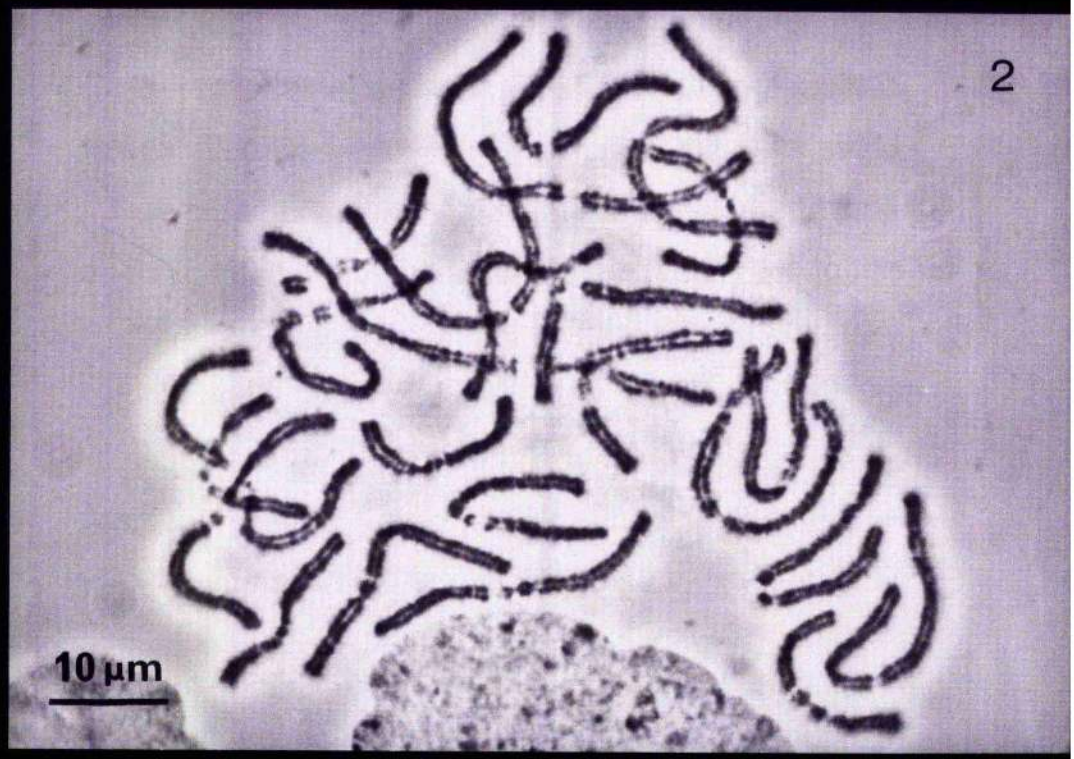
II - 1a. Phase contrast photograph of T.c. carnifex tissue culture cells growing on the flask surface. These cells are from a part of the flask where individual cells are not densely packed and show the highly extended morphology characteristic of cells in a near-confluent density.

II - 1b. Phase contrast photograph of T.c. carnifex tissue culture cells in an over-confluent condition. The parallel array of cells which forms the cell sheet is characteristic of fibroblast cell lines.


1a
50 µm


1b

II - 2. Phase contrast photograph of mitotic metaphase chromosomes from the T.c. carnifex tissue culture. These cells were cultured in medium containing BUdR (1 µg/ml.) for 8 days, colchicine being added for the last 18 hrs. of culture. The cells were hypotonically treated with 0.075 M KCl for 60 mins before fixation. Cells were squashed in 45% acetic acid. This set of chromosomes shows pericentromerically located elongated regions or secondary constrictions which were induced by culture in BUdR.


2
10 µm

CHAPTER III.

Location of the genes coding for 28S, 18S and 5S ribosomal RNA in the mitotic chromosomes of Triturus cristatus carnifex and T. vulgaris

1. INTRODUCTION

Certain achromatic secondary constrictions of chromosomes have long been known to be associated with nucleoli, and are referred to as nucleolus organizers. Having looked at the patterns of cold-inducible secondary constrictions and Giemsa bands in some newt species, and noticed that some secondary constrictions were visible in the metaphase sets with or without prior cold treatment (Chapter I; Rudak & Callan, 1976), I decided to see whether I could prove a positive correlation between the positions of these constrictions and the locations of the genes coding for 28 + 18S ribosomal RNA (rRNA).

In recent years, two techniques have been developed which allow identification of those regions of eukaryotic chromosomes which contain DNA sequences complementary to ribosomal (28 + 18S) RNA. The first technique (Gall & Pardue, 1969; John et al., 1969) involves molecular hybridization between radioactively labelled rRNA or DNA molecules which have been extracted and identified by their specific sedimentation properties, and slide preparations of cells which have been fixed and denatured prior to annealing. Under conditions of renaturation at the optimum temperature required to give molecular hybrids with maximum fidelity of base pairing, the labelled rRNA or rDNA molecules will hybridise to those regions of the chromosomes to which their pattern of base sequences is complementary. The location of the hybridized region can then be detected autoradiographically. It can be appreciated that the success of this technique for gene localization depends on several factors: only those genes with a high degree of sequence repetition and which are clustered in one or a few locations

in the chromosome set can be identified by hybridisation, essentially because there is a limit to the specific activity of the RNA or DNA obtainable by ^{3}H-labelling *in vivo* or *in vitro*. RNA labelled *in vivo*, by incubation of cells in the presence of a tritiated precursor such as ^{3}H-uridine, can be extracted and fractionated into different size-classes by standard biochemical procedures. The specific activity of *in vivo* labelled RNA is relatively low because the synthetic activity of cells is depressed to an extent which is proportional to several factors, including the specific activity of the labelled precursor with which they are incubated and also because of the size of the pool of endogenous precursor. *In vitro* labelling of rDNA with ^{3}H by the technique of "nick-translation" (Kelly *et al.*, 1970) can give a high yield of ^{3}H-rDNA with a specific activity of 10^{6} cpm/µg or more (Macgregor & Mizuno, 1976). However, this technique is regularly found to give technical difficulties which result in DNA with lower specific activity (H.C. Macgregor, personal communication) and, because it required DNA polymerase I as well as four ^{3}H-labelled deoxyribonucleotide triphosphates, it is expensive to perform.

Although ^{3}H is known to give the best autoradiographic resolution obtainable, the time required for exposure is long compared to other isotopes. I therefore decided to use a technique developed by Commerford (1971) whereby polynucleotides can be iodinated with an isotope of iodine, ^{125}I, which has a half-life of 60 days, 1/70th of that of tritium. Iodine is incorporated into RNA or single-stranded DNA as 5-iodocytosine. This incorporation has a negligible effect on the melting profile and renaturation kinetics of the DNA or RNA (Commerford, 1971), and therefore does not reduce the potential extent of hybridisation to its complement *in situ*. Iodine-labelled 28 + 18S rRNA has been successfully used to locate the positions of the ribosomal genes in the chromosomes of the

mouse (Henderson *et al.*, 1974), in the polytene chromosomes of the dipteran fly *Glyptotendipes barbipes* (Wen et al., 1974) and in the chromosomes of *Triturus cristatus carnifex* (Hennen *et al.*, 1975). I too had intended to locate the positions of the nucleolus organizing regions in the chromosomes of *T.c. carnifex* by *in situ* hybridisation and the latter study was published during the course of my research. The results of Hennen *et al.* (1975) suggested that in *T.c. carnifex*, the ribosomal genes are located in the middle of the short arm of only one homologue of chromosome IX, and perhaps in a sub-terminal position on one of a larger pair of metacentric chromosomes, probably II, III or IV. This type of labelling was seen in the chromosomes of two male animals. rRNA hybridized to lampbrush chromosomes was localized near the middle of the short arm of both chromosomes forming bivalent IX. These results indicated that further work needed to be carried out to see whether the same pattern of labelling in male animals is shown by larger numbers of individuals, or whether it was restricted to the animals used for that study, and never found in females, or whether it was due to some peculiarities of their conditions of hybridisation. I therefore decided to extend Hennen *et al.*'s investigation and look at the locations of the 28 + 18S rRNA genes by *in situ* hybridisation of *T.c. carnifex* tissue culture cells.

I have mapped the positions of the cold-induced Giemsa bands in *T. vulgaris* (Chapter I) and have found a general correspondence of their positions with the cold-inducible constriction pattern for this animal (Callan, 1942). Callan found six secondary constrictions which were usually present in metaphase cells with or without prior incubation at low temperatures. This suggests that these six constrictions are probably nucleolus organising. I thought it would be of interest to see whether I could verify this observation using the technique of in situ hybridisation and also compare the distribution of the ribosomal genes in *T. vulgaris*

and T.c. carnifex with each other and with other Triturus species: T. marmoratus (Pilone et al., 1974) and Notophthalmus (= Triturus) viridescens (Hutchison & Pardue, 1975; Pukkila, 1975). Also, using the technique of in situ hybridisation, I decided to look at the distribution of the nucleolar genes in the mitotic chromosomes of hybrid newts, formed by artifically inseminating T. vulgaris eggs with sperm from a T.c. carnifex male. In some urodele species there has been some evidence that the number of ribosomal genes and even the numbers of rDNA loci can vary from animal to animal (Macgregor & Kezer, 1975; Hutchison & Pardue, 1975). Thus, by crossing two species whose rDNA loci are already known some insight might be gained into the inheritance of ribosomal genes.

The third class of ribosomal genes found in the eukaryote genome are those coding for 5S RNA. In those eukaryotes that have been studied, there is a very high redundancy of 5S rRNA genes, the numbers varying from near equality with the number of 28 + 18S genes to a several-fold excess. At present there is no explanation to account for the functional necessity of these large numbers of 5S genes which are represented many more times in the genome than the major rRNA species. In situ hybridisation has been used to identify the chromosomal locations of the 5S genes in Drosophila melanogaster (Wimber & Steffensen 1970; Prensky et al., 1973), Chinese hamster (Amaldi et al., 1971), Xenopus laevis (Pardue et al., 1973), human (Steffensen et al., 1973; Johnson et al., 1974), Zea mays (Wimber et al., 1974), T. marmoratus (Pilone et al., 1974), and N. viridescens (Hutchison & Pardue, 1975; Pukilla, 1975). I decided to look at the chromosomal locations of the 5S RNA genes in T.c. carnifex and compare their positions with those known for other urodele species.

The second technique which has been developed to locate the positions of the ribosomal genes is based on a silver-staining technique, originally applied to human chromosomes by Howell *et al.* (1974), and later modified by Goodpasture & Bloom (1975) and Denton *et al.* (1976). This technique has proved successful in locating the nucleolus organizers on the chromosomes of human (Howell *et al.*, 1974), Denton *et al.*, 1976), nine species of mammals (Goodpasture & Bloom, 1975) and *Xenopus laevis* (Jennifer Varley, personal communication), and gives excellent resolution of the nucleolus organizer loci on metaphase chromosomes. I decided that this would be a valuable technique to use in addition to *in situ* hybridisation to check the validity of the results obtained by this latter technique.

2. MATERIALS AND METHODS

(i) Preparation of 18 + 28S ribosomal RNA from *T.c. carnifex* ovaries

Five female *T.c. carnifex* were anaesthetized in a 0.5% solution of MS222 (Sandoz) and their ovaries were removed and placed in a beaker containing about 10.0 mls of NTM buffered saline (0.1 M NaCl, 10 mM Tris, pH 7.5, 2 mM $MgCl_2$) at 0°C. Care was taken to remove all pieces of fat-body which adhere to the ovaries. The ovaries were washed briefly in NTM then homogenized in about 10.0 mls NTM in a Dounce homogenizer. The homogenate was centrifuged for 10 mins at 10,000 x *g* and 4°C. The tube and its contents were warmed to room temperature and the supernatant was placed in a clean tube, avoiding the fatty surface layer. EDTA and SDS from concentrated stock solutions, were added to the supernatant to final concentrations of 4 mM and 0.5% respectively. The tube was capped and shaken with an equal volume of tris-buffered phenol/chloroform (1:1) and centrifuged for 10 mins at 10,000 x *g* and 20°C. The cloudy, top layer was removed with a pipette and placed in a clean tube, and the phenol-extraction was repeated twice more. Any phenol remaining in the

aqueous phase was removed by extracting twice with chloroform. The RNA was precipitated by adding two volumes of 96% ethanol, and the tube was left to stand at 4°C for 2 hours.

The RNA was fractionated by sucrose density gradient centrifugation. The RNA precipitate was obtained by centrifugation for 10 mins at 10,000 x g and 4°C. This RNA precipitate was resuspended in a denaturation mixture of 0.9 ml. formamide, 0.1 ml. 10 x "Li buffer", (10 x Li buffer = 1 M LiCl, 50 mM EDTA, 0.1 M Tris pH 7.5, 2% SDS), 0.1 ml. H_2O at 37°C for 5 mins. 1.1 ml. of 1 x Li buffer was added, and the denatured solution of RNA was loaded onto two 5 - 20% linear sucrose gradients made in 1 x Li buffer containing 50% formamide. The two gradients were spun for 24 hours at 35,000 rpm and 4°C in the MSE 6 x 14 ml. Ti Swing-our rotor. The RNA fractions from each gradient were collected by puncturing the bottom of the centrifuge tube and detecting the position of the various RNA peaks by their absorbance at 260 nm (Fig. III - 1).

The RNA precipitate was resuspended in 1.0 ml. of acetate buffer. A 1 in 200 dilution of the rRNA solution gave an optical density reading at 260 nm of 0.3. Taking 1.0 O.D. to correspond to an RNA concentration of 40 µg/ml, it can be calculated that the concentration of 28 + 18S RNA obtained was (0.3 x 200 x 40) µg/ml = 2.40 mg/ml.

(ii) <u>Iodination of 28 + 18S rRNA with ^{125}I</u>

A mixture was prepared which contained 120 µl. 0.2 M sodium acetate pH 5.0, 20 µl. 0.01 M $TlCl_3$ (freshly prepared) and 10 µl. of distilled water. 15 µl. of this mixture was put into a 1.5 ml. plastic Eppendorf tube, and 5 µl. of rRNA (at 1 mg/ml.) was added. Working in a fume cupboard behind a lead screen in a room set aside for the handling of radioactive materials, 10 µl. of ^{125}Iodide (as supplied from Amersham

at 1 mCi/10 µl, pH 8.0 - 11.0) was added to the tube. The tube containing the reaction mixture was incubated in a water bath at 60°C for 30 mins. The RNA was purified to remove excess iodide on a Sephadex G-25 gel-filtration column made up in 10 mM Tris buffer pH 7.5. The passage of the front peak of excluded RNA was monitored in the column using a manual counter, and was collected by fractionation. 2-mercaptoethanol was added to a concentration of 10 mM and the tube was reheated to 70°C for 45 mins. 200 µl. of 1 mg/ml. E. coli tRNA was added as carrier, and the iodinated RNA was precipitated by the addition of 2 vols. of 96% ethanol. The RNA was stored in ethanol at -20°C for 2 days. The RNA was centrifuged for 10 mins at 10,000 x g and 4°C and the precipitate was resuspended in 0.1 M acetate buffer. The RNA was alcohol precipitated, centrifuged as before, and resuspended in 1.0 ml. of 4 x SSC/0.1 M KI. The radioactivity incorporated into the RNA was measured in a liquid scintillation counter and a value of 1.1×10^6 cpm per 5 µl RNA was obtained.

Iodine-labelled 5S rRNA was kindly given me by Dr. J. Sommerville. This RNA had been extracted and iodinated as described above.

(iii) Preparation of animal material used

T.c. carnifex: for in situ hybridization with 28 + 18S rRNA, air-dried preparations of colchicine-treated tissue culture cells were used. Logarithmically growing cells were incubated for the last 20 hours of culture in medium containing colchicine at a concentration of 1 µg/ml. The cells were hypotonically treated, fixed and slide preparations were made as described in Chapter II.

For in situ hybridization with 5S RNA I used squash preparations of testes from colchicine-treated male T.c. carnifex. The method for inducing

out-of-season spermatogonial mitoses in these animals and the details for making squash preps is described in detail in Chapter I.

T. vulgaris: 28 + 18S rRNA was hybridized to squash preparations of portions of brain tissue taken from 12 - 14 day-old larvae which had been incubated in a 0.5% colchicine solution in 1/10 Steinberg saline for 20 hours before fixation. The brains were dissected out of fixed larvae and portions were squashed in 45% acetic acid. The method for making squash preparations is described in detail in Chapter I.

T.c. carnifex x T. vulgaris hybrids

Even in captivity T.c. carnifex and T. vulgaris do not normally mate together to give hybrid offspring; fertilization had therefore to be performed in vitro. Four female T. vulgaris newts in breeding condition were injected interperitoneally with 50 i.u. of chorionic gonadotrophin. 55 hours later they were anaesthetised in a 0.5% solution of MS222 (Sandoz) together with one male T.c. cristatus. The latter animal was in full breeding condition with a large dorsal crest and well-marked tail flash. The oviducts were removed from the females and placed in a dry, large, glass petri dish, containing a piece of filter paper moistened with about 1.0 ml. of 1/10 Steinberg solution. The eggs were removed from the oviducts by tearing apart the oviduct walls with forceps. The dish was kept covered to prevent the eggs from drying out. The sperm-containing vasa deferentia was removed from the carnifex male newt, then placed in an embryo cup and finely minced in a drop of full strength Steinberg saline, using two pairs of clean watchmakers No. 5 forceps. A small portion of minced vas deferens was picked up in a pair of watchmakers forceps and touched onto each egg in turn. The petri dish was filled with 1/10 Steinberg solution and the eggs were kept at a constant temperature of 18°C. 24 hours later, 30 eggs

were showing signs of cleavage, and all of these remained healthy during their further development. The 18-day-old larvae were incubated in colchicine for 20 hrs prior to fixation in 3:1 (absolute ethanol: glacial acetic acid). Squash preparations of pieces of brain tissue were made as described in Chapter I.

(iv) In situ hybridisation

The method which I used to hybridise ^{125}I-labelled RNA to material on slides was somewhat modified from that used by other workers. Essentially, the modifications were to prevent any possibility of the denatured DNA of the chromosomes from reannealing before the ^{125}I-labelled RNA could be added.

The slides were placed flat on a hotplate at 65°C for 5 minutes. 95% formamide in 0.1 x SSC ((9.5 mls formamide (Analar), 0.45 mls H_2O, 0.05 mls 20 x SSC)) was heated to 65°C in a water bath. 40 µl. of 95% formamide in 0.1 x SSC was spotted into the centre of each squash or air-dried preparation from an Eppendorf pipette and the slides were left for 2 mins. 20 µl. of the iodinated 28 + 18S RNA at 1.1 x 10^6 cpm per 5 µl. was diluted with 480 µl. of 8 x SSC/0.2 M KI and 40 µl. of this was added into the drop of formamide on each slide, giving a total of 1.7 x 10^6 cpm in 80 µl. volume on each slide, and containing approximately 50% formamide, and 0.1 M KI in 4 x SSC. The 5S RNA was in the same concentration of formamide, KI in 4 x SSC but the final 80 µl. volume contained 2.4 x 10^6 cpm per preparation. A 22 x 22 mm (Acid-boiled) coverslip was dropped onto the preparation and the slides were removed from the hot-plate and placed in square plastic petri dishes (Falcon), 3 slides per dish, on the raised level made by two parallel glass rods. Each petri dish contained a piece of filter paper moistened

with 10 mls of 4 x SSC. The slides were incubated in these "moist chambers" for 18 hours at 37°C. After 18 hours, the coverslips were removed by rinsing each slide in a beaker containing 2 x SSC. Endogenous RNA was removed by incubating the slides in pancreatic RNAse A (Sigma) at a concentration of 50 µg/ml. for 90 mins. The RNase had been previously boiled to inactivate any DNase which might have been present. The slides were rinsed in 2 changes of ice-cold 2 x SSC for 4 hours. This was done by placing the slides in a stainless steel slide rack which was then suspended in a 3 litre Pyrex beaker about 10 cm. above the bottom. The beaker contained 1½ litres of 4 x SSC and was filled to within 3 cms of the top with crushed ice. A large, teflon-coated magnetic stirring bar was dropped into the beaker, and the beaker was placed on a magnetic stirrer during the rinsing process. Afterwards the slides were rinsed in 2 changes of distilled water and dehydrated in 2 changes each of 70% ethanol, 96% ethanol and absolute ethanol, then air dried.

(v) Autoradiography

A Kodak Wratten series II (red) safelight was used throughout this process. The emulsion used was Kodak NTB 2 nuclear track emulsion which had been stored in plastic-capped glass vials in double boxes at 4°C. The emulsion had been diluted 1:1 with distilled water. Immediately before use, the vial of emulsion was placed in a 45°C water bath in the autoradiography room for about 5 mins. The melted emulsion was poured into a glass dipping vial and the slides were individually dipped into the emulsion chamber until the preparation was just covered. (Squash preparations were always made near the end of the slide furthest from the labelled end, to economise on the amount of emulsion used to cover the preparation). Dipped slides were placed in a drying rack, labelled ends down, about two feet away from a cold air fan, the side of the slide bearing the

preparation facing away from the fan. The emulsion on the slides was dry in about an hour. Dry slides were placed in labelled, black plastic storage boxes which were then sealed with masking tape of 'Elastoplast' adhesive strip. The plastic boxes containing autoradiographs were stored in cardboard boxes at 4°C.

A "test" slide was developed after 1 day and after 7 days to see the rate of silver grain deposition. All solutions used during the development of autoradiographs were prepared at least 3 hours before use, and left in the autoradiography room to equilibrate to room temperature. Developer was always used 'fresh'; fixer could be stored for up to 7 days. Exposed autoradiographs were developed in full strength Kodak D 19 developer for 2½ mins in complete darkness, rinsed in distilled water, fixed in Kodak 'Unifix' for 5 mins, then rinsed for 30 mins in filtered tap water. The slides were rinsed briefly in distilled water and stained with Giemsa in 0.05 M phosphate buffer pH 7.1 for 10 - 20 mins. Autoradiographs generally take longer to stain staisfactorily than do preparations not covered with emulsion. Stained slides were examined under the microscope unmounted, immersion oil being applied directly to the preparation. The preparations were photographed as described in Chapter 1.

(vi) Location of the nucleolus organizers by a silver staining technique

The technique of Denton et al. (1976) was used to stain the nucleolus organizing regions of the chromosomes of T.c. carnifex and T. vulgaris. 3 drops of silver nitrate solution (1 gm. $AgNO_3$) in 2 ml. deionized water) were pipetted onto a dry chromosome preparation and a coverslip was applied over the drop. The slide waspplaced under a photoflood light (Phillips Photoflood 240/250 v. 275 W) for 10 mins. After cooling, the coverslip was washed off the slide with deionised water and the preparation was

air-dried. Two drops of 3% neutralised formalin (3 mls 40% formaldehyde + 3.2 gms $CH_3COONa.3H_2O$ in 100 mls distilled water) and two drops of ammoniacal silver (8 gms $AgNO_3$ dissolved in 10 ml. H_3O then added to 15 ml. concentrated NH_4OH) were simultaneously added to the preparation which was then covered with a coverslip. The progress of the staining reaction was watched under the microscope using phase contrast objectives. After about 15 - 20 secs, when the preparation was a pale yellow-orange colour the coverslip was washed off the slide with tap water and the slide was air-dried. Preparations were examined unmounted, and counts were made of the number of nucleoli present in interphase cells.

(vii) Location of the nucleoli in interphase cells by staining with methyl green/pyronine

Air-dried preparations of T.c. carnifex were stained with methyl green/pyronine in acetate buffer for 20 mins. The staining solution was made by extracting 0.5 gm. methyl green (G.T. Gurr) twice with chloroform, to remove any methyl violet, then 0.15 gm. of the dried residue was dissolved in 100 mls acetate buffer, pH 4.7 (102 ml. 0.1 M CH_3COOH + 98 ml. 0.1 M CH_3COONa). This solution was shaken with chloroform in a separator and the chloroform tapped off. 0.25 gm. pyronine (Geigy) was dissolved in 100 mls of the buffer containing methyl green, then 2.5 mls of 95% ethanol was added. This staining solution should only be used on the day of preparation because it quickly deteriorates. After staining, the slides were rinsed in distilled water, and excess pyronine was removed by rinsing the slides in 95% ethanol for about 1 - 2 minutes. The stained slides were air-dried, and examined unmounted, oil being applied directly to the preparation.

3. RESULTS

(i) Triturus cristatus carnifex: localization of the DNA sequences complementary to 28 + 18S ribosomal RNA.

Homologous 28 + 18S rRNA extracted and iodinated *in vitro* hybridizes to two loci in the mitotic set of *T.c. carnifex* tissue culture cells. The labelled loci appear to be in identical positions on both homologues of a small submetacentric pair of chromosomes, about 2/5 of the way down the long arm from the centromere. Figures III - 2 to III - 4 show examples of the labelling pattern. The position of the labelled locus on one of the chromosomes' arms is shown particularly well in fig. III - 2a. One of the labelled chromosomes in fig. III - 2b shows good resolution of the label over each chromatid. The interphase cells in fig. III - 4c have two labelled areas per cell, which probably correspond to the position of nucleoli. Fig. III - 5 is an outline drawing of the chromosome set in fig. III - 2a, with a diagrammatic karyotype constructed from cut-out chromosomes. In squash preparations, chromosomes IX, X and XI are frequently difficult to identify because of their similarity in size and the variations encountered due to differential stretching of regions of some chromosomes. However, the air-dried chromosome preparation in fig. III - 2a appears to be uniformly flattened and arms of these chromosomes can be identified with a high degree of certainty. I have therefore assigned the position of the 28 + 18S ribosomal genes to chromosome X in *T.c. carnifex* tissue culture cells.

(ii) Triturus cristatus carnifex: Localization of the DNA sequences complementary to 5S ribosomal RNA.

Homologous 5S rRNA binds to two specific regions on one pair of T.c. carnifex chromosomes. All chromosome preparations were obtained from testis follicles and are spermatogonial mitoses. The labelled regions are about halfway down the longer arms of a small pair of submetacentric chromosomes. In figs III - 6a and III - 7a and 7b, the labelled regions are halfway along the long arm of the chromosome. In fig. III - 6b and III - 8a the label is localized at about 2/5 of the way down the long arm from the centromere. The variable resolution of the clusters of silver grains do not permit a more accurate localization of the 5S genes. Fig. III - 8b is a diagrammatic karyotype of the chromosome set in Fig. III - 8a. In this particular chromosome set it is difficult to identify which pair of chromosomes IX, X or XI bears the 5S genes. I have tentatively assigned them to chromosome X. Non-dividing spermatogonia show either two areas of label or one large area, the latter being probably due to a close association of the 5S genes on both homologues.

(iii) Triturus vulgaris: Localization of the DNA sequences complementary to 28 + 18S ribosomal RNA.

I have used non-homologous 28 + 18S rRNA extracted from T.c. carnifex ovaries and iodinated in vitro to locate the 28 + 18S ribosomal genes in T. vulgaris. The RNA preparation was that used to locate the 28 + 18S ribosomal genes in T.c. carnifex. All the chromosome preparations of T. vulgaris were obtained from larval brain tissue. I have attempted to analyse the labelling patterns of the mitotic metaphase chromosomes of four larvae. The pattern of labelling is complex and varies between individual larvae. Up to six chromosomes (probably two pairs and two

"others") and eight loci have regions complementary to T.c. carnifex 28 + 18S ribosomal RNA. For each animal, the pattern of labelling is more or less consistent, but it is impossible to rule out completely the possibility of real variations in labelling between the cells from the same animal.

Metaphase cells from animal A2 (figs III - 9 to III - 11) show labelling over all eight of the possible loci of the 28 + 18S genes. There are large clusters of silver grains near to the telomeres of both of a pair of large metacentric chromosomes. Hereafter, this chromosome will be referred to as chromosome II, since the subterminal position of the silver grains corresponds to a secondary constriction, regularly found near the telomere of the second longest chromosome in normal mitotic metaphase cells (Callan, 1942). There are also large clusters of silver grains halfway down the long arm and smaller grain clusters at the ends of the long arms of a small acrocentric chromosome, which could be either chromosome X or XI. I have identified the labelled chromosomes as X on the basis of their slightly longer length. The other two chromosomes which are regularly labelled, do not appear to be a pair. One large metacentric chromosome has a labelled region near to the centromere of one of its arms, and a slightly smaller submetacentric chromosome is labelled about halfway down its longer arm. Figure II - 9a is a partial mitotic metaphase set of 23 chromosomes and showing 5 labelled chromosomes. It is assumed that one of the usually-labelled chromosomes II is missing. Figure III - 9b, 10a and 11, show 6 labelled chromosomes with all 8 labelled loci. The chromosomes in fig. III - 10b are labelled at both loci on chromosome X and perhaps near to the centromere of a large metacentric chromosome but there are no other significantly labelled regions. If this is a result of inefficient hybridization to some regions of the slide, it would indicate that the loci

on chromosomes X and on one large metacentric chromosome probably contain a greater number of ribosomal genes than do any of the other loci. Partially labelled metaphase sets, as in fig. III - 10b, were frequently seen in the autoradiographs of all the animals studied.

Animal B 12 has 4 labelled chromosomes, with 5 - 6 labelled loci (figs. III - 12 and 13). Generally, both chromosomes X are labelled, each at both loci. There are regions of label on one of chromosome II, and halfway down the long arm of one medium-sized sub-metacentric chromosome. However, the chromosome preparations of this animal are quite lightly labelled, which makes any analysis difficult, therefore the results are only tentative.

Figure III - 14 is a partial metaphase set from animal B 20. One chromosome X has label over only one chromatid at both loci, while its homologue is labelled at both loci on both chromatids. There is also label near the telomere of one large metacentric chromosome and perhaps some halfway down an arm of another chromosome. The difference in labelling over the two chromatids of chromosome X is not found in all metaphase sets. Although the labelling specificity was generally poor for this animal, as far as I can analyse, the pattern of labelling is similar to that of animal B 12.

Animal B 10 has only two labelled chromosomes (figs III - 15 and 16). There are clusters of silver grains over both loci on both chromosomes X, with no other significantly labelled regions.

(iv) T.c. carnifex x T. vulgaris hybrid animals: Localisation of the DNA sequences complementary to 28 + 18S ribosomal RNA.

The results obtained after hybridising T.c. carnifex 28 + 18S rRNA to preparations of the mitotic chromosomes of two T.c. carnifex x T. vulgaris hybrid animals are shown in figs III - 17 and 18. None of the preparations are easily analysable, but an overall impression suggests that there are regions complementary to 28 + 18S rRNA 1/3 of the way down the long arm of one or perhaps two small, acrocentric chromosomes. There also appears to be a labelled region near the telomere of another (smaller) acrocentric chromosome. In addition, animal B 2 (fig. III - 17) appears to have a cluster of silver grains halfway down one arm of one large sub-metacentric chromosome, while animal C 7 (fig. III - 18) has a labelled region near to the telomere of one arm of one, large, sub-metacentric chromosome.

(v) Estimation of the number of nucleoli per cell in T.c. carnifex and T. vulgaris by methyl green/pyronine staining and "silver staining".

When the cells were stained with methyl green/pyronine, bright pink nucleoli were clearly visible against a background of bluish-pink nucleoplasm. In T.c. carnifex tissue culture cells, the number of nucleoli per cell ranged from a minimum of one to a maximum of twelve, the vast majority of cells containing 3 - 5 nucleoli. These results are shown in figure III- 19a. The histogram enclosed by unbroken lines in figure III - 19b shows the results obtained by counting the numbers of nucleoli in "silver stained" T.c. carnifex tissue culture cells. The results are essentially the same as those in figure II - 19a, with most of the cells containing from 3 - 5 nucleoli. In the same figure, the histogram enclosed

by the dotted lines shows the results obtained after "silver staining" spermatogonial cells from one T.c. carnifex adult animal. Most of the spermatogonia contain two or three nucleoli and never more than five. "Silver stained" T. vulgaris larval brain cells contain from 1 - 8 nucleoli, with most cells having from 3 - 5 (fig. III - 19c). Using this technique I have obtained differential staining of the nucleolar organizer loci in human mitotic metaphase chromosomes from lymphocyte culture (fig. 20), yet, with identical conditions of staining, not in T.c. carnifex or T. vulgaris mitotic chromosomes. In general, the results obtained with this staining technique are variable and difficult to standardize. After the ammoniacal silver nitrate and formalin developer are added to the preparation, the staining reaction proceeds by first staining the cells a pale lemon colour, the yellowness increasing with staining time until the cells are dark orange/brown and will eventually become black. Accompanying this, the nucleoli begin to stain more and more darkly, with maximum differentiation when the nucleoli are deep brown/black and the rest of the cell is yellow/orange. If staining is prolonged beyond this point, the chromocenters begin to stain deeply and become indistinguishable from the nucleoli. Similarly, regions of the metaphase chromosomes which show constrictions or C-bands after suitable treatment (Chapter I), begin to stain deeply (fig 21). Ideally, the staining solutions should be rinsed off the slide before the chromocenters begin to stain, or these may be confused with the nucleoli.

4. DISCUSSION

Using the technique of *in situ* hybridization, I have located the position of the 5S rRNA genes in the mitotic chromosome set of *T.c. carnifex* on both homologues of chromosome X, about halfway down the long arm. This result is in agreement with Pilone *et al.* (1974) who mention that they have located the 5S genes in a similar position on chromosome X both in *T. marmoratus* and *T. cristatus carnifex*, but do not illustrate their results for the latter species. When 5S rRNA was hybridized to the lampbrush chromosomes of *T. marmoratus*, regions of localized labelling were found at, or very near to, a dense matrix loop landmark found in an intermediate position on the long arm of chromosome X. The lampbrush chromosome X of *T.c. carnifex* bears two similarly conspicuous loops in positions such that either could correspond to the 5S loci. This site may also be analogous to two of a possible 10 5S loci observed by Hutchison & Pardue (1975) and Pukkila (1975) in *T. viridescens*. In *T. viridescens*, the 5S genes are located at or very near to the centromeres of four pairs of subtelocentric chromosomes, with a fifth cluster of 5S genes in an intermediate position on the long arm of a small submetacentric pair of chromosomes, probably chromosome X. The *T. viridescens* chromosome X is almost identical in size and centromere position to chromosome X from *T.c. carnifex* and *T. marmoratus*. *T. cristatus* and *T. marmoratus* are taxonomically very closely related and have been classed together as a superspecies (Mayr, 1931). *T. viridescens*, the North American spotted newt, is a rather more distant relation, and has two less chromosomes in its diploid set ($2n = 22$). If chromosome X can be considered homologous in these species it is interesting that during the course of evolution, the position of the 5S genes on chromosome X has remained constant, yet in *T. viridescens*, additional 5S loci have become pericentrically located.

The results I have obtained concerning the location of the 28 + 18S genes in T.c. carnifex are not easy to explain in the light of previous work. In the mitotic chromosomes of T.c. carnifex female tissue culture cells, I have located two regions which contain DNA complementary to 28 + 18S rRNA. These regions are approximately 3/5 of the way down the long arm of both homologues of chromosome X, which means that the 28 + 18S genes are either in the same position, or very close to, the location of the 5S genes. This position does not agree with results of Hennen et al. (1975), who located the positions of the T.c. carnifex nucleolus organizers in spermatogonial mitoses, using iodinated rDNA from Xenopus laevis. They found that the rDNA hybridized to a region halfway along the shorter arm of one chromosome IX, and perhaps also to a region near the end of a large metacentric chromosome. However, in lampbrush chromosomes, they found labelling on lampbrush chromosome IX at a position corresponding to the one on mitotic chromosome IX, but on both homologues, and nowhere else. Filter hybridization experiments to estimate the proportion of rDNA in the animals whose tissues they used for in situ hybridization indicated that both the males and the female contained approximately the same number of ribosomal genes. Hennen et al. (1975) therefore suggested an explanation for the lack of hybridization to one of the chromosomes IX in males, which argued that the latter phenomenon was due to some kind of "differential hybridizability" of the two nucleolus organizers and not due to a deletion of one of them. The "differential hybridizability" was interpreted to be a consequence of differential packing of the chromatin at the two organizer loci, possibly also influenced by differential synthetic activity of each organizer at the preceding interphase.

The location of the 28 + 18S genes on chromosome IX by Hennen et al. (1975) is partially in agreement with Mancino et al. (1972) who described the positions of attachment of nucleoli to the lampbrush chromosomes of

semi-albino and wild-type T.c. carnifex. By the criterion of nucleolar attachment he assigned the position of the nucleolus organizers to two pairs of chromosomes. Nucleoli were attached near the end of the short arm of chromosome VI and halfway down the shorter arm of chromosome IX. By the same criterion, Callan (1966) had earlier established homology between mitotic and oocyte lampbrush nucleolar organizers of the axolotl, Ambystoma mexicanum, and a similar correlation has been described for Plethodon cinereus (Kezer & Macgregor, 1973). In T.c. carnifex, the nucleolar attachment described by Mancino et al. (1972) agree with the locations of the secondary constrictions I have regularly seen in mitotic chromosome sets in the many squash preparations I have made of pieces of tissue from this species. In the cells I have looked at, I have never seen all four constrictions present in any one chromosome set. The T.c. carnifex larva whose Giemsa bands and constrictions pattern are shown in figures I - 2a and I - 10a showed a cell to cell variation in the number of nucleolus organizer constrictions visible at metaphase. Metaphase cells in brain tissue had nucleolar constrictions on either one or two chromosomes IX but only on one chromosome VI, while metaphase cells in gut tissue showed nucleolar constrictions on one or two chromosomes VI, but only one on chromosome IX. Neither myself nor Hennen et al. (1975) have found evidence from in situ hybridization experiments that there are ribosomal genes on chromosome VI in male or female animals. Consequently, it is debatable whether the presence of secondary constrictions in mitotic cells and/or the attachment of nucleoli to certain loci in lampbrush chromosomes are definitive criteria for distinguishing nucleolus organisers, unless the lack of hybridisation over chromosome regions which fulfil these last two criteria is again due to some kind of "differential hybridizability" related to their synthetic activity, or lack of it. I think the latter is unlikely for several reasons, which I will discuss later.

Hutchison & Pardue (1975), found sites on three pairs of chromosomes of T. viridescens male animals which bound ^{3}H-rRNA. Among the animals they studied, they never found hybridization to all six of the potential sites, one on each chromosome. Each animal showed a specific pattern of labelling, with differing levels of hybridization over the different rDNA sites, but the labelling pattern and levels of hybridization were constant in all metaphase cells of each animal. Hutchison & Pardue compared the in situ hybridization patterns of each animal with the results of saturation hybridization of rDNA extracted from the same newts, and, in direct contrast to the situation described by Hennen et al. for T.c. carnifex they found that individual T. viridescens newts vary quite widely in the number of ribosomal genes they possess. However, there was no direct correlation between the number of labelled loci and the amount of rDNA. These results suggest that the phenomenon of heterozygosity of nucleolar organizer loci, is in fact, real in T. viridescens. In different individuals of T. vulgaris I have found different but specific patterns of hybridization to 28 + 18S rRNA, rather similar to the situation found in T. viridescens. In situ hybridization of T. vulgaris mitotic chromosomes from larval brain tissue showed that there was a maximum of eight possible 28 + 18S loci, with two sites on the long arm of chromosome X, one site near the end of chromosome II, and two other sites on non-homologous chromosomes, one near the centromere on one arm of a large metacentric chromosome, and one about halfway down the longer arm of a slightly smaller submetacentric chromosome. The chromosomes from one animal had labelling over all eight possible sites, and yet another animal had only four labelled regions, two on each chromosome X. This variation between individuals would seem to parallel the observations of Hutchison & Pardue (1975) on T. viridescens. Thus, heterozygosity and in some cases, deletion

of some of the nucleolus organizer loci is of common occurrence in both T. viridescens and T. vulgaris. An alternative explanation to that of "differential hybridizability is therefore possible for the results of Hennen et al., (1975) who located a site of rDNA on only one chromosome IX in T.c. carnifex, yet the male animal which showed this pattern of labelling had as much rDNA as a female with labelled loci on both chromosomes IX. It is quite possible that this can be explained in terms of a real heterozygosity for one organizer locus on chromosome IX, which could have arisen as a result of unequal crossing-over of sister chromatids at some time prior to fertilization. The concept of homologous pairing is based on the idea that an attraction exists between regions of the chromosomes with nearly identical base sequences. The ribosomal genes coding for 28 + 18S RNA are arranged in tandemly repeated units, where the sequences complementary to 28S and 18S rRNA alternate, but are interspersed with DNA which is not homologous to either gene (spacer sequence). It has been estimated that T.c. carnifex contains about seven times as many ribosomal genes as Xenopus laevis, or about 6,000 (Rosbash et al., 1974). In regions of a chromosome which contain tandemly arranged genes with identical base sequences, the probability of unequal exchanges occurring between chromatids is very high. Mis-pairing of bases has been found to occur in mitotic chromosomes (Taylor et al., 1957) Taylor et al. studied the pattern of DNA replication in individual chromosomes of Vicia faba using ^{3}H-thymidine. Differential labelling of the chromatids suggested that frequent exchanges between two chromatids of the same chromosome had occurred during the S-phase of the mitotic cycle. Such exchanges during the mitotic and meiotic cycles might not appear to have any advantageous effect, since sister chromatids are assumed to be absolutely identical. However, in regions of the chromosomes where repetitive gene sequences occur, any

unequal exchange between chromatids would lead to a deletion of a number of genes from one chromatid, and the incorporation of this segment into the sister strand. Carrano & Wolff (1975) have found that the distribution of sister chromatid exchanges in the euchromatin and heterochromatin of the Indian Muntjac are distributed at random in each of the chromosomes, thus there is no reason to suppose that such exchanges do not occur at the nucleolus organizer loci. Wellauer et al. (1976) have looked at the sizes of the fragments excised from the ribosomal genes of Xenopus laevis by EcoR1, and have found a heterogeneity in the non-transcribed spacer lengths which fall into two size classes. This heterogeneity was found to differ between animals, yet was a heritable factor (Reeder et al., 1976). Such differences in spacer lengths could have arisen as a result of unequal crossing-over in the spacer segments. This is, of course, on a much smaller scale than that required for an unequal sister chromatid exchange, but the mechanism implied is the same. Unequal exchanges which occurred in either ovary or testis cells by mitotic or meiotic recombination prior to fertilisation would determine the number, position and size of the organizer loci found in all descendent cells of the fertilized egg. The less frequently found cell to cell variations in the individual could be a result of sister chromatid exchanges between unequal numbers of ribosomal genes during mitosis. The methods used to study sister chromatid exchanges influence the number of exchanges seen to a certain extent (for greater detail, see Chapter IV) but it is generally accepted that they are a naturally occurring phenomenon at low frequency in mitotic cells. Nucleolar variants are well-known in several species of animals, including the "bobbed" mutant of Drosophila melanogaster (Stern, 1927; Ritossa et al., 1966) formed by a deletion of some of the nucleolar genes, and the axolotl, Ambystoma mexicanum, where a reduction in size of the nucleolus organizer constrictions and a smaller nucleolar volume is linked with a loss of some rDNA (Humphrey, 1961)

Sinclair *et al.*, 1974). A similar mechanism has been suggested by Carroll & Brown (1976a, b) to explain the presence of adjacent 5S genes in *Xenopus laevis* which have different lengths. They explain the length variations in terms of unequal crossing-over in the AT-rich spacer segments in which the sub-repeats are essentially homologous.

A correlation has never been shown to exist between a *lack* of hybridization with rRNA at a particular nucleolus organizer locus, yet the *presence* of a secondary constriction in that position in other cells from the same animal. While there is so much evidence that variation in nucleolar organizer number, position and size can occur in some urodeles, it is quite likely that the chromosomes of animals which lack some of the possible nucleolar organizer loci would never have shown secondary constrictions in those positions, had the latter been examined. It is interesting to note here that *T.c. cristatus*, an extremely close relative to *T.c. carnifex* which shows essentially the same pattern of Giemsa banding, has secondary constrictions on different chromosomes from *T.c. carnifex* (Chapter I). The chromosomes of *T.c. cristatus* regularly show secondary constrictions near the end of the long arm of chromosome X and about 3/5 of the way down one arm of a large metacetric chromosome. All of the mitotic cells from one *T.c. carnifex* larva whose chromosome complement I examined in some detail, had a secondary constriction on both chromosomes X but on only one of the large metacetric homologues.

One outcome of my results which I have not discussed so far is the fact that Hennen *et al.* (1975) have partially confirmed the observations of Mancino *et al.* (1972) by locating the nucleolus organizer in *T.c. carnifex* on one chromosome IX, although on neither homologue of chromosome VI. Therefore it was somewhat disconcerting to find that my *in situ* hybridisation results showed that ribosomal genes were present about half way down

the long arm of chromosome X, very near to the 5S gene locus. There are several ways in which this result might be explained. Despite the care taken in isolating the 28 + 18S rRNA by fractionation following sucrose density gradient centrifugation, it is not completely unlikely that a 5S contaminant might have been present with the 28 + 18S rRNA. However, it is unlikely that the 5S contaminant could have been present at such a high concentration that it hybridized to the 5S loci without the vastly higher concentration of 28 + 18S rRNA showing any signs of hybridization to their loci during the exposure time. The results obtained after hybridizing this 28 + 18S rRNA preparation to the chromosomes of T. vulgaris also support the assumption that the RNA was predominantly 28 + 18S. Hybridization was seen in T. vulgaris chromosomes at positions which correspond to secondary constrictions which were originally described by Callan (1942) and therefore presumed to be nucleolus organizing. In the light of reports by Pardue et al. (1973) and Ford & Mathieson (1976), it is possible that the transformation of T.c. carnifex cells in culture, which were used for the in situ hybridization experiments with 28 + 18S rRNA, was accompanied by a translocation of the 28 + 18S genes from the nucleolar organizer locus to the position of the 5S genes. Pardue et al. (1973) and Ford & Mathieson (1976) have described the reverse of this situation for a Xenopus laevis cell line, where some of the 5S genes, present at the telomeres of many if not all of the chromosomes, have been translocated to the nucleolar organizer locus. Another alternative is that the chromosomes in T.c. carnifex tissue culture cells to which 28 + 18S rRNA will hybridize, are in fact, chromosomes IX which have undergone pericentric inversion to give a pair of chromosomes whose arm lengths are similar to those of chromosome X. The results obtained after hybridizing 28 + 18S rRNA to the mitotic chromosomes of T.c. carnifex x T. vulgaris newts, shown in figures III - 15 and 16, do not really give any further insight into the inheritance of ribosomal

genes in these species. The labelled regions which can be distinguished appear to correspond to 28 + 18S loci seen in T.c. carnifex and T. vulgaris. The labelled region on the long arm of a small acrocentric chromosome is probably homologous to one of the 28 + 18S loci in T.c. carnifex, while the other regions of label more or less correspond to T. vulgaris counterparts.

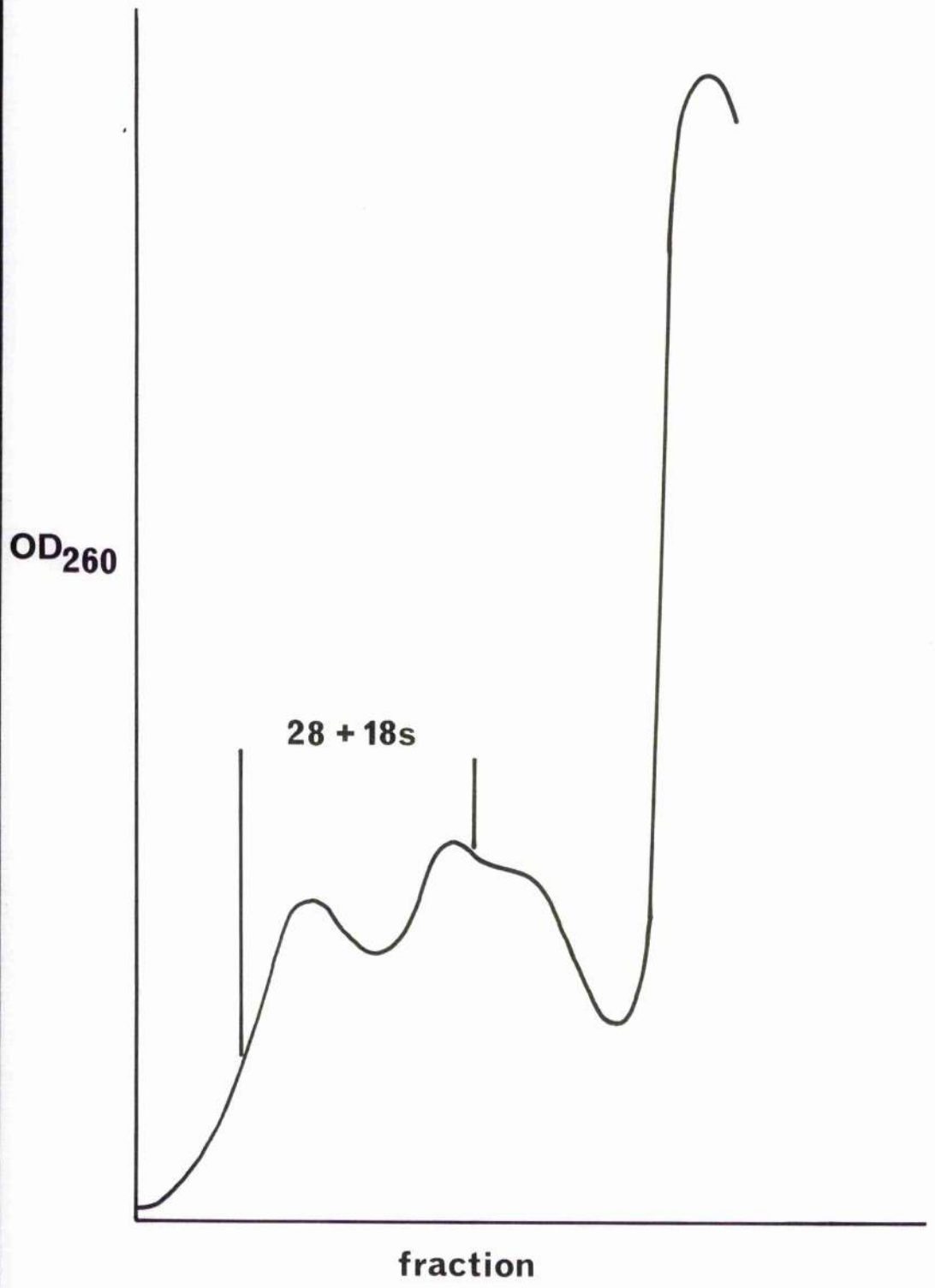
The counts of nucleoli that I have made in non-dividing spermatogonia and tissue culture cells of T.c. carnifex show that the majority of tissue culture cells contain from 3 - 5 nucleoli, while spermatogonia contain from 2 - 3 (fig. II - 17a and b). In tissue culture cells, it is difficult to see how there can be a direct relationship between the number of active nucleolar organizer loci and the number of nucleoli in interphase cells, which has previously been assumed to exist in eukaryote cells. In situ hybridization results show that there are only two clusters of 28 + 18S rRNA genes in T.c. carnifex tissue culture cells, in both metaphase and interphase nuclei, so the numbers of nucleoli found (greater than the "expected" two) must have originated by fragmentation of already synthesised nucleolar material from the two original nucleoli. The larger numbers of nucleoli seen are no doubt, in part, due to the higher ploidy of some of the cells. The spermatogonial cells from a male animal showed consistently fewer nucleoli per cell than the female tissue culture cells, most cells containing from two to three. This again could be due to fragmentation or even fusion of nucleoli, depending on the number of clusters of 28 + 18S genes that were characteristic for that particular animal. Most T. vulgaris interphase cells from larval brain tissue contained about the same numbers of nucleoli as T.c. carnifex tissue culture cells, yet in situ hybridization showed that some T. vulgaris individuals contained twice as many as 28 + 18S rDNA loci. However, since each histogram is the result obtained

from one animal only, and with the knowledge that the number of organizer loci in T. vulgaris can vary from four to eight in the study of just four animals, nucleolar counts in many more individuals should be performed before any generalizations can be made about these results.

Apart from the Drosophila melanogaster "bobbed" mutant (Ritossa et al., 1966), it would appear that all of the other reports of variation in number and/or size of nucleolus organizers in animals, come from studies on amphibians; Xenopus laevis (Wallace & Birnstiel, 1966; Miller & Brown, 1969), Plethodon cinereus (Macgregor & Kezer, 1973; Kezer & Macgregor, 1973), Ambystoma mexicanum (Sinclair et al., 1974), Notophthalmus (= Triturus) viridescens (Hutchison & Pardue, 1975), and T. vulgaris (this study). Amphibians, and particularly the Urodeles, contain large amounts of DNA per haploid genome and all contain ribosomal DNA with a high level of redundancy. It is unlikely that all 6,000 or so 28 + 18S genes present in each diploid cell of T.c. carnifex, as measured by filter hybridisation experiments (Rosbash et al., 1974), are actually used during transcription. If this is the case, the loss of genes from nucleolar organizer loci by deletion or the rearrangement of these genes among the chromosomes will therefore only be deleterious if the number at the normally "active" locus falls below a certain critical value. Such rearrangements appear to be tolerated in amphibian cells, probably because there is such a high degree of redundancy of ribosomal genes as to make these effects negligible. In mammalian species, in situ hybridisation experiments to locate the 28 + 18S ribosomal genes have not shown any variation between individuals. The low C-values and the fewer numbers of ribosomal genes might account for this. Selection pressures would favour constancy in numbers of ribosomal genes if they were present in such numbers that required most of them to be functional for most of the time. In the

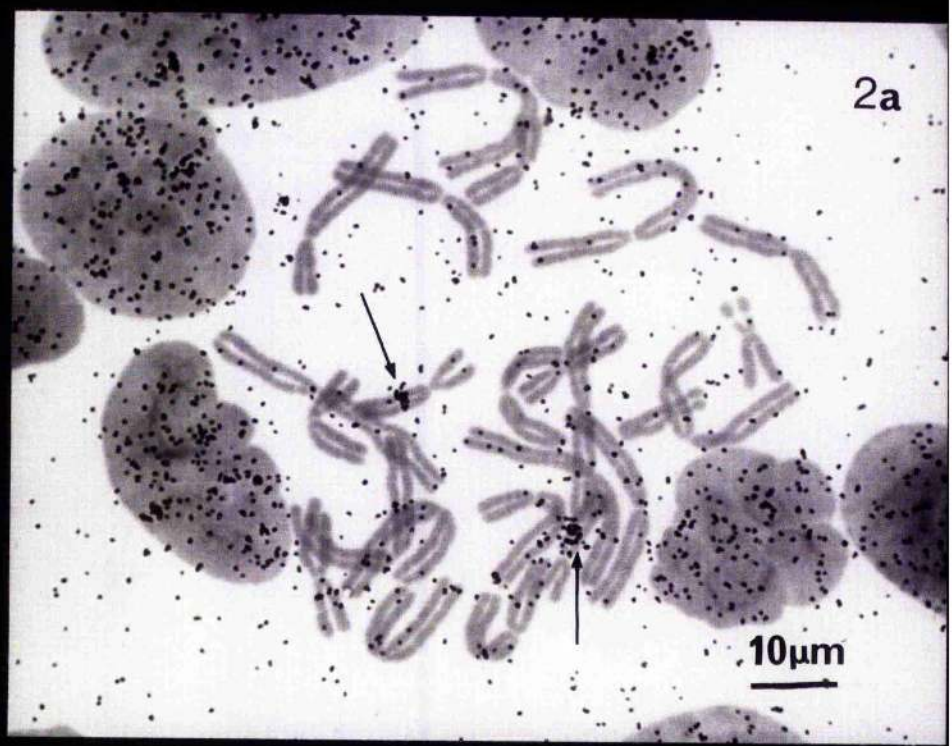
latter situation, almost any structural rearrangement would be deleterious, if not lethal.

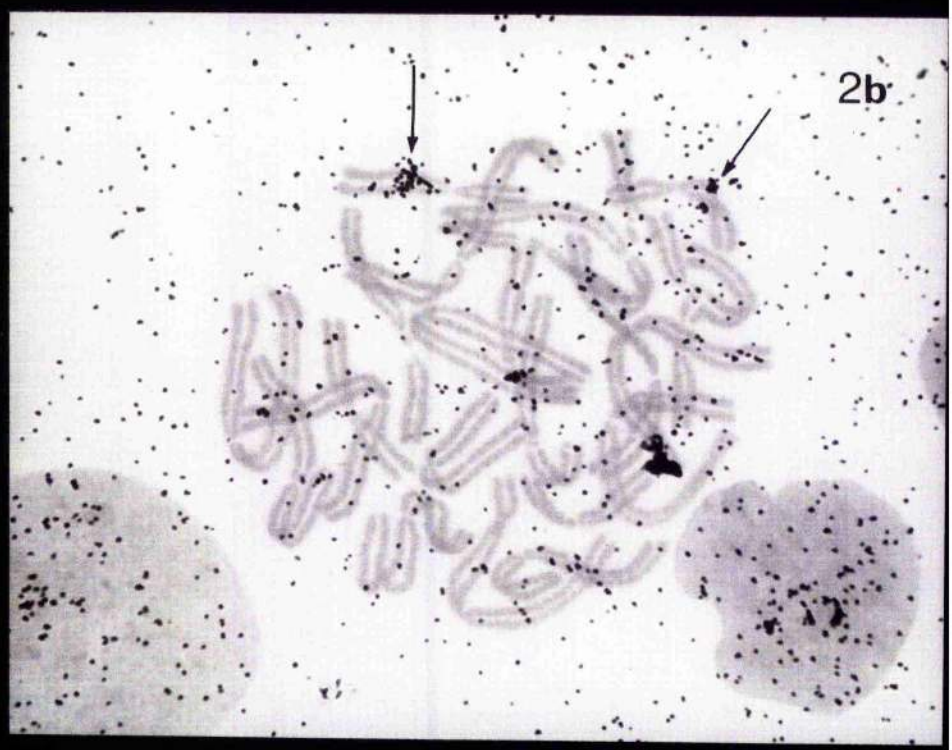
III - 1. An enlarged and expanded copy of the trace reading from an LKB Uvicord recorder of T.c. carnifex RNA fractionated on a 5 - 20% linear sucrose gradient containing 50% formamide. The position of the various RNA peaks were detected by their absorbance at 260 nm. The 28 + 18S peaks were pooled for iodination and were used for in situ hybridization to the chromosomes of T.c. carnifex and T. vulgaris.


OD260
28 + 18s
fraction

III - 2a. An autoradiograph of a mitotic chromosome set of a T.c. carnifex tissue culture cell hybridised with ^{125}Iodine-labelled 28 + 18S RNA from T.c. carnifex ovaries. Two chromosomes are clearly and specifically labelled (arrowed) at a position about 2/5 down the long arm from the centromere. The labelled chromosomes are thought to be chromosomes X (see fig. II - 5).

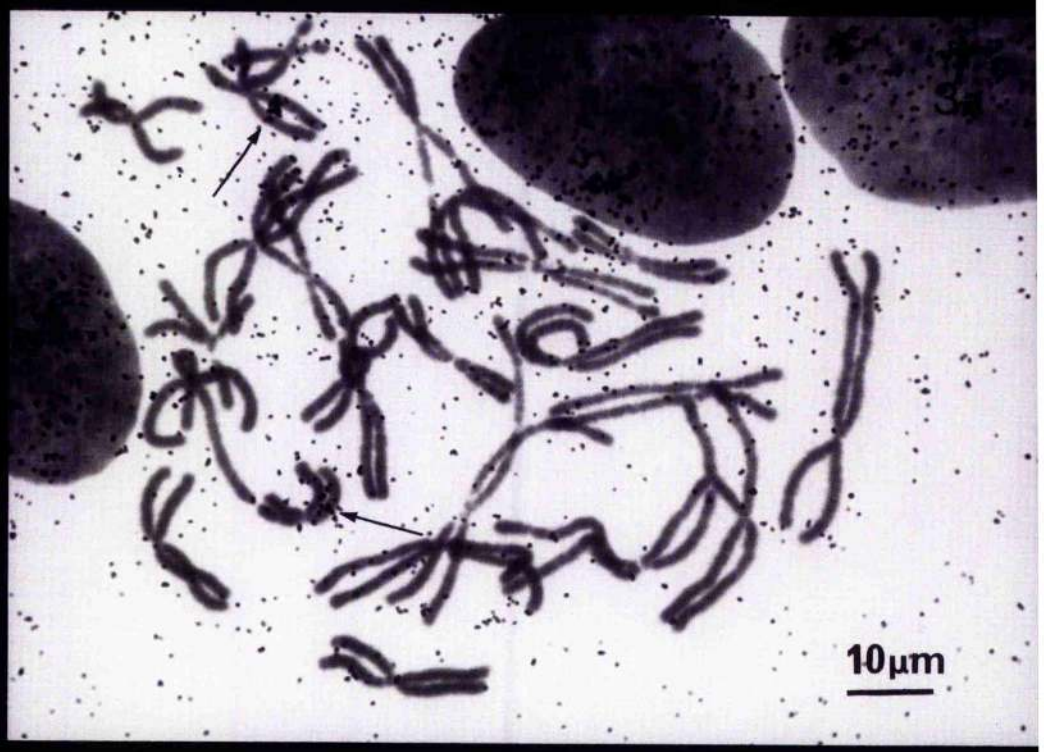
III - 2b. As fig. III - 2a.

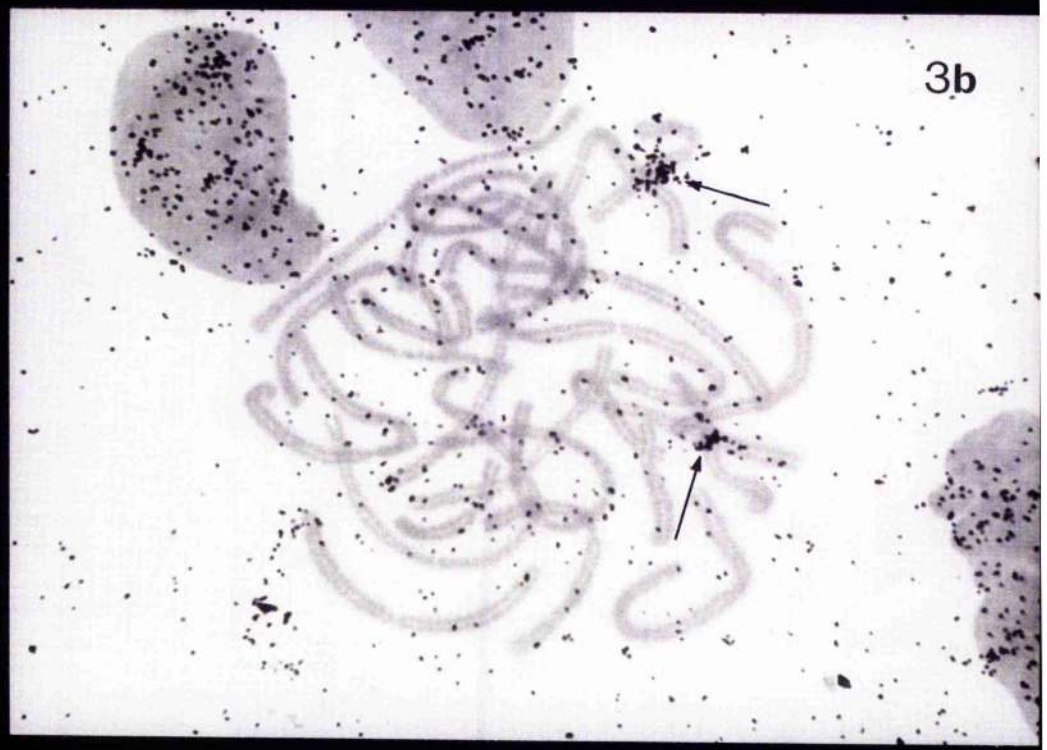

2a
10µm


2b

III - 3a. As fig. III - 2a.

III - 3b. As fig. III - 2a.

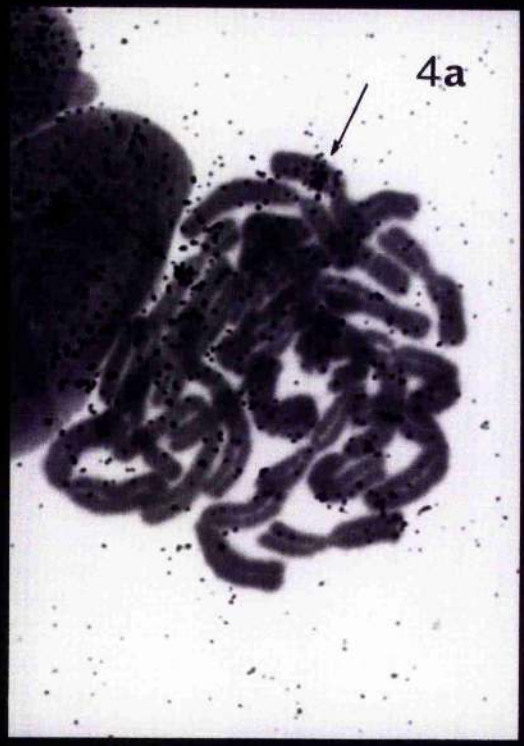

10µm

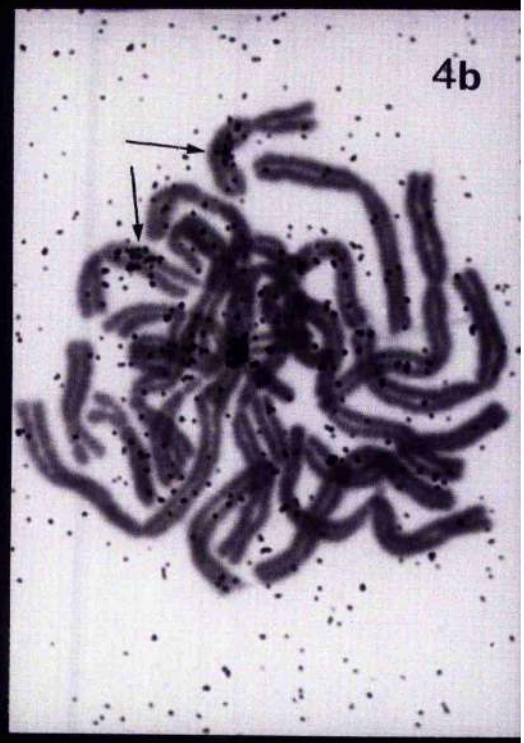

3b

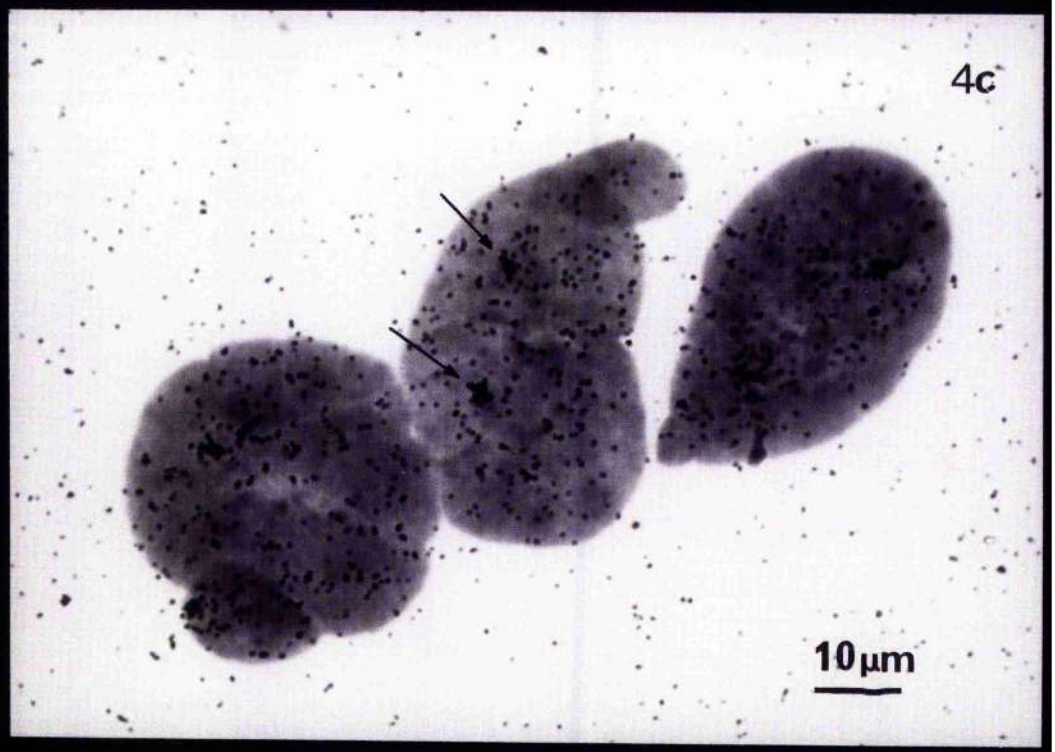
III - 4a. As fig. III - 2a.

III = 4b. As fig. III - 2a.

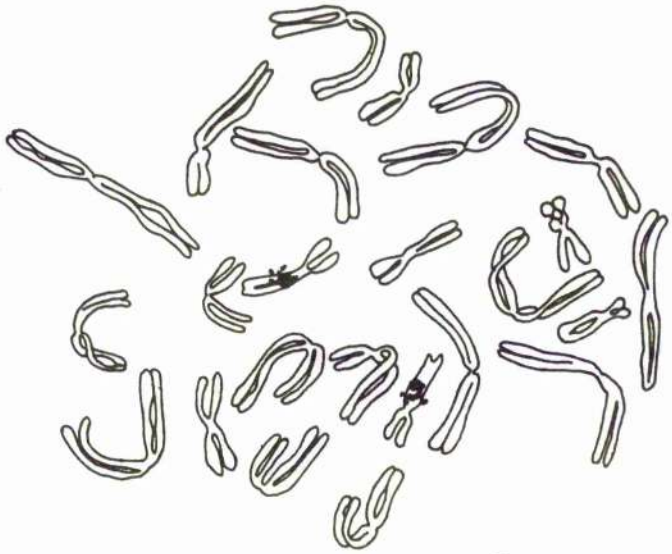
III - 4c. An autoradiograph of interphase cells from the T.c. carnifex tissue culture hybridized with ^{125}Iodine-labelled 28 + 18S RNA fro. T.c. carnifex ovaries. Two regions of label are visible in each cell (arrowed).

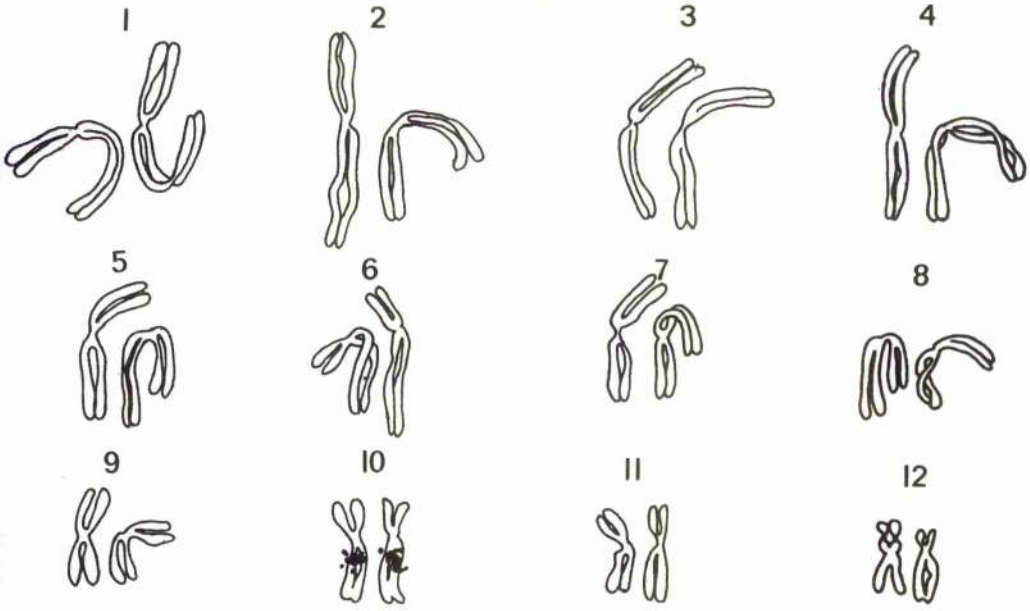

4a


4b


4c
10µm

III - 5a. A diagrammatic representation of the chromosome set in fig. II - 2a. The karyotype of paired chromosomes suggests that the regions of the chromosomes complementary to 28 + 18S RNA are on the long arms of chromosomes X.




1
2
3
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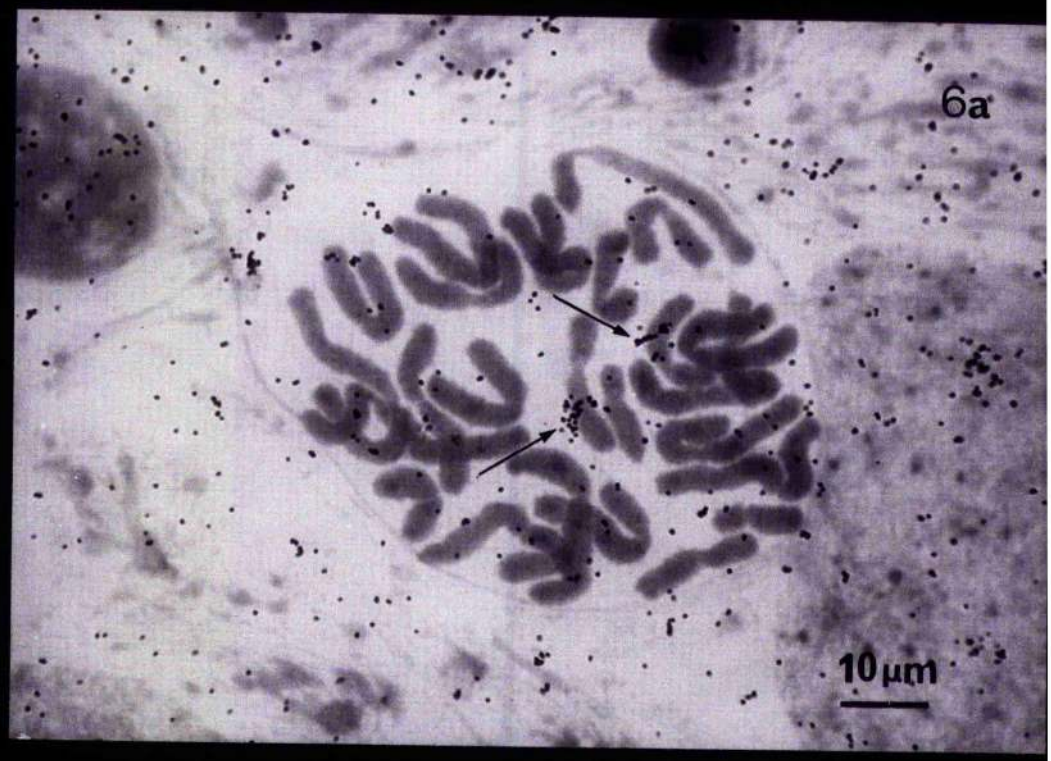
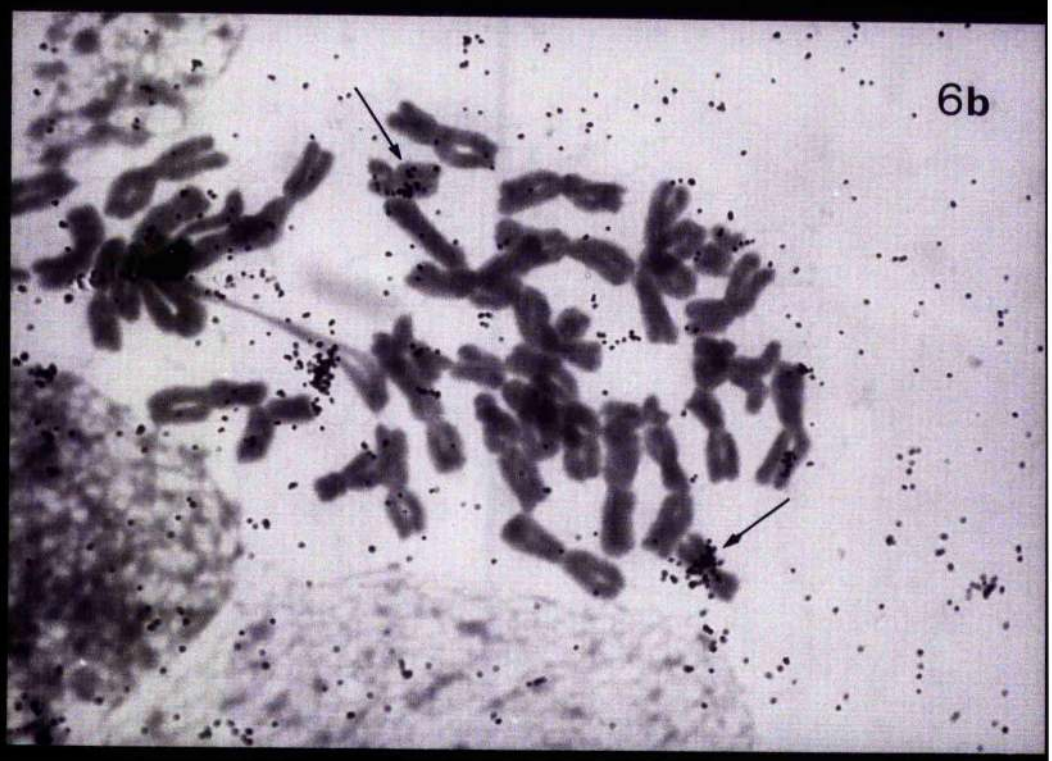
11
12

III - 6a. An autoradiograph of mitotic metaphase chromosomes of a T.c. carnifex spermatogonium hybridized with ^{125}Iodine-labelled 5S RNA. Two chromosomes are clearly and specifically labelled. These are thought to be both chromosomes X.

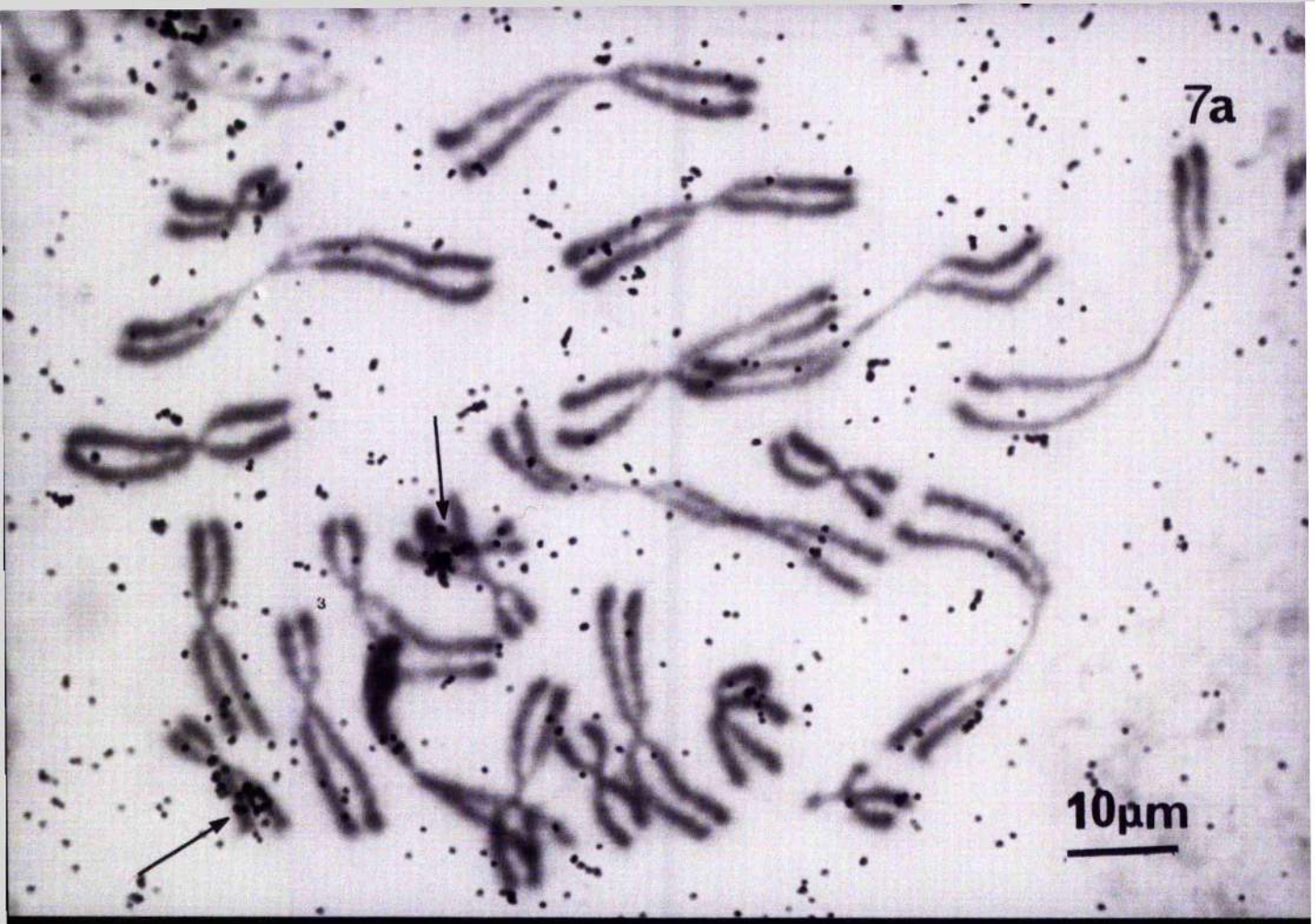
III - 6b. As fig. III - 6a.

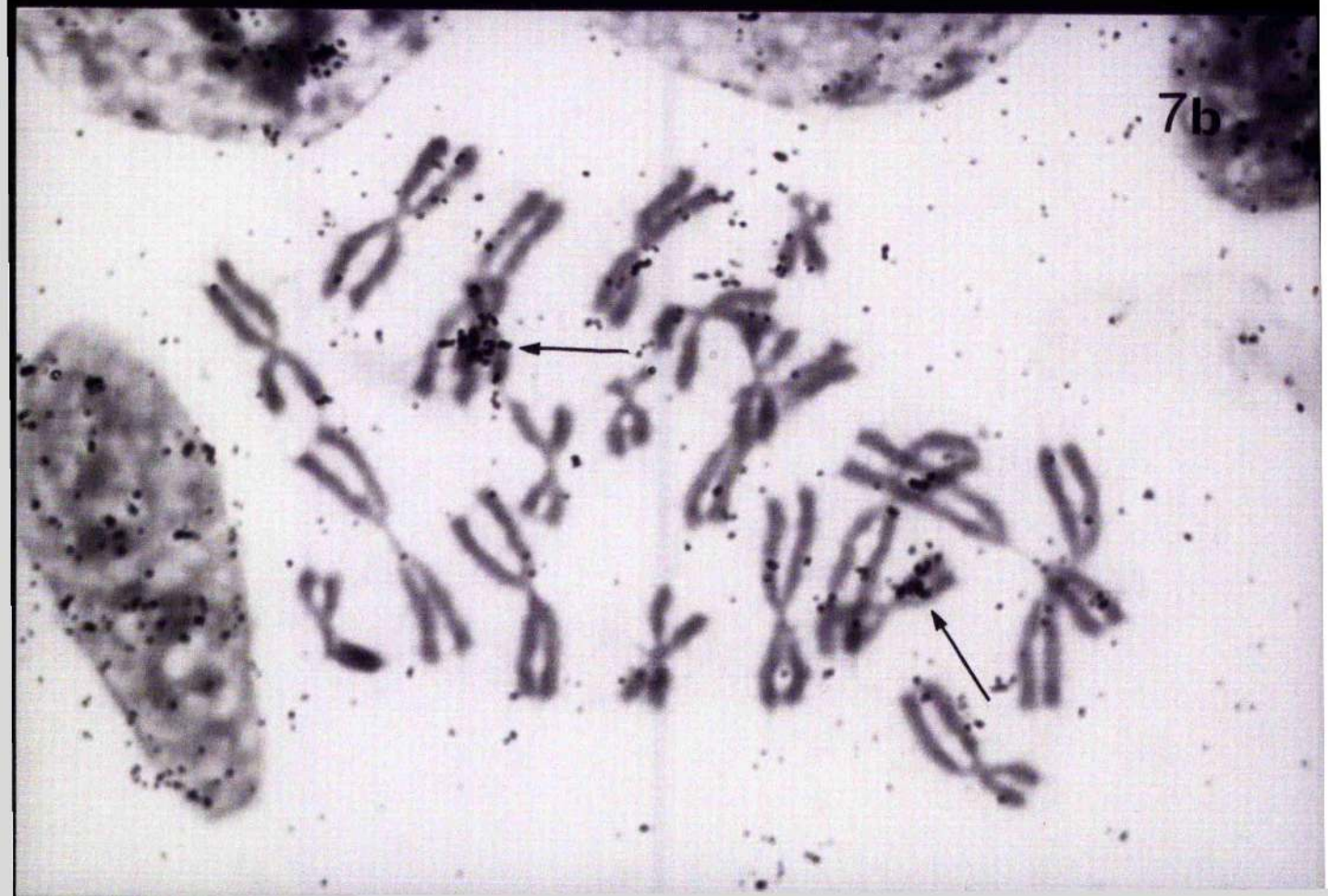

6a
10 µm


6b

III - 7a. As fig. III - 6a.

III - 7b. As fig. III - 6a.

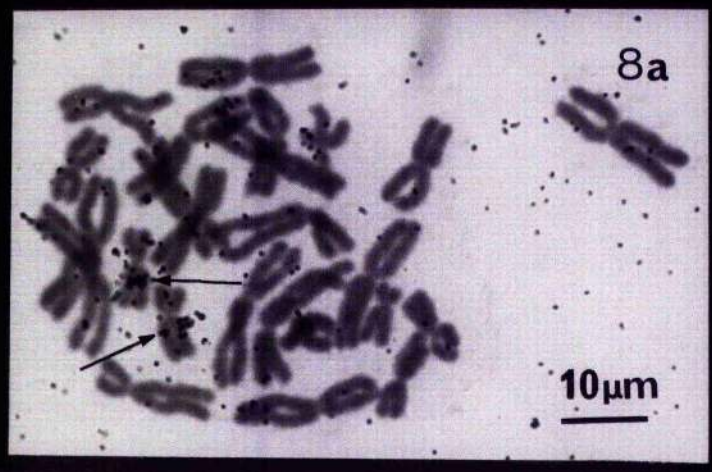

7a
10µm

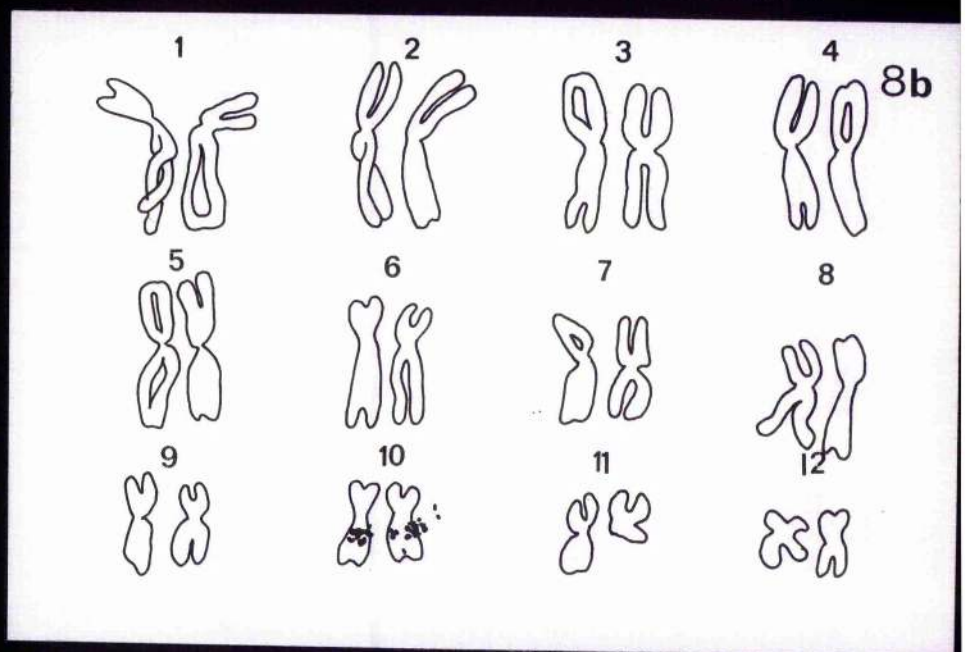

7b

III - 8a. As fig. III - 6a.

III - 8b. Diagrammatic karyotype of the chromosome set in fig. III - 8b. The 5S genes appear to be located on both chromosomes X.

III - 8c. An autoradiograph of non-dividing spermatogonia of T.c. carnifex hybridized with ^{125}Iodine-labelled 5S RNA. Two spermatogonia show two labelled regions (one cell is arrowed) and two show one labelled region.


8a
10µm


1
2
3
4
8b
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8
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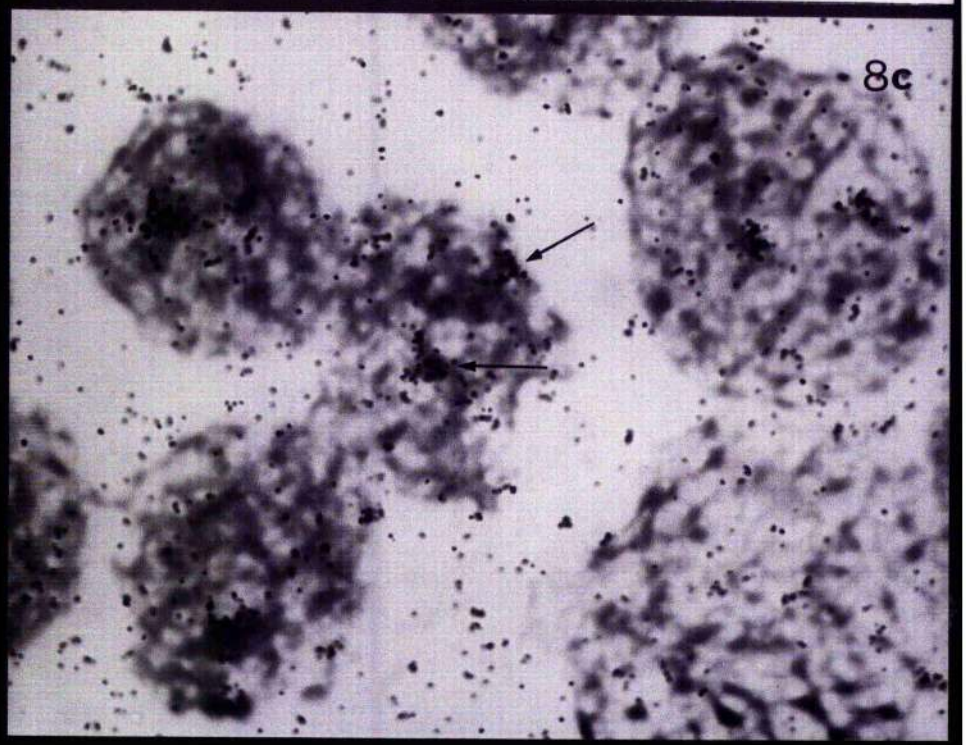
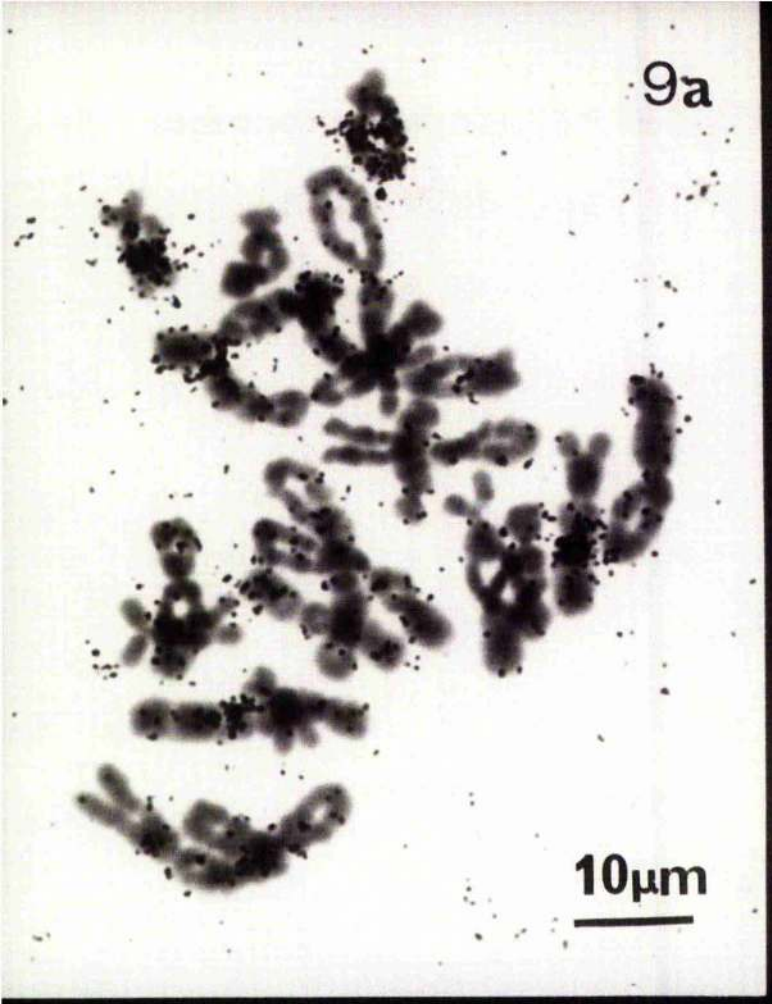
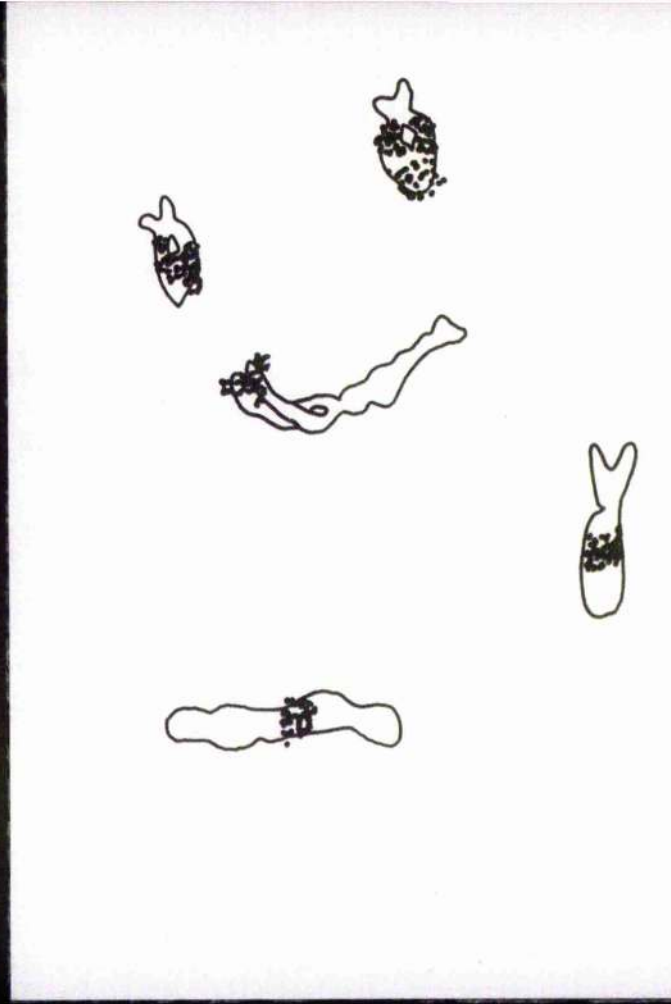
10
11
12

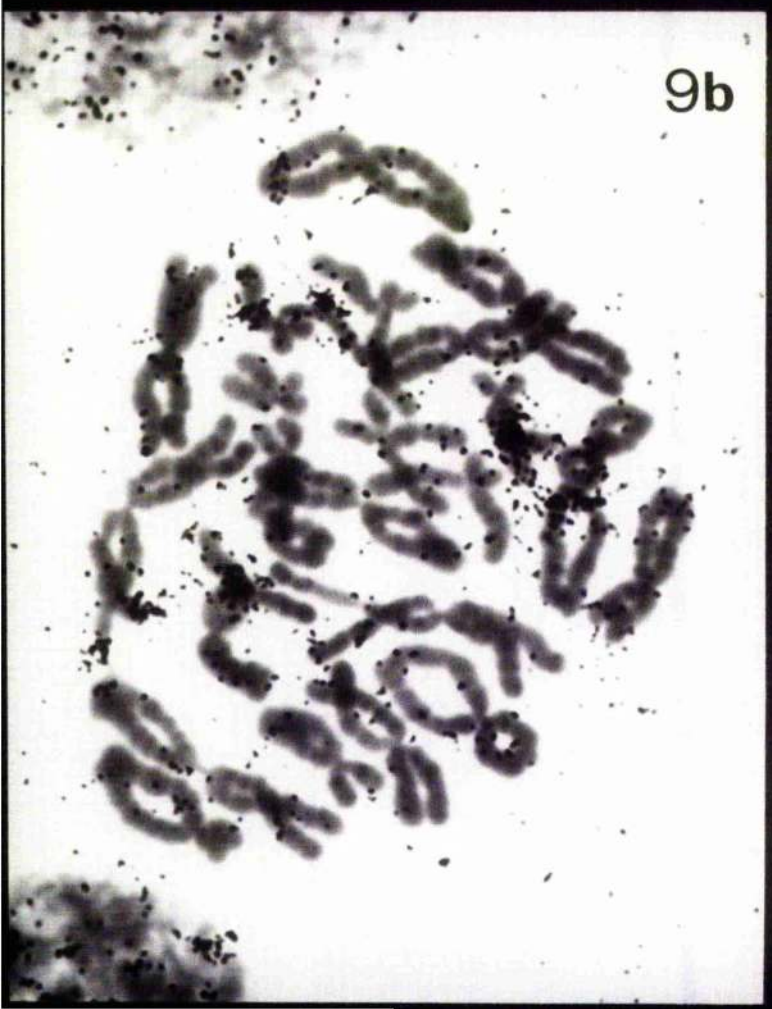

8c

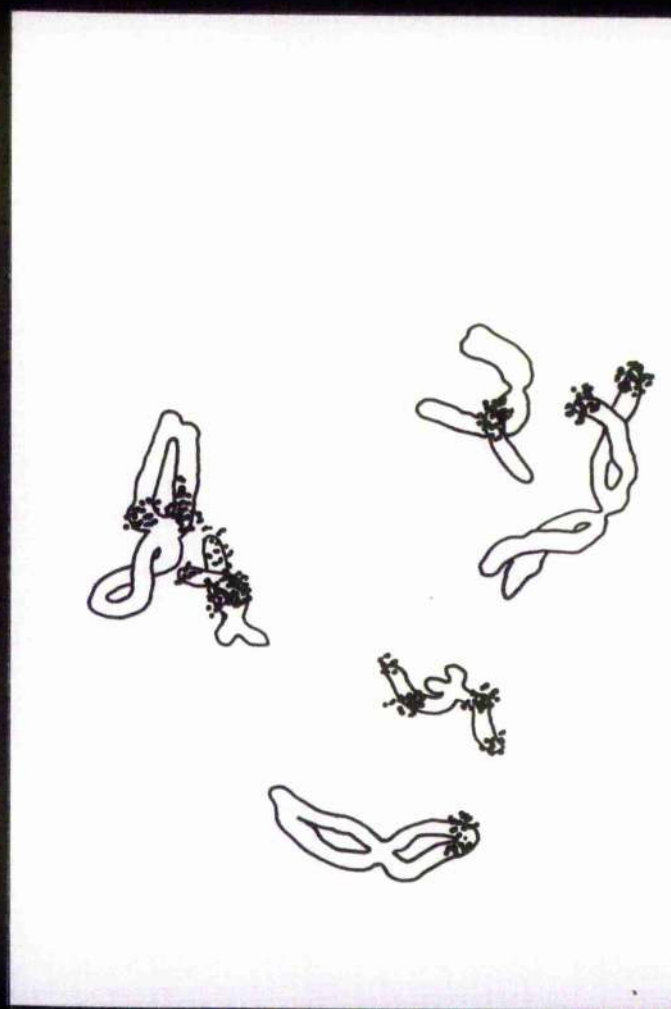
III - 9a. Au autoradiograph of mitotic metaphase chromosomes of a brain cell of T. vulgaris larva "A2" hybridized with ^{125}Iodine-labelled 28 + 18S RNA from T.c. carnifex ovaries. In general, metaphase cells from animal "A2" showed labelling over all eight of the possible loci of the 28 + 18S genes (see text). Regions of the chromosomes complementary to 28 + 18S RNA are (i) near the telomeres of both chromosomes II, (ii) halfway down the long arms and at the ends of the long arms of both chromosomes X, (iii) near the centromere of one large metacentric chromosome, and (iv) halfway down the long arm of a medium-sized submetacentric chromosome. The diagram accompanying each autoradiograph shows the labelled chromosomes.

III - 9b. As fig. III - 9a.


9a
10µm

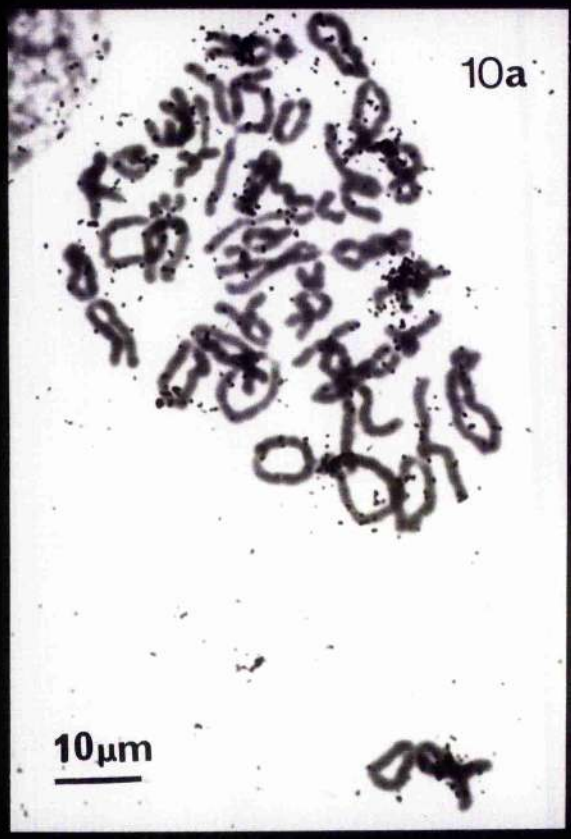


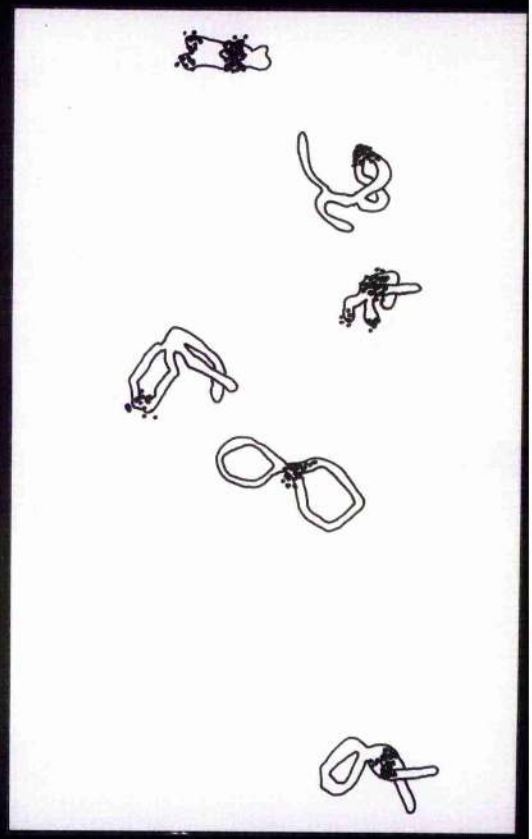

9b

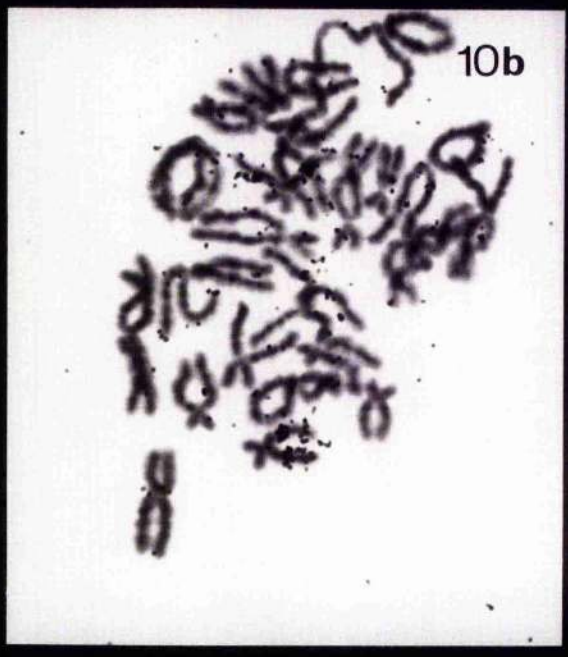


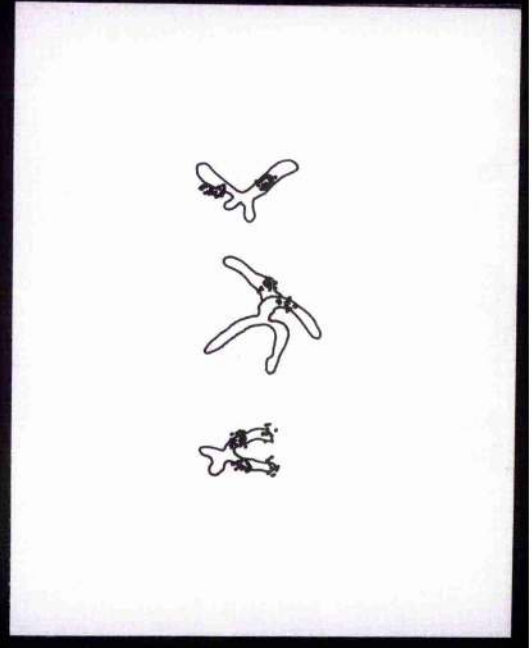
III - 10a. As fig. III - 9a.

III - 10b. As fig. III - 9a. Only the three chromosomes appear labelled.

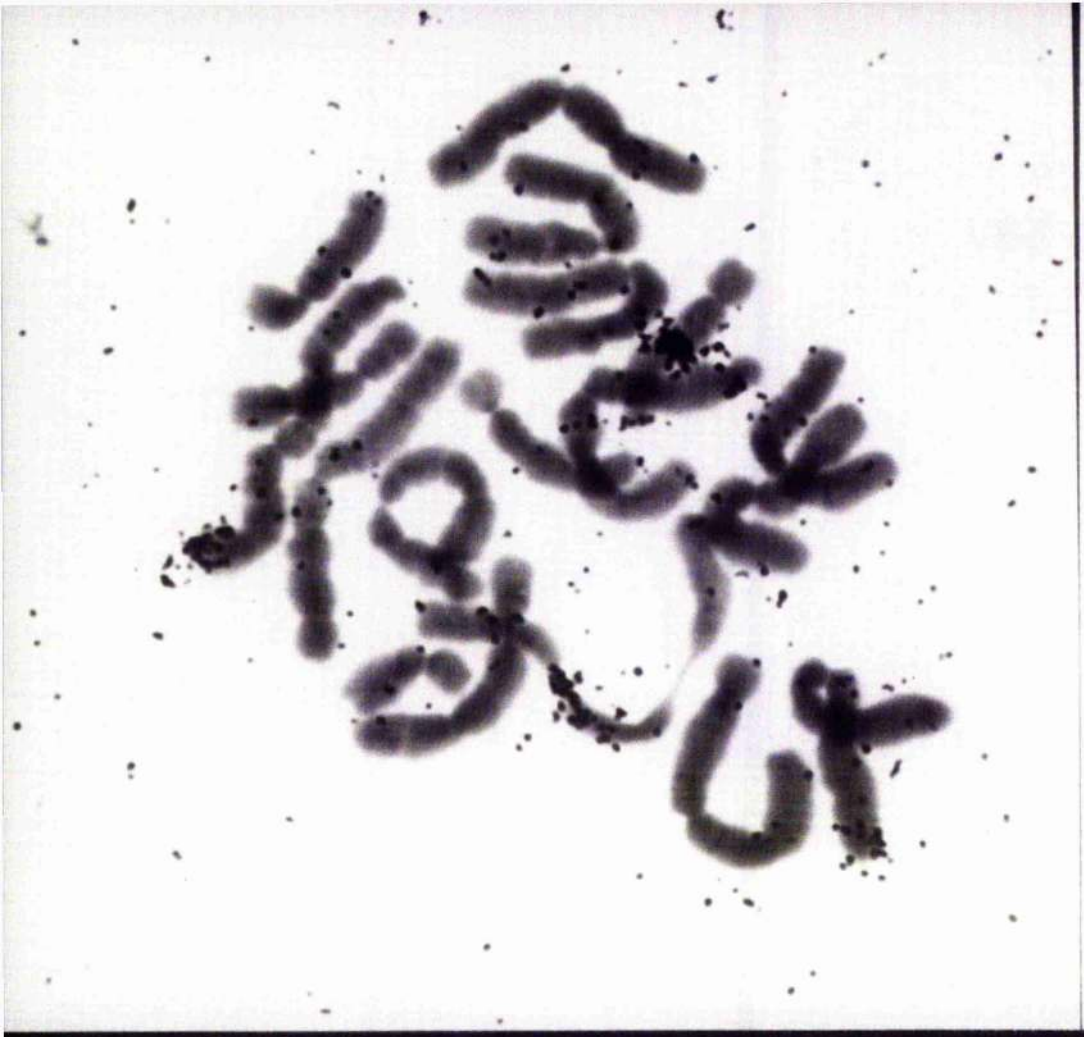

10a
10µm

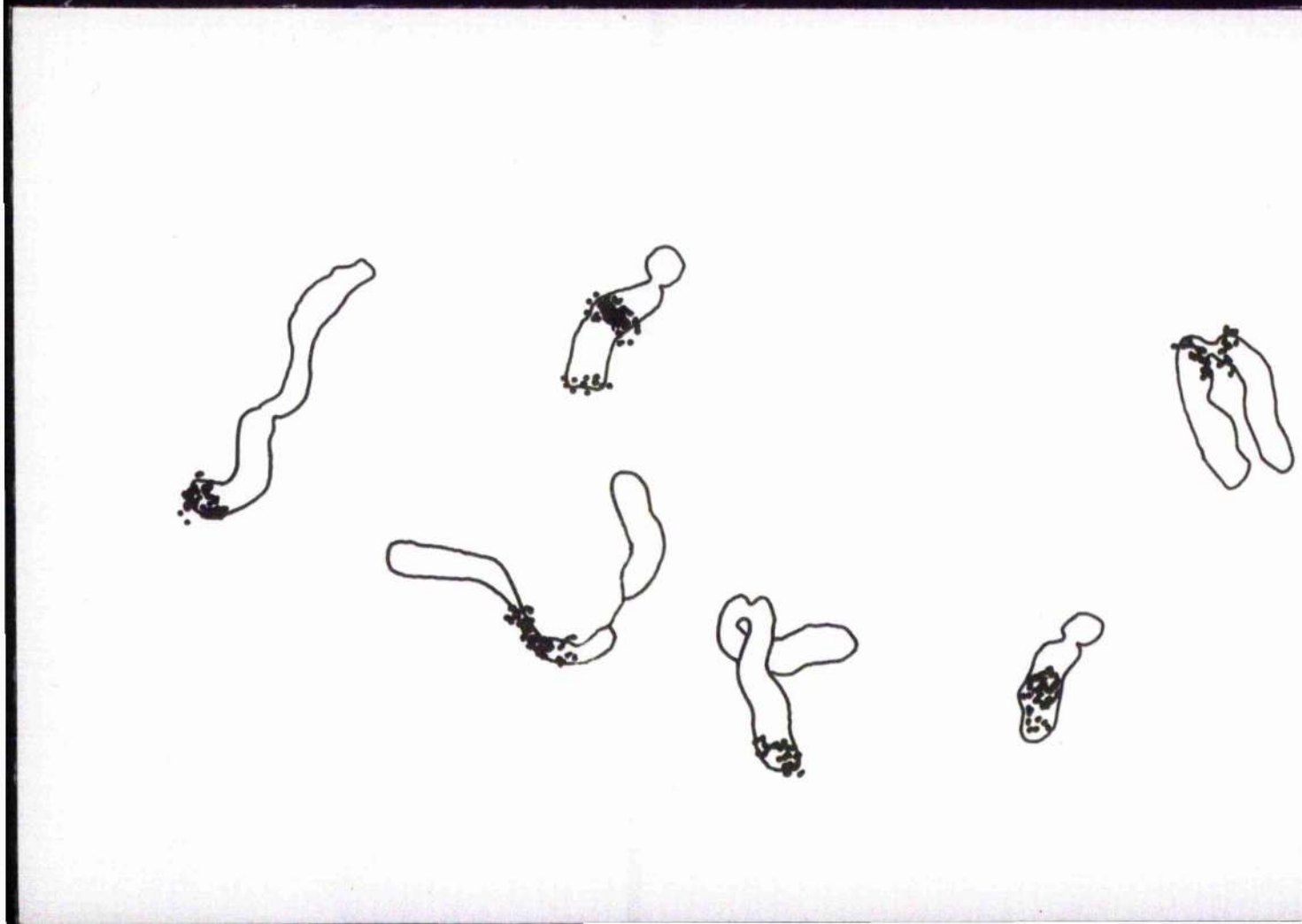



10b



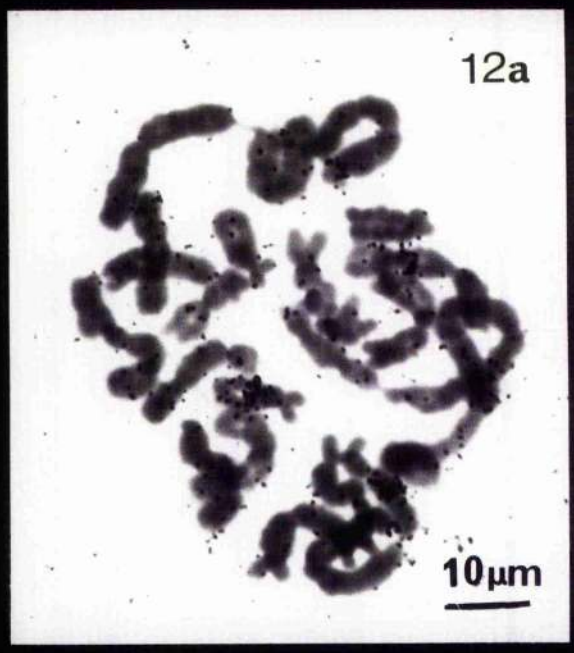
III - 11. As fig. III - 9a.

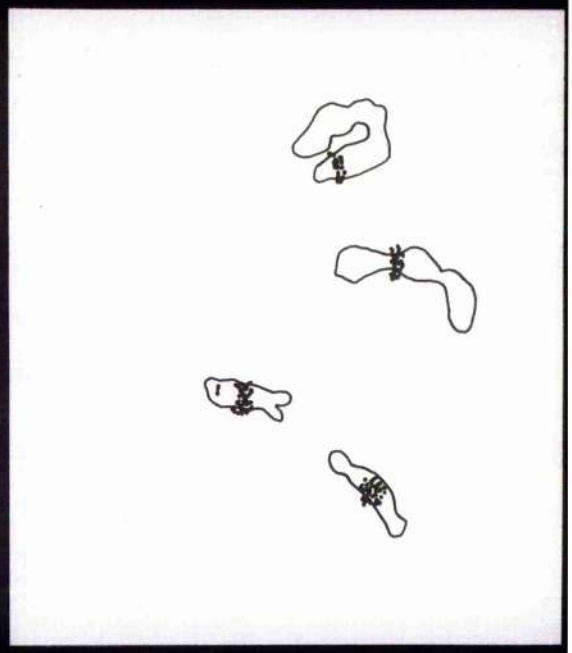


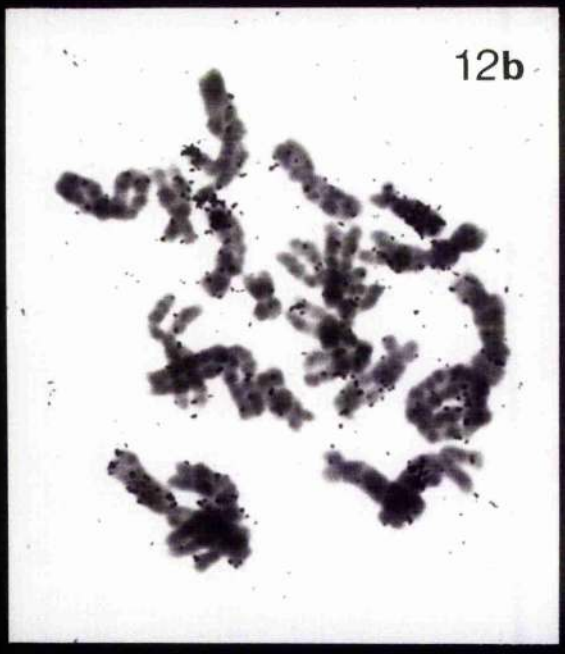


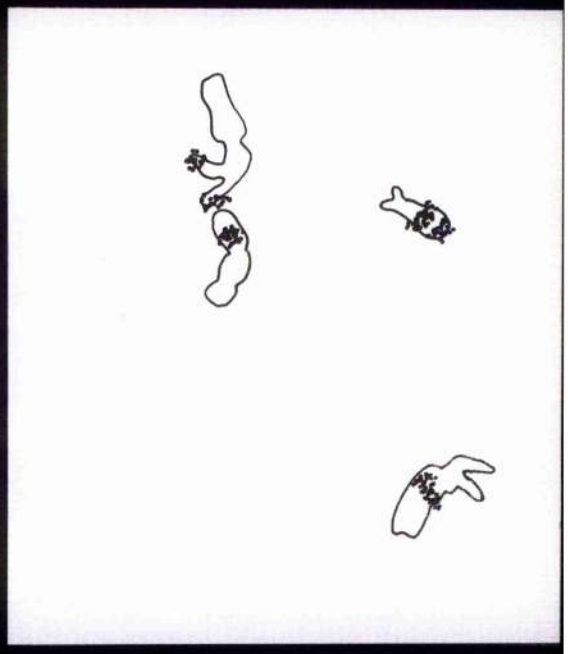
III - 12a. An autoradiograph of mitotic metaphase chromosomes of a brain cell of T. vulgaris larva "B12" hybridized with ^{125}Iodine-labelled 28 + 18S RNA from T.c. carnifex ovaries. Cells from animal "B12" showed labelling at both loci on both chromosomes X, at the end of one chromosome II and halfway down the long arm of one medium-sized submetacentric chromosome.

III - 12b. As fig. III - 12a.

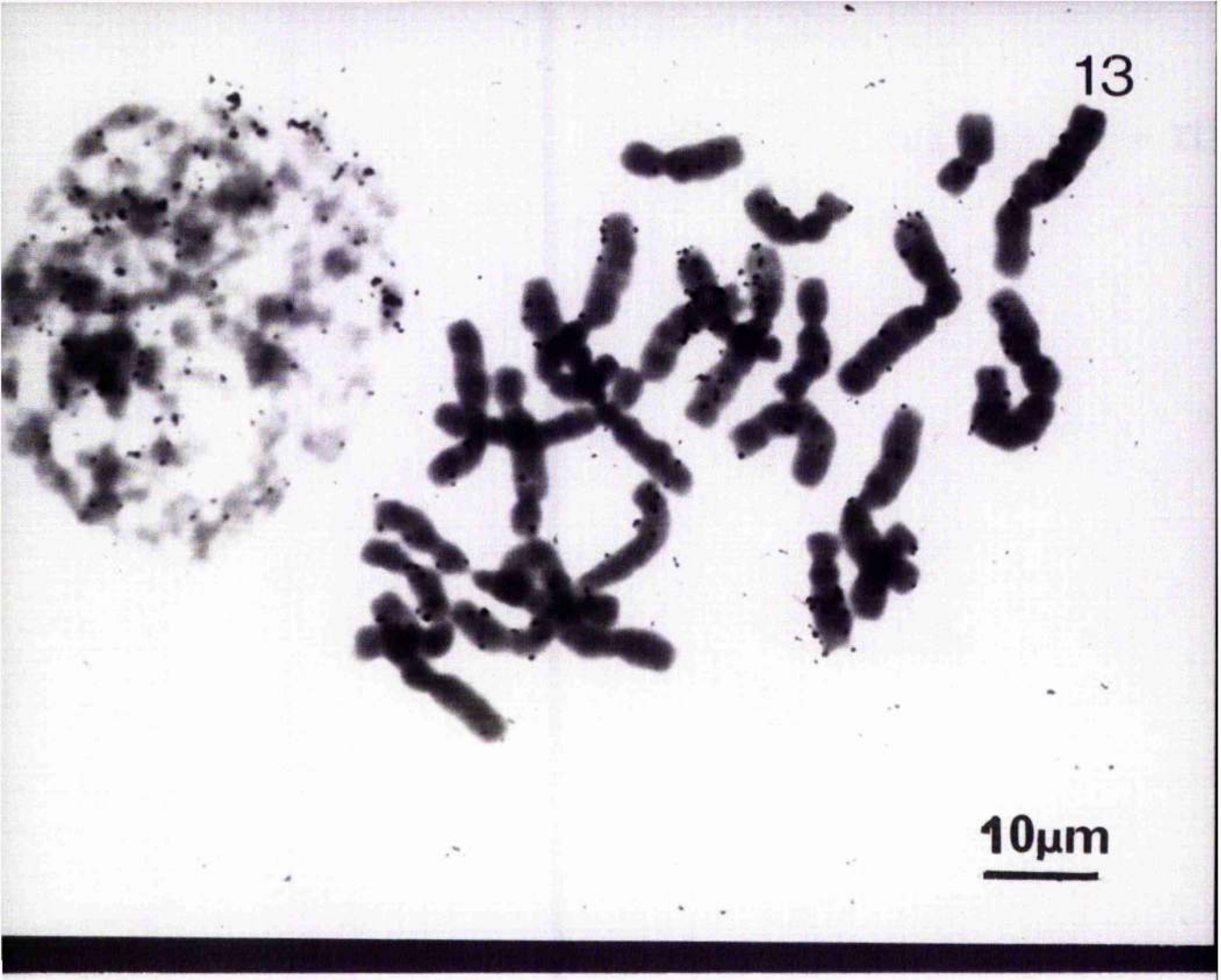

12a
10µm

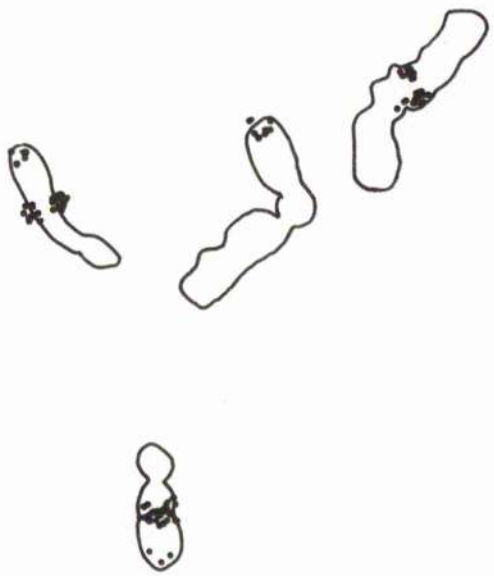



12b



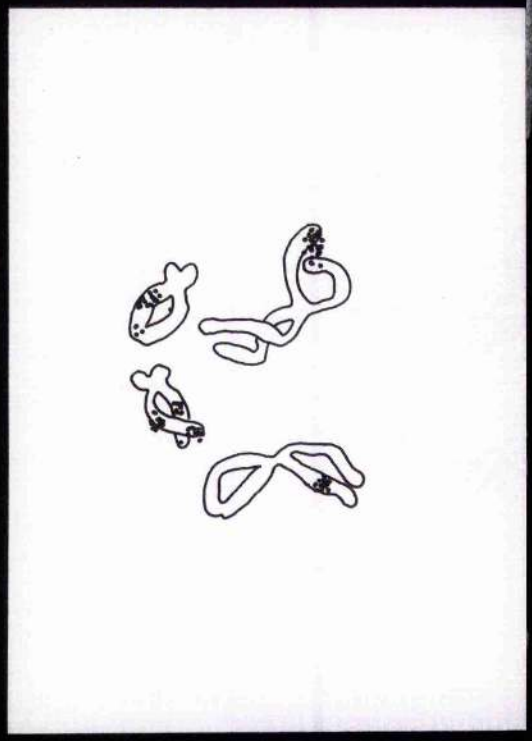
III - 13. As fig. III - 12a.


13
10μm

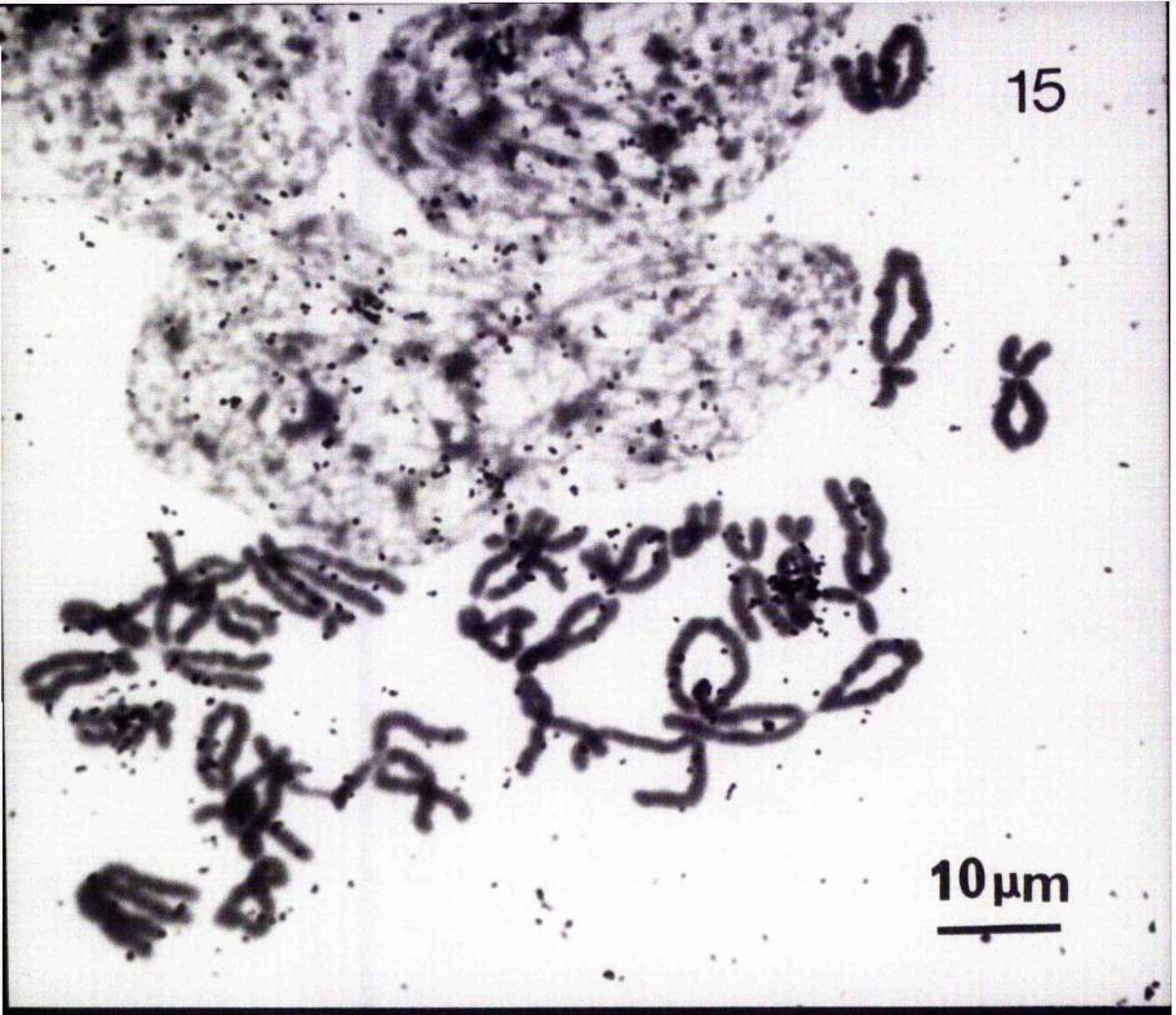


III - 14. An autoradiograph of a partial set of mitotic metaphase chromosomes of a brain cell of T. vulgaris larva "B20" hybridized with ^{125}Iodine-labelled 28 + 18S RNA from T.c. carnifex ovaries. One chromosome X has label over only one chromatid at both loci while its homologue is labelled at both loci on both chromatids. This difference in labelling between the two chromatids of one chromosome X is not found in all metaphase cells.

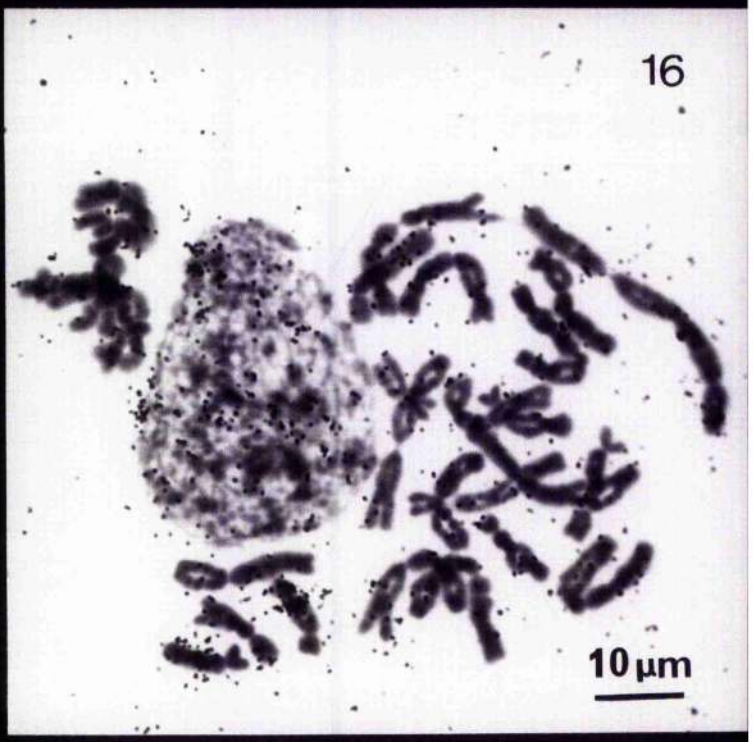

14
10µm



III - 15. An autoradiograph of a set of mitotic metaphase chromosomes of a brain cell of T. vulgaris larva "B10" hybridized with ^{125}Iodine-labelled 28 + 18S RNA from T.c. carnifex ovaries. There are labelled regions over both loci of chromosomes X, with no other significantly labelled areas.


15
10µm

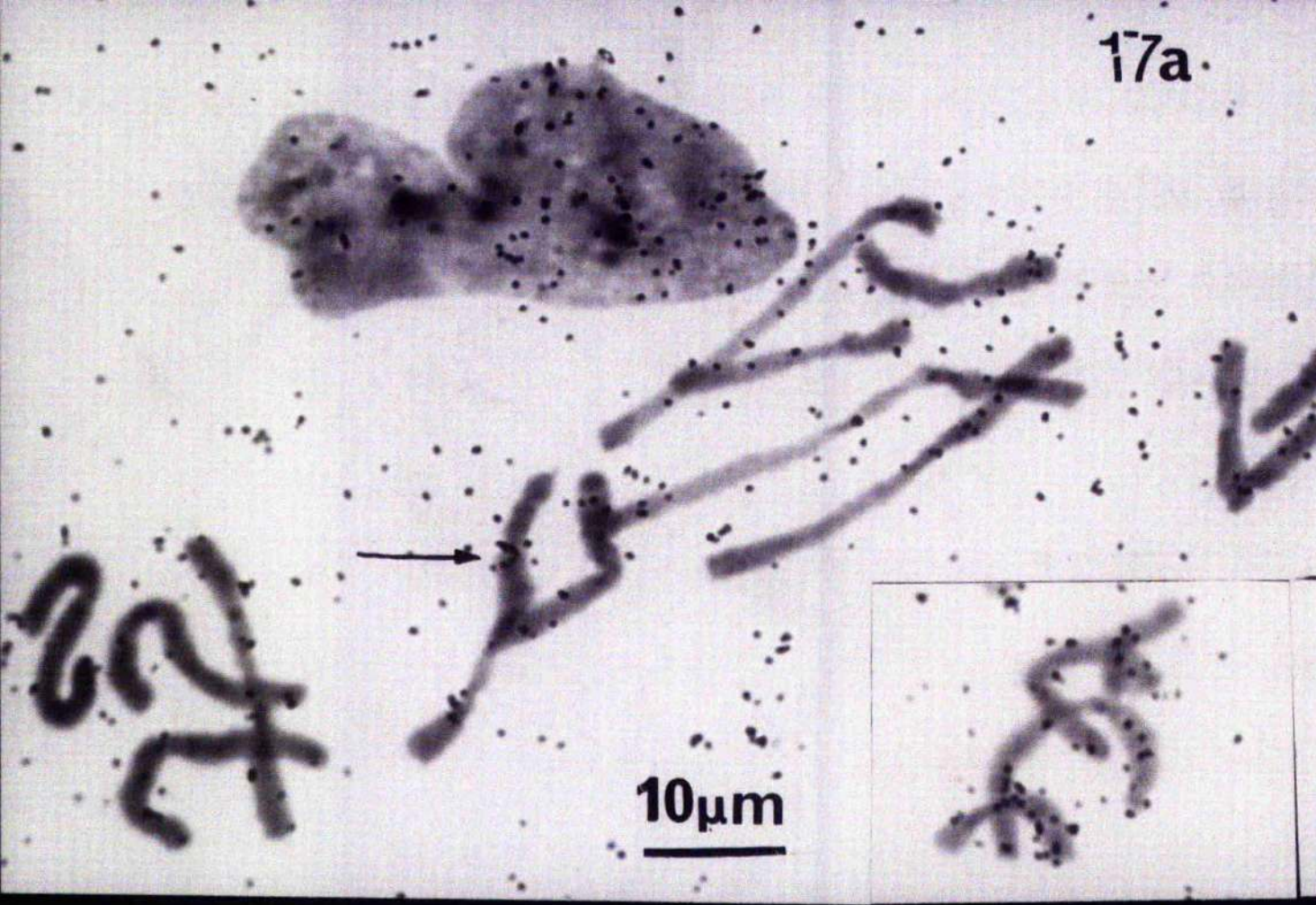
III - 16. As fig. III - 15.

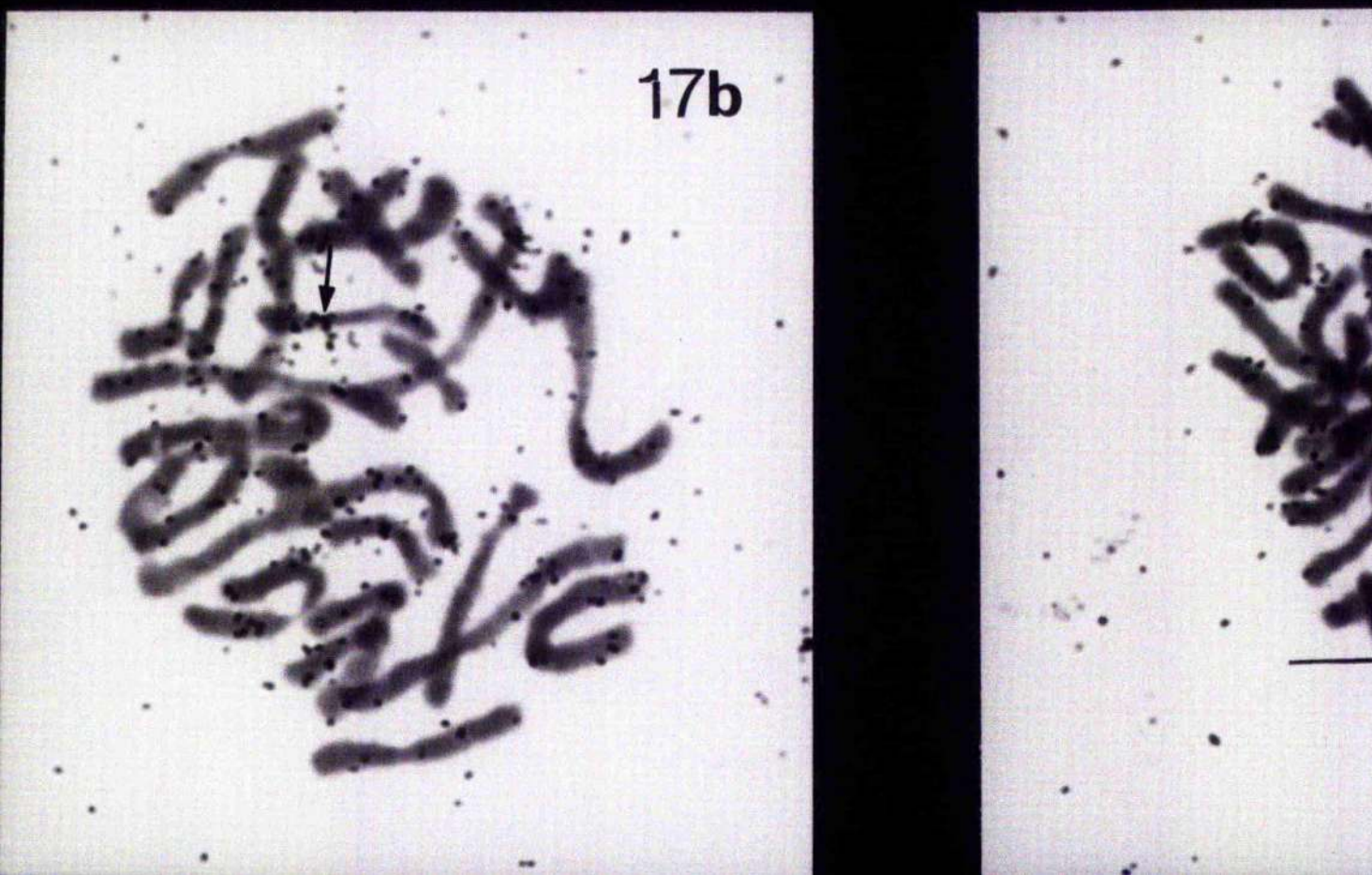

16
10 µm

III - 17a An autoradiograph of a set of mitotic metaphase chromosomes of a brain cell of T.c. carnifex x T. vulgaris hybrid larva "B2" hybridized with ^{125}Iodine labelled 28 + 18S RNA from T.c. carnifex ovaries. Labelled regions are difficult to distinguish but in general, there appears to be regions complementary to 28 + 18S RNA 1/3 of the way down the long arm of one small acrocentric chromosome (arrowed), near the telomere of a smaller acrocentric chromosome, and halfway down one arm of one large submetacentric chromosome (arrowed).

III - 17b. As fig. II - 17a.

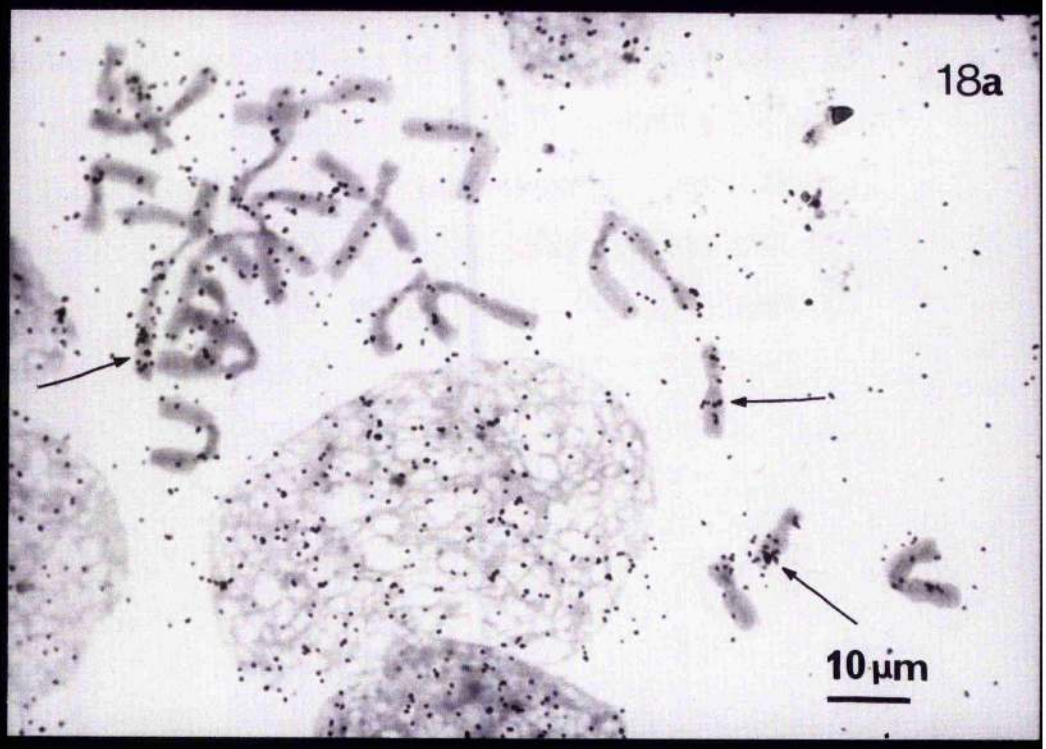
III - 17c. As fig. III - 17a.

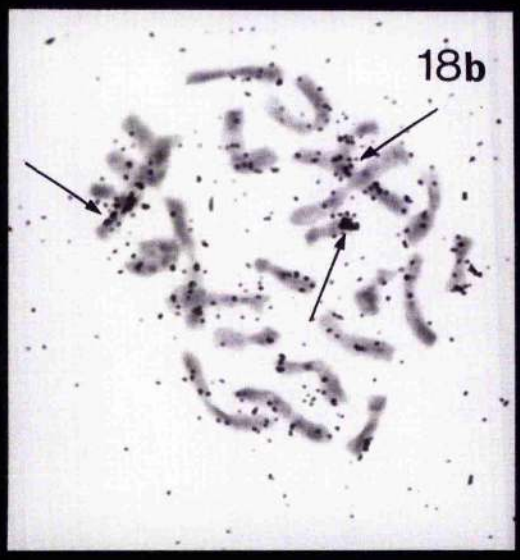

17a
10µm


17b

III - 18a. An autoradiograph of a set of mitotic metaphase chromosomes of a brain cell of T.c. carnifex x T. vulgaris hybrid larva "C7" hybridised with ^{125}Iodine-labelled 28 + 18S RNA from T.c. carnifex. Labelled regions are as described in fig. II - 17a, except animal "C7" has a labelled region near the telomere of one arm of one, large, submetacentric chromosome instead of halfway down one arm of one large submetacentric chromosome.

III - 18b. As fig. III - 18a.

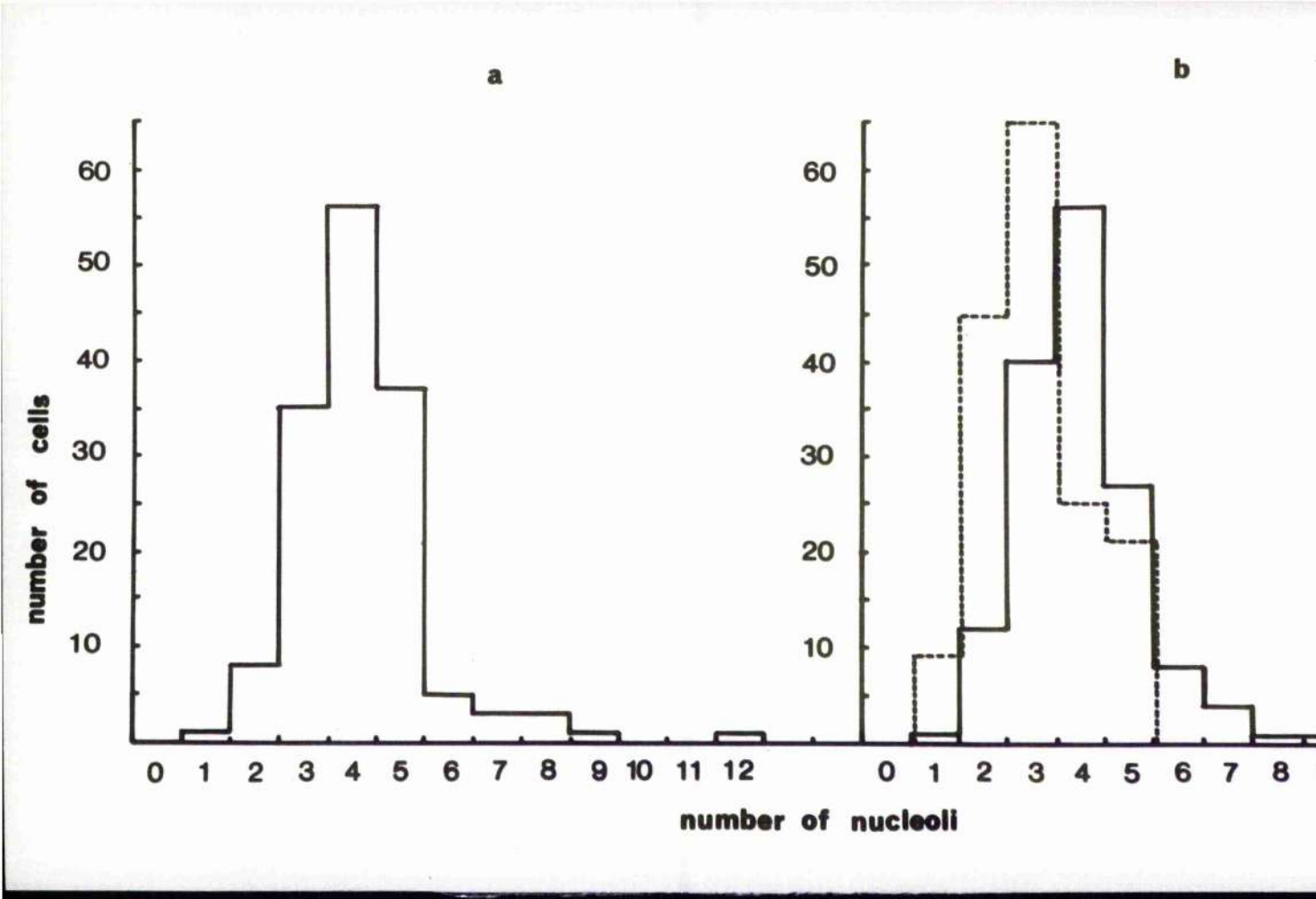

18a
10 µm

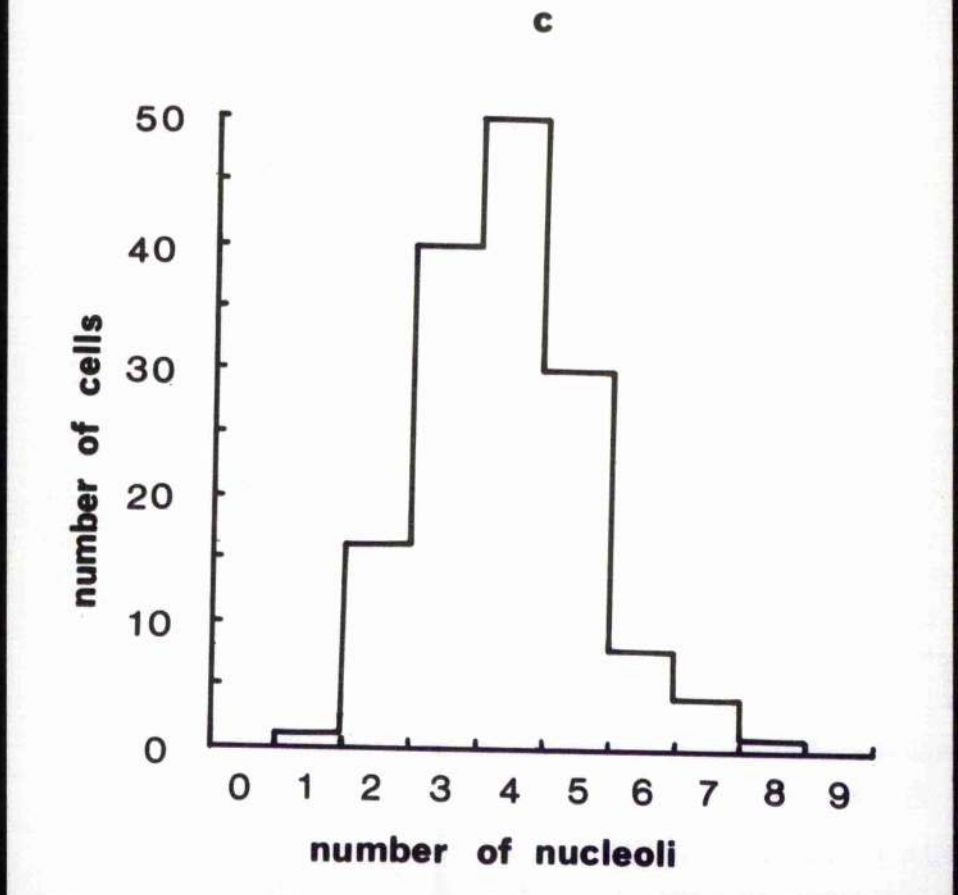

18b

III - 19a. Histogram to show the numbers of nucleoli in T.c. carnifex tissue culture cells stained with methyl green/pyronine.

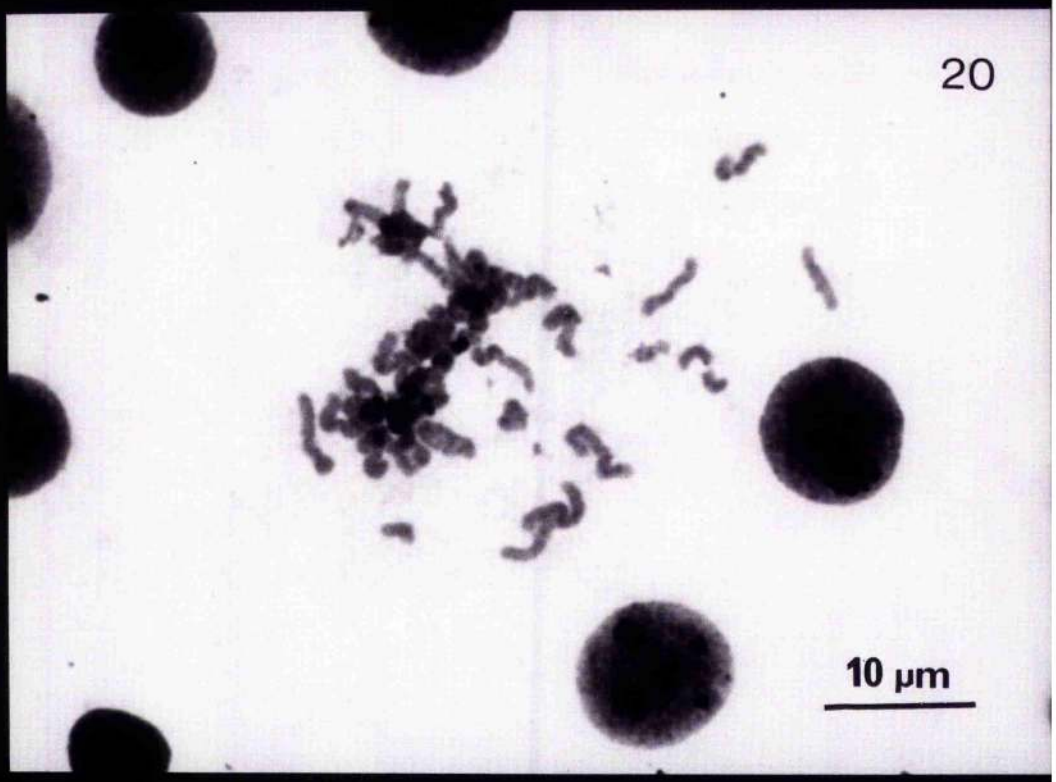
III - 19b. Histograms to show the numbers of nucleoli in T.c. carnifex tissue culture cells (unbroken lines) and T.c. carnifex spermatogonia (broken lines) after "silver staining".

III - 19c. Histogram to show the numbers of nucleoli in T. vulgaris larval brain cells after "silver staining".


a
b
60
50
40
30
20
10
number of cells
0 1 2 3 4 5 6 7 8 9 10 11 12
0 1 2 3 4 5 6 7 8
number of nucleoli


c
50
40
30
20
10
0
number of cells
0 1 2 3 4 5 6 7 8 9
number of nucleoli

III - 20. Mitotic metaphase chromosome set and interphase cells from human female lymphocyte culture. These cells were "silver stained" by the technique of Denton et al. (1976). The nucleolar organizer regions of the chromosomes and nucleoli in interphase cells stain black.


20
10 µm

III - 21. Mitotic metaphase chromosomes of a T.c. carnifex spermatogonium "silver stained" by the technique of Denton et al. (1976). Nucleolar organizer loci are not differentially stained yet the G-banding regions of the chromosomes stain black.


21
10 µm

CHAPTER IV.

Sister chromatid exchanges in the mitotic chromosomes of Triturus.

1. INTRODUCTION

In eukaryotes, recombinational events between homologous DNA sequences were long thought to be confined to the zygotene or pachytene phase of the meiotic cycle where the events leading to the breakage and subsequent rejoining of homologous but non-identical (non-sister) chromatids of a pair of homologous chromosomes (Whitehouse, 1963; Holliday, 1964) is a genetic mechanism whereby material and paternal genes are reassorted. However, in 1957, Taylor, Woods & Hughes described results which showed that physical exchange could occur between identical (sister) chromatids during mitosis. This latter phenomenon was observed in the mitotic metaphase chromosomes of Vicia faba which had undergone two periods of DNA synthesis, the first being spent in medium containing ^{3}H-thymidine. Each chromosome examined at the second metaphase after labelling had one labelled and one unlabelled chromatid, one of the first strong pieces of evidence in favour of the theory of semiconservative DNA replication and segregation. However, some chromosomes showed a discontinuity in their labelling pattern which could be accounted for if a reciprocal exchange between labelled and unlabelled sister chromatids had occurred. The visualisation of sister chromatid exchanges (SCEs) using ^{3}H-thymidine labelling was later reported for several other eukaryote species, but it was suspected that endogenous radiation from the tritium incorporated into the DNA was in part, if not totally responsible for the exchanges seen (Wolff, 1964). Geard & Peacock (1969) described results which suggested that the frequency of SCEs in Vicia faba is independent of the level of incorporated tritium, yet Gibson & Prescott (1972) found that at low levels of incorporated radioactivity, there was a direct relationship between the level of incorporated tritium

and the number of exchanges observed in rat kangaroo cells. The frequency of SCEs, as monitored after tritium labelling, was found to increase following exposure to physical agents such as X-rays (Marin & Prescott, 1964) and UV light (Rommelaere et al., 1973). It was generally accepted that the majority of sister chromatid exchanges were radiation-induced, yet the possibility that they were naturally-occurring phenomena could not be ruled out completely.

A major breakthrough in the study of SCEs was made by Latt in 1973. Latt (1973) found that chromosome preparations of PHA-stimulated human lymphocytes which had undergone two rounds of DNA synthesis in medium containing the thymidine analogue 5-bromodeoxyuridine (BUdR), when stained with the fluorochrome 33258 Hoechst had one chromatid which fluoresced brightly (unifilarly substituted) and another which fluoresced dully (bifilarly substituted) when examined by UV microscopy. When 33258 Hoechst is bound to BUdR-containing DNA, its fluorescence is reduced to an extent proprotional to the degree of substitution (Latt, 1973). Thus, DNA with BUdR substituted for thymine residues in both strands fluoresces weakest while unsubstituted DNA fluoresces brightest. The discovery that such preparations can be made permanent using a technique involving exposure to light, heating and Giemsa-staining, is the latest development in methodology to visualize SCEs (Fluorescent plus Giemsa (FPG) technique) (Perry & Wolff, 1974; Wolff & Perry, 1974; Korenberg & Freedlender, 1974). When this technique is used successfully, chromatids monofilarly substituted with BUdR (which fluoresce brightly with 33258 Hoechst) stain intensely with Giemsa, while bifilarly substituted chromatids (which fluoresce dully with 33258 Hoechst) stain less intensely. This technique has been used to demonstrate that the frequency of SCEs increases also following the exposure of cells to chemical mutagens (Kato, 1974a; Perry & Evans, 1975). The level of resolution attained by this technique is far superior to that offered by

tritium autoradiography, allowing SCEs to be analysed in detail. Apparently isolabelled chromosome segments seen in many ^{3}H-thymidine-labelled preparations (Taylor, 1958; Peacock, 1963; Walen, 1965; Gibson & Prescott, 1973; Kato, 1974b) as a single autoradiographic image over homologous regions of sister chromatids can now be explained as being the result of several SCEs occurring within a short stretch of chromosome, yet not far enough apart from each other to be resolvable by autoradiography (Latt, 1974a; Wolff & Perry, 1974; Korenberg & Freedlender, 1974; Kato, 1974b).

Unfortunately, similar problems to those encountered in studies involving tritium incorporation are attached to the use of BUdR in visualising SCEs, namely that BUdR is itself known to induce exchanges (Kato, 1974b; Latt, 1974; Wolff & Perry, 1974), but there is evidence to suggest that when BUdR is used at low concentrations (0.1 - 1.0 μg/ml) a "spontaneous" level of SCEs is seen (Kato, 1974b). A study of the SCE frequency in the mitotic chromosomes of persons homozygous for the recessive gene bl/bl which is clinically manifest as Blooms' syndrome, has demonstrated that the chromosome breaks and rearrangements characteristic of this disorder (German, 1969; German et al., 1965; German et al., 1974) are associated directly with a considerable increase in the number of SCEs above that of normal cells (Chaganti et al., 1974). The breaks and rearrangements characteristic of Bloom's syndrome chromosomes are similar to those found at low frequencies in cells from normal persons. These results support the idea that SCEs are spontaneous in normal cells, and are not necessarily induced by the effects of incorporated radioactivity or BUdR.

With the knowledge that SCEs might be a naturally occurring phenomenon, I decided to look at their frequency in the mitotic chromosomes of Triturus cristatus carnifex using the FPG technique of Perry & Wolff (1974). The treatment of cells with BUdR in vivo can only be performed with success by

permanent subcutaneous infusion (Pera & Mattias, 1976) because BUdR introduced into the animal by injection is rapidly inactivated by debromination in the liver. Because such a method is impossible to perform on newts I have used mitotic cells from the T.c. carnifex fibroblast culture for my studies; these could be easily incubated with BUdR simply by addition to the tissue culture medium.

The DNA of eukaryotes is known to replicate in discrete, tandemly arranged units, the numbers and lengths of which are peculiar to the species of animal and to developmental stage in the life-cycle (e.g. Callan, 1971, 1973; McFarlane & Callan, 1973). At a given temperature there is a direct relationship between the duration of the S-phase and the distance between consecutive initiation sites for DNA replication. In T. vulgaris embryos at 18°C, the intervals between initiation sites are shortest in late blastulae/early gastrulae (25 - 30 μm, (Callan, unpublished results), S-phase = 2 3/4 hrs. (Pamela McFarlane, 1976, unpublished results)), becoming longer in neurulae (40 - 50 μm (Callan, 1972), S-phase = 6 3/4 hrs or longer (Pamela McFarlane, 1976, unpublished results)), and longer still in somatic cells (T.c. carnifex) in culture at 25°C (about 200 μm, S-phase = about 2 days (Jean H. Priest, cited in Callan, 1972)). There is evidence which suggests that SCEs occur only during or shortly after the S-phase of the mitotic cycle (Kato, 1973, 1974d; Perry & Evans, 1975) as a result of a type of recombination repair process. This process was originally suggested by Rupp & Howard-Flanders (1968) to be the mechanism used by excision-defective bacteria to repair regions of DNA containing pyrimidine dimers which had been induced by UV light. Thus, if DNA replication and the formation of SCEs are temporally related, there might be some relationship between the number of junctions between neighbouring replication units (the potential sites for recombination between sister chromatid DNA strands)

and the number of SCEs observed. It follows from this that for a given concentration of BUdR and at a constant temperature, SCEs should be more frequent in early embryonic stages than in tissue culture cells. I decided to test this hypothesis using different stages of T.c. carnifex and T. vulgaris embryo cells, and T.c. carnifex tissue culture cells.

The staining behaviour of T.c. carnifex metaphase chromosomes after culture at low temperature has been shown to be related to the degree of chromatin condensation in certain regions of the chromosomes (Rudak & Callan, 1976). I therefore decided to compare the degree of chromatin packing in cells grown for two periods of DNA synthesis in medium containing BUdR and that of cells grown in normal medium, to see whether a similar relationship between staining behaviour and chromatin packing could be found.

2. MATERIALS AND METHODS

(i) Analysis of SCE frequency in T.c. carnifex tissue culture cells

The T.c. carnifex tissue culture cells were grown and maintained as described in Chapter II. The confluent cell layers from three 75 cm^2 flasks were trypsinized, and seeded into 12.0 mls of fresh culture medium in twelve new flasks. An additional 0.5 ml. volume of amphibian wash solution (AWS) containing BUdR in the following concentrations was added to each flask:

	Final Concentration BUdR
0.50 ml. 2.50 mg/ml. BUdR + 0.00 ml. AWS	100 µg/ml.
0.37 ml. 2.50 mg/ml. BUdR + 0.13 ml. AWS	75 µg/ml.
0.25 ml. 2.50 mg/ml. BUdR + 0.25 ml. AWS	50 µg/ml.
0.12 ml. 2.50 mg/ml. BUdR + 0.38 ml. Aws	25 µg/ml.
0.50 ml. 0.25 mg/ml. BUdR + 0.00 ml. AWS	10 µg/ml.
0.05 ml. 0.25 mg/ml. BUdR + 0.45 ml. AWS	1 µg/ml.

Each different BUdR treatment was given to each of two flasks of cells. The final volume of medium containing BUdR in each flask was 12.5 mls. To prevent photolysis of BUdR-containing DNA, which would result in SCEs extra to those induced by the BUdR, the flasks were wrapped in aluminium foil to exclude all light. The cells were cultured at 25°C.

After seven days, colchicine at a final concentration of about 1 μg/ml. in AWS was added to each flask (0.15 ml. of 100 μg/ml. colchicine). Eighteen hours later, the cells were trypsinized, hypotonically treated, fixed, and slide preparations made by the air-drying technique described in Chapter II.

(ii) Fluorescence plus Giemsa (FPG) staining technique (Perry & Wolff, 1974).

The air-dried slide preparations were stained for 12 mins in 33258 Hoechst (1.0 μg/ml.) in deionised water, then rinsed and mounted in deionised water under a coverslip. The edges of the coverslip were sealed with rubber solution (Dunlop). Some preparations were examined with UV illumination using a Zeiss fluorescence microscope fitted with a x 100 Fl-Neofluar oil objective, and excitor filter I and barrier filter 50. This combination gives a green/pale green fluorescence of 33258 Hoechst-stained material, with a dark brown background. Photographs were taken on Kodak Tri-X Pan film, with an exposure time of 15 secs. The fluorescence fades quickly when exposed to UV light (in 10 - 15 secs) therefore longer exposure times result in poorer differentiation between the chromatids.

The slides were placed on a window ledge, near a fluorescent strip light, for 20 - 24 hours. The coverslips were removed and the slides placed in deionised water at 60°C in glass coplin jars in an oven for 2 hours. The slides were then removed from the coplin jars, cooled, and stained with

Giemsa in 0.05 M phosphate buffer, pH 7.1, for 10 mins, rinsed with tap water and air dried. The stained preparations were examined unmounted, immersion oil being applied directly to the slide. Photographs were taken on Ilford Pan F film using a Zeiss x 100 planapochromatic oil objective.

(iii) Analysis of SCE frequency in T.c. carnifex and T. vulgaris embryo cells.

I would like to state here that although I could not obtain any differential staining of sister chromatids in the mitotic metaphase chromosomes from embryo cells, for reasons which I will disucss in the Results section of this chapter, I still wish to present the methods I have used so that they may be used as a guide for further experimentaion along similar lines.

Female T.c. carnifex in breeding condition were purchased from the Zoological Station, Naples. Female T. vulgaris were caught during the breeding season (April/May) from several ponds in Tentsmuir Forest, Fife. The newts were kept at 18°C in large glass aquaria containing Canadian pondweed, Elodea. Eggs laid on the pondweed were collected daily and placed in 1/10 Steinberg saline in small plastic tanks. Developing eggs were sorted into stages in accordance with the numbered stage series proposed by R.G. Harrison for Ambystoma mexicanum (cited by Hamburger, (1966)). According to this classification, blastulae are stages 6 - 8, gastrulae are stages 9 - 12 and neurulae are stages 13 - 20.

The outer membranes were removed from the embryos before they were incubated in saline containing FUdR or BUdR. To do this, the embryos were placed in full strength Steinberg (FSS) saline in a large glass petri dish with a layer of black wax at the bottom. The outer gelatinous membrane was removed from the embryo by piercing the membrane at the top, going through the gelatinous layer, and coming out at the bottom and into the wax

with one point of a pair of stainless steel No. 5 watchmakers' forceps. Keeping the forceps still, the point of a tungsten needle was brought close to the embedded forceps point, and both were quickly drawn across each other in a scissor-like motion. When this was done successfully, the embryo in its vitelline membrane was released from the gelatinous capsule. The embryo was then transferred using a siliconised glass pipette with a bent, wide-bored end, to a small plastic petri dish containing FSS with a thin layer of 2% agar in FSS on the bottom. The vitelline membrane was removed from the embryo by grasping the membrane at the top with a pair of stainless steel no. 5 watchmakers' forceps, then inserting the tip of a tungsten needle at this point and making a tear in a downwards direction, thus tipping out the ball of cells onto the agar surface. Cells in this state were transferred to agar-coated petri dishes containing BUdR etc. using a siliconised wide-bored pipette with a bent end.

The lengths of time required for incubation of the embryos in saline containing BUdR for two periods of DNA replication was roughly estimated taking into account some previous work on the rates of DNA replication and the lengths of the replicating units in Triturus (Callan, 1972). Late blastulae/early gastrulae were thought to have a cell cycle of less than 4 hours, while the cell cycle duration in neurulae was estimated at greater than 4 hrs. Thus, in order to look at mitotic metaphase cells which had replicated their DNA twice in the presence of BUdR and arrested at the second metaphase by treatment with colchicine, late blastulae/early gastrulae should have a total incubation time in BUdR of 8 - 9 hours, and neurulae 9 - 11 hours. Both of these estimates allow for the slight increase in cell generation time caused by the BUdR. After some preliminary experiments when neither differential fluorescence with 33258 Hoechst nor differential Giemsa staining was observed in embryonic metaphase cells after incubation

TABLE IV - 1. - To show the lengths of time spent in FUdR + uridine, BUdR, and colchicine by the embryonic stages of T.c. carnifex. Numbers refer to time in hours.

Embryo stage number (after Harrison)	Embryo stage	FUdR (1 µg/ml.) + uridine (0.5 µg/ml)	BUdR		BUdR + colchicine	Total time in BUdR (hrs)
			1 µg/ml.	2 µg/ml.		
7 - 8	Blastula		6		3¼	9¼
9 - 10	Gastrula		6		4	10
9 - 12		2		6	3	9
10 - 11		2		6 1/3	3	9 1/3
12 - 16		2		6 1/3	3	9 1/3
13 - 15		1		5	6	11
14 - 16	Neurula	1		6	4	10
14 - 20		2		6	4	10
15 - 18			5½		4¼	9 3/4
15 - 18		1		6¼	4	10¼
15 - 19		1		6¼	4	10¼

During the course of these experiments, I was given further information concerning the cell cycle duration in Triturus embryos (Pamela McFarlane, personal communication) which were considerably longer than previously assumed. Late blastulae cells were estimated to have a generatinn time of 4½ hrs, and neurulae, 8 hours or longer. Therefore, for the next series of experiments performed on T. vulgaris embryo cells, I increased the times of incubation in BUdR.

TABLE IV - 2. - To show the lengths of time spent in FUdR + uridine, BUdR and colchicine by the embryonic stages of T. vulgaris Numbers refer to time in hours.

embryo stage number (after Harrison)	Embryo stage	FUdR (1 µg/ml.) + uridine (0.5 µg/ml.)	BUdR 10 µg/ml.	BUdR + colchicine	Total time in BUdR (hrs)
9	Gastrula	1	7½	2	9½
10 - 11		1	9	2	11
13 - 14		1	16	2	19
14 - 17	Neurula	1	18½	5	23½
15 - 16		1	17	3	20
18 - 19		1	18½	5	23½

After the period of colchicine treatment, the cells were fixed in freshly prepared 3:1 and squash preparations of the cells on slides were made as described in Chapter I. Dehydrated and air-dried preparations of cells were stained by the FPG technique of Perry & Wolff (1974) in the manner described above.

with BUdR, the cells were treated with FUdR to enhance the uptake of BUdR and to reduce the effect of the large endogenous thymidine pools of the embryonic cells which depresses BUdR uptake.

FUdR, uridine and BUdR solutions were made up in FSS to the required concentrations. The experimental regime followed is summarized in tables IV - 1 and IV - 2.

(iv) EM analysis of chromatin packing in 'normal' and BUdR-treated T.c. carnifex tissue culture cells.

Cultures of T.c. carnifex cells were grown as described in Chapter II. Some of the cells were cultured in medium containing BUdR (20 µg/ml.) for 8 days. The last 18 hrs of the culture period of all cells were spent in medium containing colchicine (1 µg/ml.). (The fixation and dehydration of the cells was performed in 15.0 ml. conical Pyrex centrifuge tubes; thereafter the cells were embedded in plastic "Beem" capsules. Normal and BUdR-treated cells were fixed, dehydrated and embedded identically. After each step in the process the cells were pelleted by centrifugation at 1000 rpm for 10 mins and the supernatant was decanted off). The cells were harvested by trypsinization and fixed in buffered 2.5% gluteraldehyde (90 ml. 0.02 M phosphate buffer, pH 7.8 + 10 ml. 25% gluteraldehyde + 0.6 gm. sucrose) for 30 mins, rinsed twice in washing buffer (100 ml. 0.02 M phosphate buffer pH 7.8 + 2 gm. sucrose), then fixed in buffered osmium (5 mls of (100 ml. 0.02 M phosphate buffer pH 7.8 + 4 gm. sucrose) + 5 ml. 2% osmium)) for 30 mins, then dehydrated through 50%, 60%, 70%, 80% and 96% ethanol, each for 10 mins, then absolute ethanol for 1 hour and propylene oxide for 1 hour. The cells were embedded in 2 parts propylene oxide : 1 part Araldite for 30 mins, 1 part propylene oxide : 2 parts Araldite for 30 mins, then in "full" Araldite at room temperature for 4 hrs. The Araldite was then polymerized at 60°C for 2 days. Sections of the embedded cells were cut on

an LKB Ultratome III and mounted on copper grids. The grids were stained in a saturated solution of uranyl acetate in 50% ethanol for 2 hrs and in Reynolds' lead citrate (Reynolds, 1963) for 10 mins. The grids were examined with a Phillips 301 electron microscope at 60 kV.

3. RESULTS

(i) Analysis of SCE frequency in T.c. carnifex tissue culture cells.

Fig. IV - 1 illustrates the effect of increasing BUdR concentration on the SCE frequency shown by mitotic metaphase chromosomes of T.c. carnifex tissue culture cells. These cells were stained by the FPG technique of Perry & Wolff (1974). Over a hundred-fold increase in BUdR concentration (1 μg/ml. to 100 μg/ml. or 3.25 μM to 325.0 μM) the SCE frequency rises linearly from about 20 exchanges per cell at 1 μg/ml. BUdR to about 50 exchanges per cell at 100 μg/ml. BUdR. The regression line cuts the ordinate at about 20 SCEs per cell, which probably reflects the spontaneous exchange frequency. However, the number of SCEs seen in a set of mitotic chromosomes at the second metaphase (M_2) after culture in medium containing BUdR for the two preceding periods of DNA synthesis (S_1 and S_2) includes those exchanges which occurred during (or shortly after) S_2 as well as half the number of exchanges which occurred during (or shortly after) S_1. The latter exchanges are seen because each M_2 cell contains all of the DNA synthesized and SCEs formed in S_2, but only half of the DNA and therefore half the exchanges generated in S_1 in its parent cell. Thus, the number of exchanges seen at M_2 is approximately 33% more than the actual number of exchanges that occurred in S_2, providing that the rate of SCE formation remains constant from one cell generation to another. However, Miller et al. (1976) have found that the latter assumption is not valid for some mammalian cell

lines grown in the presence of BUdR, where approximately twice as many exchanges occur during S_1 as occur during S_2. In T.c. carnifex then, the value of 20 exchanges/cell cycle/diploid cell which is thought to measure the spontaneous incidence of SCEs is an overestimate; 10 - 14 exchanges/cell cycle/diploid cell is probably nearer the actual frequency. Figures IV - 2 to IV - 5 illustrate the staining behaviour of metaphase cells grown in medium containing BUdR (concentrations given in figure legends) and stained by the FPG technique. The pale-staining chromatids (bifilarly substituted) in fig. IV - 3b show considerable under-condensation when compared to their darkly-staining sisters (monofilarly substituted). The chromosomes from cells grown in high concentrations of BUdR (100 μg/ml.) shown in figs. IV - 4a and 4b have a high frequency of SCEs, and also show structural aberrations, notably chromatid breaks. These breaks occur at the point of sister chromatid exchange and sometimes result in a joining of two of these breaks between non-homologous chromosomes. Two such figures are shown in figure IV - 4b (arrowed). Identical patterns of staining are sometimes seen in short stretches of sister chromatids. Examples of this are shown in fig. IV - 5a and 5c. This type of staining is analogous to the "isolabelled" segments seen in tritium autoradiographs. However, in this case, the apparent staining behaviour is due to the difficulty with which the end region of one chromatid can be identified when bifilarly substituted with BUdR and therefore staining weakly with Giemsa. Thus, the phenomenon of "isostaining" is only apparent and not real.

Although Carrano & Wolff (1975) have found that SCEs are distributed at random in the chromosomes of the Indian Muntjac, some of the chromosomes of T.c. carnifex occasionally show many more exchanges than do other chromosomes in the same set (fig. IV - 5b and 5c).

(ii) Analysis of SCE frequency in T.c. carnifex and T. vulgaris embryo cells.

I have been unable to obtain either differential fluorescence with 33258 Hoechst or differential Giemsa staining of the mitotic metaphase chromosomes from T.c. carnifex and T. vulgaris embryo cells which had been cultured for an estimated two periods of DNA synthesis in saline containing BUdR. When these cells were stained with 33258 Hoechst and examined by UV-microscopy, the chromosomes showed a uniformly bright fluorescence. Similarly, when the cells were stained with Giemsa after being heated to 60°C for 2 hours, both chromatids stained uniformly. An additional problem was encountered when the embryonic cells were squashed in 45% acetic acid prior to making permanent preparations of these cells on slides. Squashing the material resulted in manh fragmented chromosome sets, with very few metaphase complements remaining intact. There were also very few metaphase sets visible in most preparations, probably due to the adverse effects of BUdR on embryonic cells. BUdR has been shown to inhibit differentiation of specific cell types and prevents normal development in sea-urchin embryos (Kotzin & Baker, 1972). Thus, treatment of the embryos with FUdR coupled with their sensitivity to BUdR might have showed the synthetic activities of the cells to such an extent that they had only undergone one round of DNA synthesis before colchicine treatment and fixation.

(iii) Chromatin packing in 'normal' and BUdR-treated T.c. carnifex tissue culture cells.

Fig. 6a shows some Giemsa-stained T.c. carnifex cells which have been grown in medium containing BUdR for two periods of DNA synthesis, then treated according to the FPG technique of Perry & Wolff (1974). These cells are from an air-dried preparation. Most of the cells stain uniformly mauve with a deeper staining network of chromatin within the nucleus, and bright

pink chromocenters (one cell in fig. IV - 6a). When fixed and preapred for electron microscopy, these cells can be seen to have the internal organisation and structure characteristic of most eukaryote fibroblast cells (fig. IV - 6b). The cytoplasm of the cell contains an extensive network of endoplasmic reticulum with attached ribosomes, mitochondria and other cytoplasmic inclusions. The nucleus is often lobed (not shown in fig. IV - 6b) with a layer of highly condensed chromatin next to the nuclear membrane, as well as other condensed areas of chromatin in the nuclear interior.
Figures IV - 7 to 11 compare the chromatin packing in interphase and metaphase nuclei from normally-cultured cells (top photographs) and cells grown in medium containing BUdR for two periods of DNA synthesis (bottom photographs). In general, interphase nuclei from both "normal" and BUdR-treated cultures can vary quite widely in general form. Either type of culture can contain cells with more or less smoothly spherical nuclei (fig. Iv 6b and 7b) at one end of the scale or nuclei with several lobes and indentations (fig. IV - 7a) at the other. Fig. IV 7 b shows a nucleus from a BUdR-treated cell culture. In this nucleus, the highly condensed chromatin is restricted to a sheet in the central portion of the nucleus with very little being associated with the nuclear membrane. This type of chromatin distribution is, however, atypical. Figs. IV - 8a and 8b compare the chromatin compaction in the nuclei from 'normal' and BUdR-treated cells. There appears to be a greater amount of highly condensed chromatin associated with the nuclear membrane in 'normal' nuclei (fig. IV - 8a) than in nuclei from BUdR-treated cells (fig. IV - 8b) but this is probably because the highly condensed chromatin in the latter cells is rather more tightly packed. This difference is chromatin packing is not necessarily due to incorporated BUdR but could reflect the stage in interphase that the cell had reached at the moment of fixation.

The structure of metaphase chromosomes from 'normal' and BUdR-treated cells is very similar, the only difference being the presence of regions of differentially condensed chromatin along many of the chromatids from BUdR-treated cells (fig. IV - 10b). Chromosomes from BUdR-treated cells do not show any differential packing of the chromatin between sister chromatids which would correspond to the substitution of BUdR for thymine residues in one or both DNA strands. Although it is impossible to identify the metaphase cells in terms of the number of rounds of replication their DNA has undergone in the presence of BUdR (either one or two after eight days of culture) I have looked at sections of the chromosomes from nine different metaphase cells and none of them shows differences in chromatin packing between sister chromatids. After eight days of culture in medium containing BUdR, about 80% of the metaphases seen following colchicine treatment have undergone 2 rounds of DNA replication. Thus, by the methods I have used to fix and stain cells for electron microscopy, no difference in chromatin packing between sister chromatids from cells grown in the presence and absence of BUdR can be observed.

4. DISCUSSION

The increase in SCE frequency in T.c. carnifex tissue culture cells (C = 29 pg) grown for two periods of DNA replication in medium containing increasing concentrations of the thymidine analogue, BUdR, is greater (20 at 1 µg/ml. - 50 at 100 µg/ml.) than has been observed over the same range of concentrations of BUdR in human lymphocytes (C = 3.65 pg) (11 at 1.8 µg/ml. - 18 at 28.9 µg/ml. (Latt, 1974a); 27.3 at 200 µg/ml. (Dutrillaux et al. 1974), Chinese hamster D-6 cells (C = 8.3 pg) 2.3 at 0.1 µg/ml. - 12.7 at 40 µg/ml. (Kato, 1974d), CHO cells (7.9 at 0.08 µg/ml. - 15.4 at 6.1 µg/ml. (Wolff & Perry, 1974)) and Vica faba root tips (c = 24 - 28 pg) (20.6 at 30.7 µg/ml. (Kihlman & Kronborg, 1975)).

The results of the above studies suggest that there is no direct relationship between SCE frequency as estimated by the FPG technique, and the DNA content of the cell. However, longer chromosomes have more SCEs than do shorter ones in the same cell (Taylor, 1958; Carrano & Wolff, 1974; Kihlman & Kronborg, 1975). This is in keeping with the idea that the SCE frequency may be related to the number of replication initiation sites in the chromosomes, which are characteristic for each eukaryote species and are not related to C-value.

The numbers of chromosomal aberrations and SCEs is known to increase after exposure of cells to UV-light. When mammalian cells in culture are irradiated with UV light a certain amount of "unscheduled" and non-semi-conservative DNA replication is observed (e.g. Kofman-Alfaro & Chandley, 1971; Chandley & Kofman-Alfaro, 1971). This DNA synthesis is associated with the repair of lesions induced in the DNA by UV light. Such lesions only result in exchanges when the cells pass through another S-phase (Rommelaere *et al.*, 1973; Kato, 1973; Wolff *et al.*, 1974). This would seem to suggest that SCEs arise as a result of post-replication repair of DNA damage. Repair synthesis is much increased if the DNA thymine is replaced by bromouracil (Rommelaere *et al.*, 1974). UV-irradiation of BUdR-substituted DNA results in the formation of a uridylic radical accompanied by a single chain break; the subsequent repair synthesis involves closure of the chain breaks and a limited degradation and resynthesis of DNA before the strand is sealed. In excision-defective bacteria, Rupp *et al.* (1968) have suggested that UV-induced pyrimidine dimers in replicating DNA induce the formation of gaps in the daughter strands and that these gaps are repaired by a recombinational event involving pairing between the normal and defective daughter strands. Repair synthesis would correct the defective DNA strand using the corresponding intact region of the daughter strand as a template. Fujiwara (1972) has suggested that this type of mechanism could operate in mammalian

cells to repair UV-induced lesions in the DNA. However, such a mechanism depends on the occurrence of single strand exchanges between DNA duplexes in sister chromatids. If this occurred in cells grown for one S-period in the presence of BUdR so that each chromatid contained one BUdR-sbustituted DNA strand (B) and thymine in the complementary DNA strand (T) (each chromatid = BT), any single strand exchanges would result in chromosome regions composed of TT- and BB-containing DNA. These regions would be recognized at the first metaphase after labelling with BUdR (M_1) because TT regions would stain deeply with Giemsa and BB regions would stain lightly. Such staining behaviour is never seen. The formation of a double strand break in the region of sister chromatid exchange is supported by my observation that in T.c. carnifex tissue culture cells, chromosomal abberrations resulting in breakage and rejoining of non-homologous chromatids almost always occur at the points of sister chromatid exchange (fig. IV - 4b). Both of the aberrant chromosome figures in fig. IV - 4b (arrowed) have a small piece of bifilarly substituted chromatid (palely stained) associated with them, which must have been excised from its original location.

Moore & Holliday (1976) have found that hybrid DNA is formed when Chinese hamster cells are grown in medium containing BUdR for one round of replication and then transferred to normal medium. Moore & Holliday found that the amount of hybrid (BB) DNA increased when increasing concentrations of mitomycin C, a DNA crosslinking agent, was added to the medium. The addition of mitomycin C gave a corresponding increase in the numbers of SCEs scored at metaphase. Rommelaere and Miller-Faurés (1975) have also found a correspondence between the presence of hybrid DNA and the formation of SCEs.

The conflicting evidence concerning the formation of hybrid DNA associated with sister chromatid exchanges (Rommelaere & Miller-Faurès,

1975; Moore & Holliday, 1976) yet the inability to detect this DNA by the FPG technique, can be resolved, in part, if it be assumed that the hybrid DNA is formed as a result of two single strand exchanges, each of which occurs between one DNA strand of one chromatid and the strand of the same polarity on its sister. One of the two exchanges would occur between DNA strands going in one direction while the second exchange would occur between DNA strands going in the other. The region between the two exchanges, after rejoining of DNA strands, would be composed of hybrid DNA. The distance between the two exchanges would have to be below the resolution of the light microscope and thus undetectable after staining by the FPG technique. This type of recombinational mechanism has been proposed by Holliday (1964) as the basis for the formation of meiotic exchanges but there is no reason why it should not also occur as part of the events leading to mitotic exchange. Moore & Holliday (1976) have estimated that the regions of hybrid DNA formed in Chinese hamster cells are 15,000 - 20,000 base pairs in length (5 - 7μ of DNA), yet the relationship they calculated between the number of SCEs observed and the amount of hybrid DNA formed suggests that these figures are 17 - 25 times too small. To resolve this discrepancy, Moore & Holliday suggest that the hybrid DNA formed in excess of that which could be accounted for by SCE formation might be due to a high frequency of non-reciprocal exchange.

A diploid cell of T.c. carnifex contains approximately 58 pg DNA or about 20 metres (1 pg = 9.5×10^8 base pairs; 3000 base pairs = 1μ). One T.c. carnifex chromosome I is 0.06 of the total length of all 24 chromosomes, therefore, assuming that chromatin packing does not vary widely between chromosomes, a single chromatid of one chromosome I contains 1.2 metres of DNA. If one SCE occurred on chromosome I, and the length of hybrid DNA formed as the result of a single SCE is 7 μm (200,000 base pairs) Moore &

Holliday, 1976) per single chromatid, this length is 5.8×10^{-6} of the total length of the DNA in the chromosome. Such a small fraction is indeed below the level of resolution of the light microscope!

Caffeine is known to inhibit post-replication repair in eukaryote cells therefore UV-induced lesions in the DNA cannot be eliminated in caffeine-treated cells. After caffeine treatment, UV-irradiated Chinese hamster cells show reduced survival (Rommelaere & Errera, 1972) while irradiation before caffeine treatment results in a decrease in SCE frequency but an increase in the frequency of deletion-type chromatid aberrations (Kato, 1973). This would seem to provide yet more support to the suggestion that SCEs occur during repair synthesis of the DNA and are therefore evident as chromatid aberrations if repair synthesis is inhibited. In contrast to these results, Kihlman (1975) found that Vicia faba root tips grown in the presence of BUdR have an increased aberration frequency when treated with caffeine, but the number of SCEs is not affected. Thus, it is not yet possible to describe the exact mechanism whereby SCEs are generated, although there is much evidence in favour of a recombination-like mechanism.

SCEs occur with a lower frequency than expected, on the basis of a Poisson distribution, in the heterochromatic regions of the chromosomes. Carrano & Wolff (1975) and Bostock & Christie (1976) have found that SCEs "expected" to occur within heterochromatic or C-band regions actually occur at the junction between C-band and non-C-band areas. I have found this to be generally true for T.c. carnifex. Figure IV - 12a and 12b compares the same set of metaphase chromosomes from a T.c. carnifex tissue culture cell grown in medium containing BUdR, after staining with 33258 Hoechst (fig. IV - 12a) and after heating for 2 hrs at 60°C followed by Giemsa staining

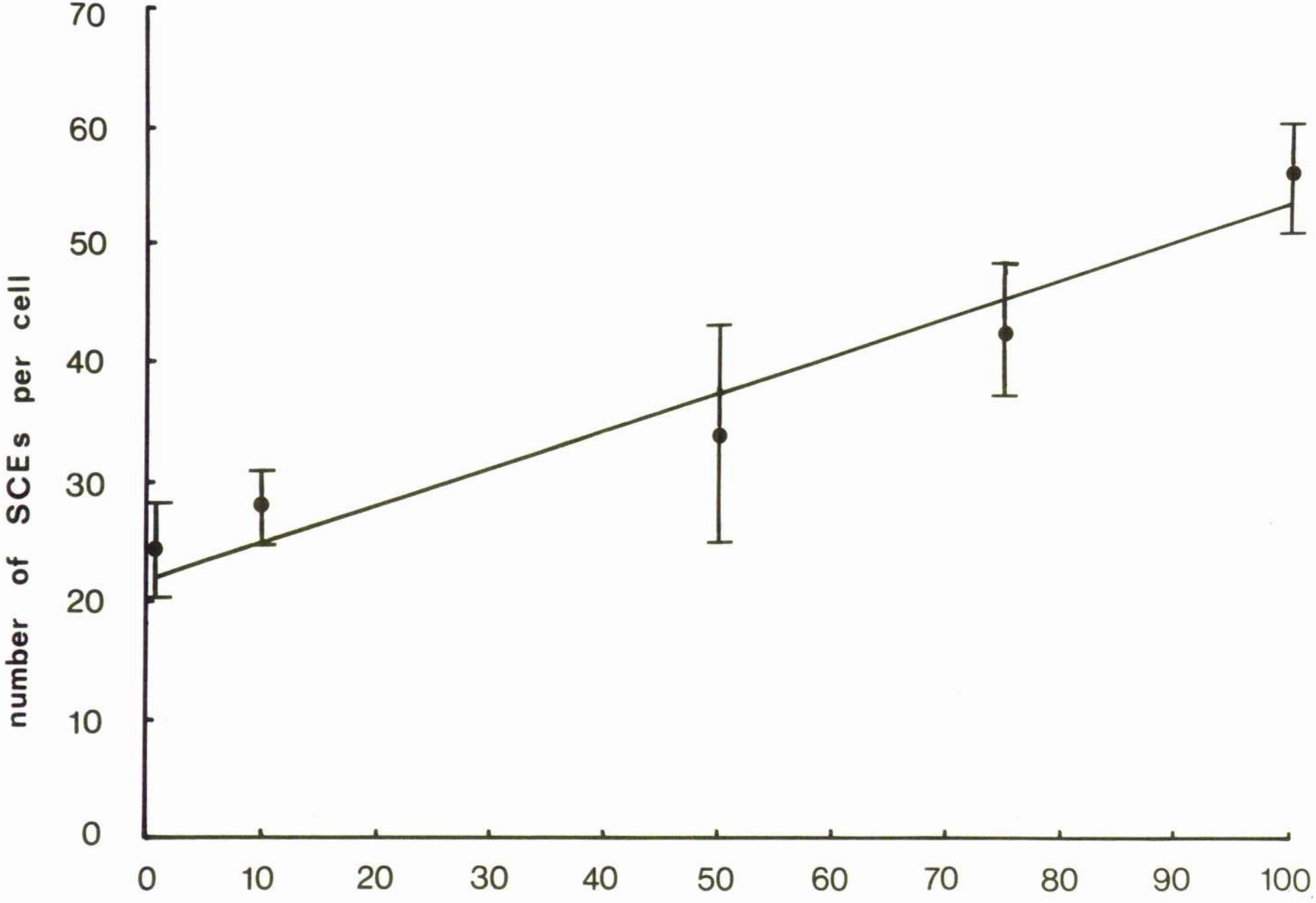
(fig. IV - 12b). A very faint differentiation between sister chromatids is visible after Giemsa staining (Fig. IV - 12b) which the most obvious feature of staining is the presence of the pericentromeric deeply-staining regions or G-bands. Only one exchange has occurred within any of the heterochromatic regions (arrowed).

The effect of BUdR on chromosome spiralization has been known for many years. Mammalian cells grown in medium containing BUdR have elongated centromeric regions and secondary constrictions, and have a high number of chromatid breakages which may or may not be translocated (Hsu et al., 1961). Human cells grown in medium containing BUdR have elongated secondary constrictions on chromosomes 1 and 9. (Kaback et al., 1964). Secondary constrictions have been induced in the mitotic chromosomes of T.c. carnifex by culture in medium containing BUdR (figs I - 10b and II - 2) in positions identical to those induced by culture at low temperature. Thus, BUdR inhibits spiralization in late replicating chromosome regions (Zakharov & Egolina, 1972) after one round of replication in its presence. At the second metaphase after labelling Chinese hamster cells with ^{3}H-BUdR for one DNA synthesis period, the labelled chromatids show a stronger spiralization delay (Zakharov & Egolina, 1972). An analogous result is shown in fig. IV - 3b where chromatids bifilarly substituted with BUdR are less spiralized or undercondensed compared to their monofilarly substituted sisters.

BUdR incorporated into the DNA is distributed uniformly between "repetitive", "intermediate" and "unique" sequences at concentrations where greater than 90% of the thymine residues have been substituted (100 µm BUdR), while at low concentrations (0.1 µg BUdR) is incorporated predominantly within the "repetitive" and "intermediate" DNA fractions (Schwartz et al., 1974). With the knowledge that BUdR-substitution into the DNA causes differential spiralization along whole chromatids, I expected to find some difference in the chromatin packing between monofilarly substituted and bifilarly

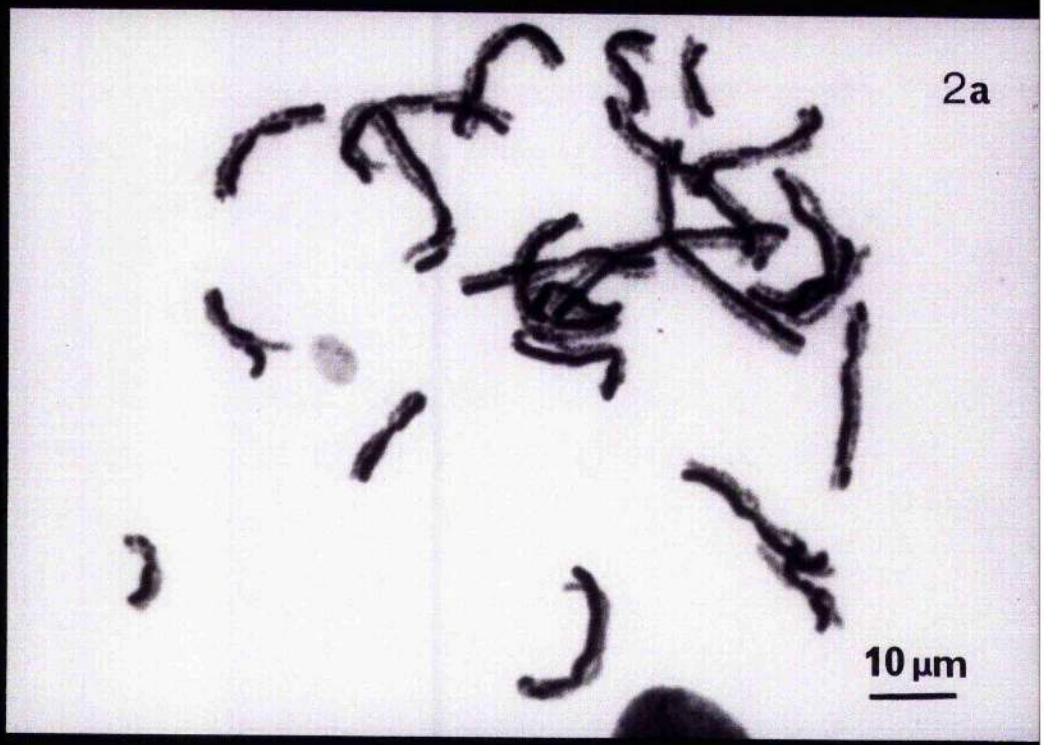
substituted chromatids by growing cells in medium containing BUdR at such a concentration (65 μM) that it ensured a high degree of thymine substitution throughout all classes of DNA sequence. I could not see any gross difference in the chromatin packing in metaphase chromosomes of T.c. carnifex cultured with the without BUdR. I have looked at the chromatin packing in about nine metaphase cells from BUdR treated cultures and the only differences in DNP packing that I have observed is that the peripheral regions of the chromosomes are generally less condensed and appear more "spotty" than do the chromosomes from normally-cultured cells. As I have mentioned before, my failure to observe differences in chromatin packing between sister chromatids could be because all of the metaphase cells I have looked at have been through only one round of DNA replication in the presence of BUdR. This could be elucidated by looking at more metaphase cells from BUdR-treated cultures.

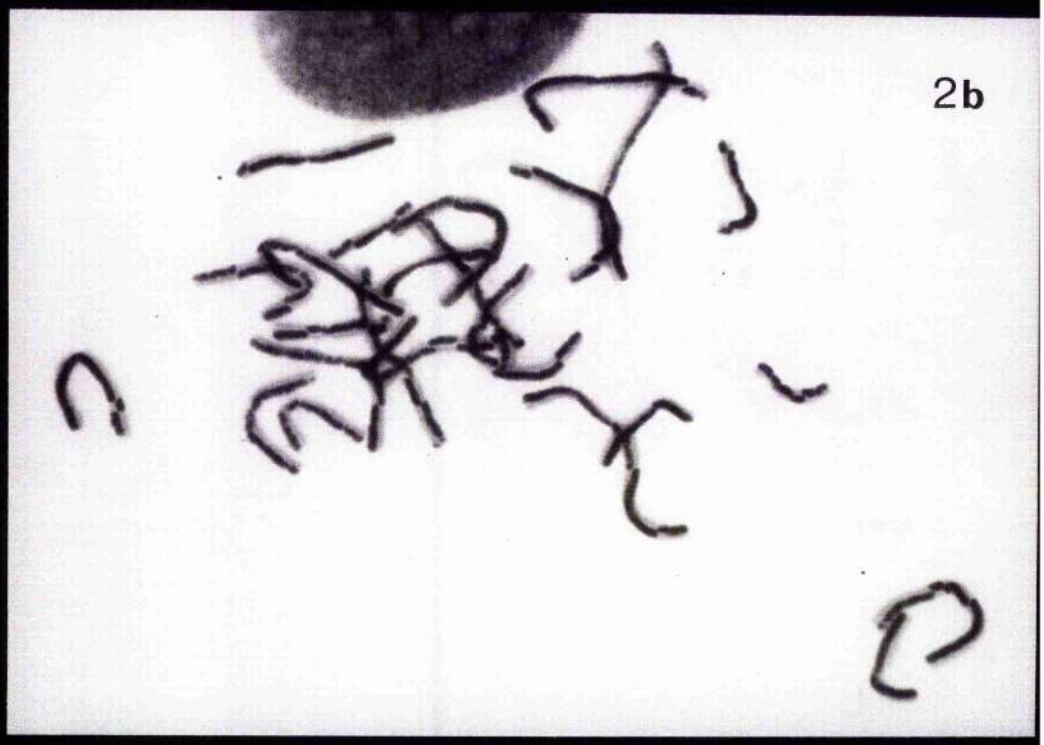
IV - 1. Graph to show the effect of increasing BUdR concentration on the number of sister chromatid exchanges seen in T.c. carnifex tissue culture cells. The regression line cuts the ordinate at about 20 exchanges per cell which probably reflects the spontaneous exchange frequency.


number of SCEs per cell
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100

IV - 2a. Mitotic metaphase chromosomes of a T.c. carnifex tissue culture cell grown in medium containing BUdR (1 μg/ml.) for 8 days, then stained by the FPG technique of Perry & Wolff (1974). Monofilarly substituted chromatids stain darkly while bifilarly substituted chromatids are pale. Points of sister chromatid exchange are clearly visible.

IV - 2b. As fig. IV - 2a.

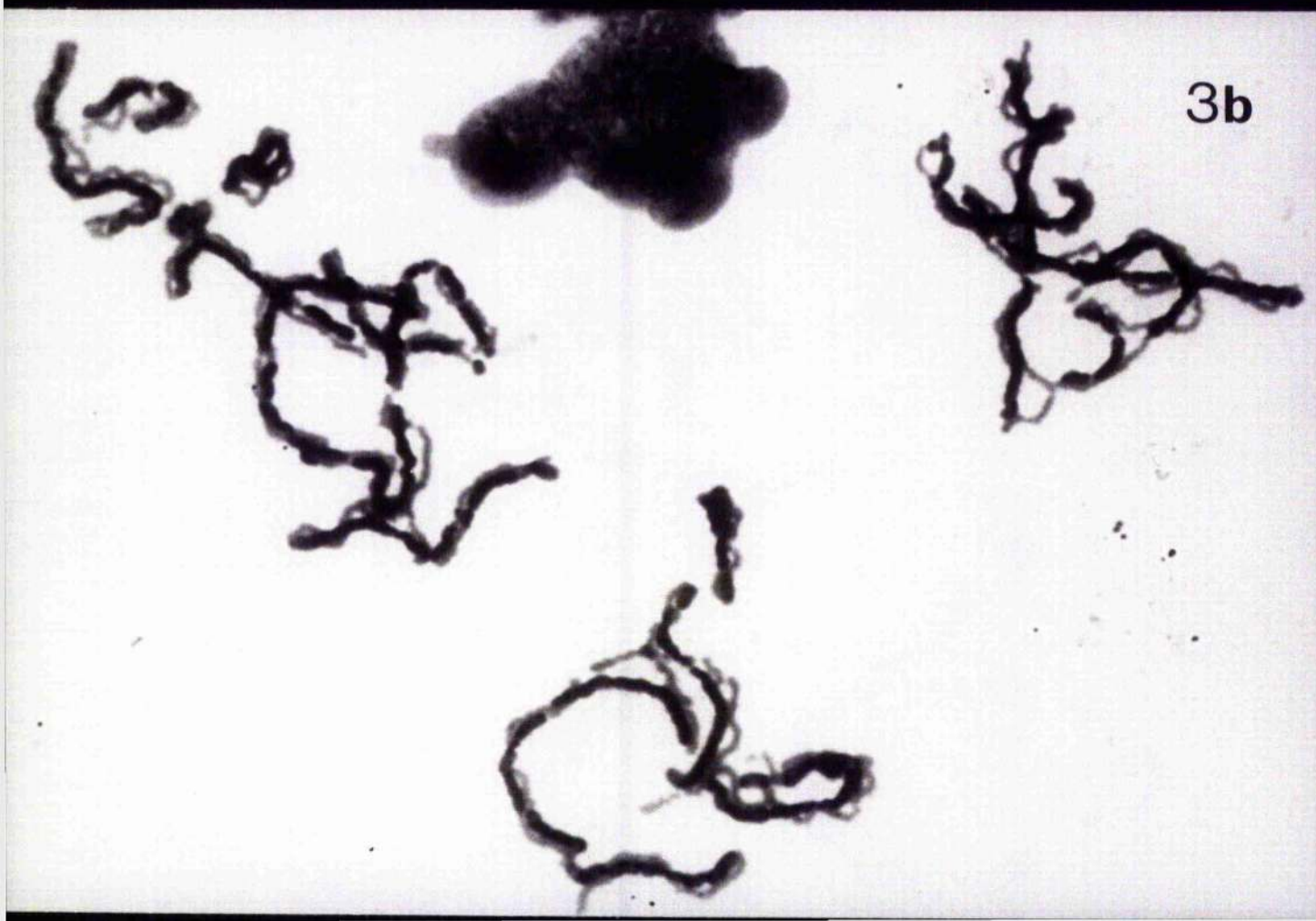

2a
10 µm


2b

IV - 3a. As fig. IV - 2a but the cells were grown in medium containing BUdR (10 µg/ml.).

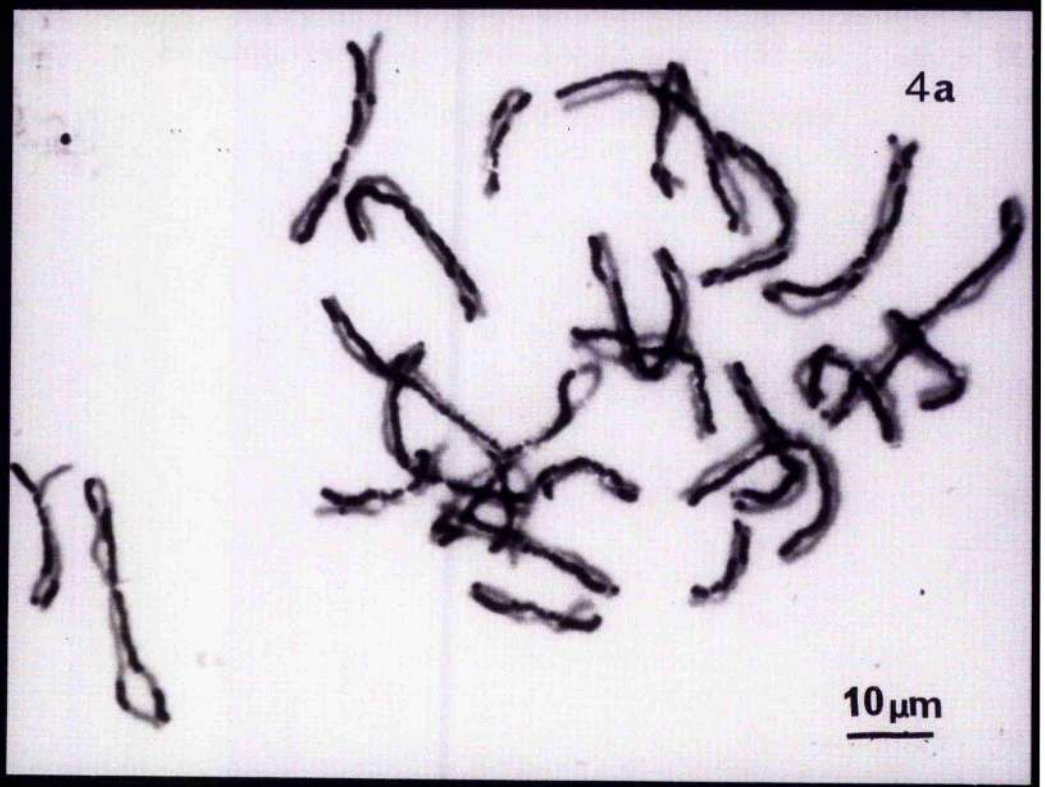
IV - 3b. As fig. IV - 2a but the cells were grown in medium containing BUdR (75 µg/ml.). Bifilarly substituted chromatids are less condensed compared to their monofilarly substituted sisters.

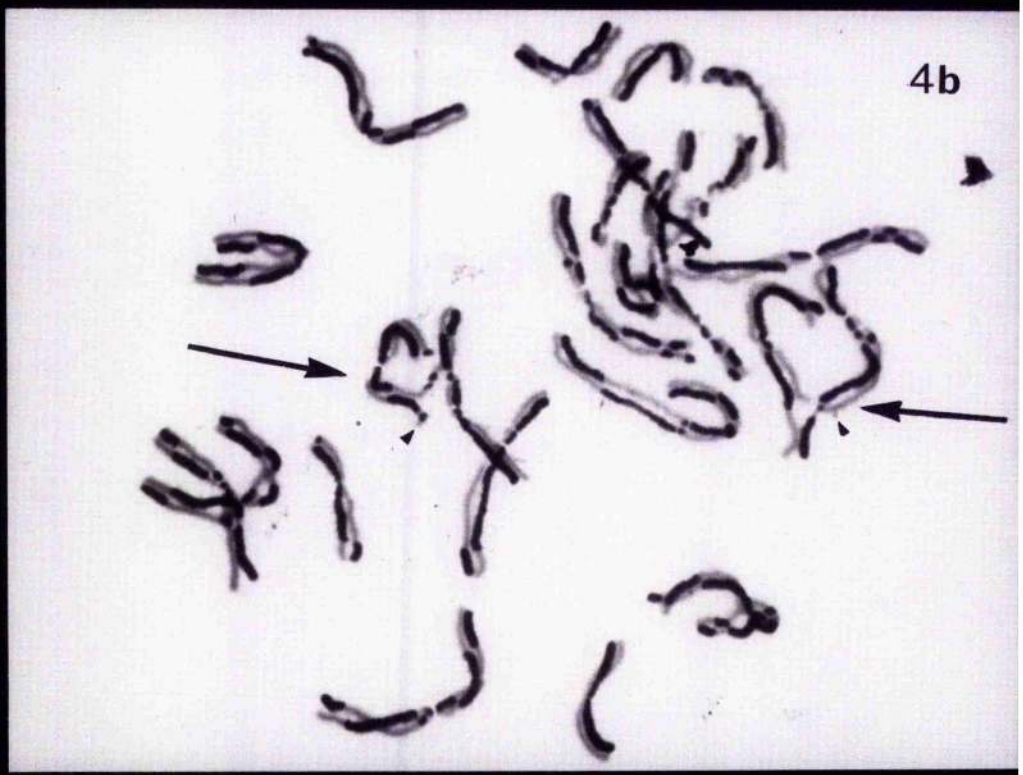

3a
10 µm


3b

IV - 4a. As fig. IV - 2a but the cells were grown in medium containing BUdR (100 μg/ml.)

IV - 4b. As fig. IV - 4a. Two aberrant chromosome figures (large arrows) have resulted from chromosome breakage at some points of sister chromatid exchange with subsequent rejoining between non-homologous chromatids. Some small pieces of bifilarly substituted chromatin have been excised from their original locations in these aberrant figures (small arrow heads).

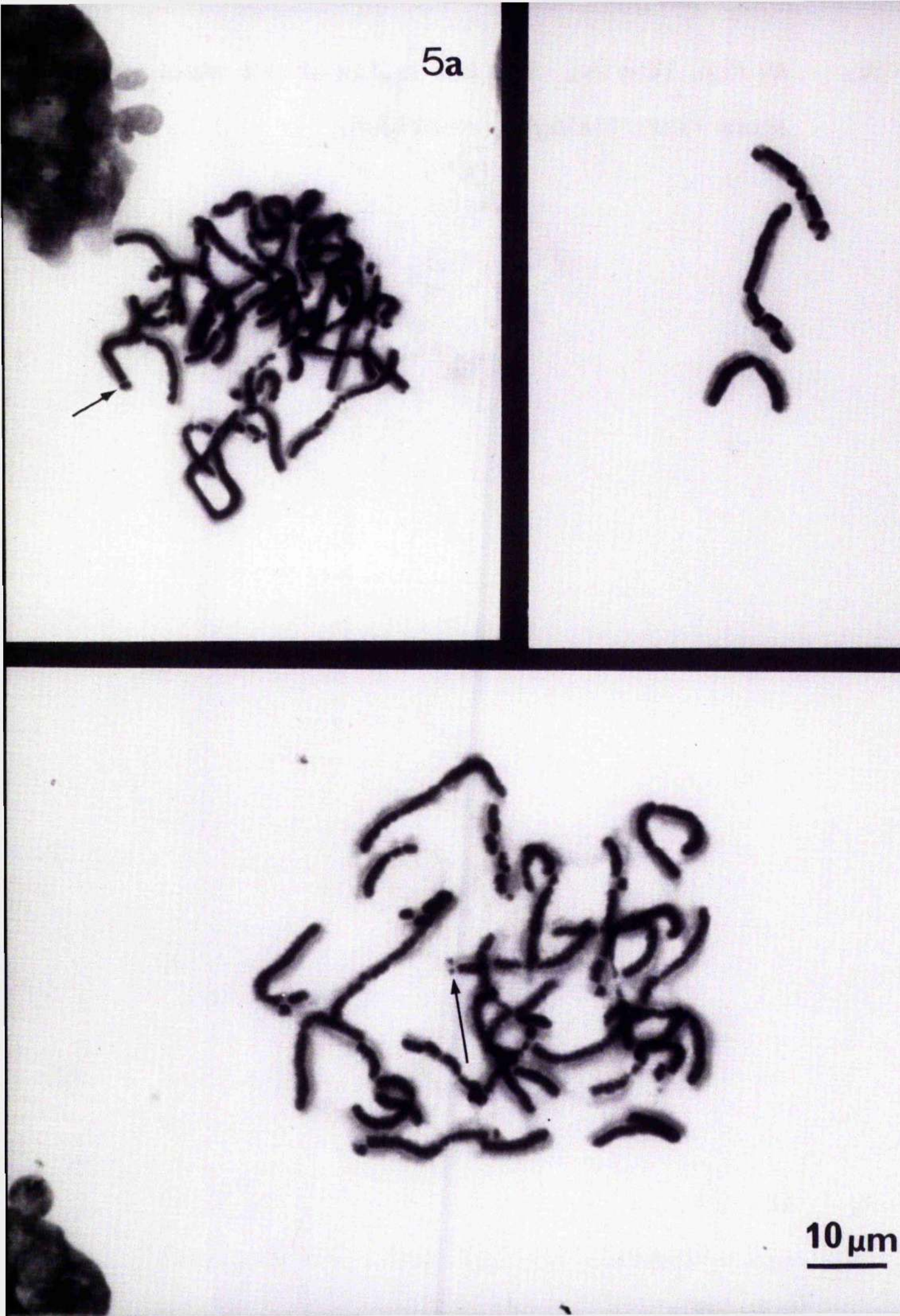

4a
10 µm


4b

IV - 5a. As fig. IV - 2a. The end region of one chromosome shows "isostaining" (arrowed).

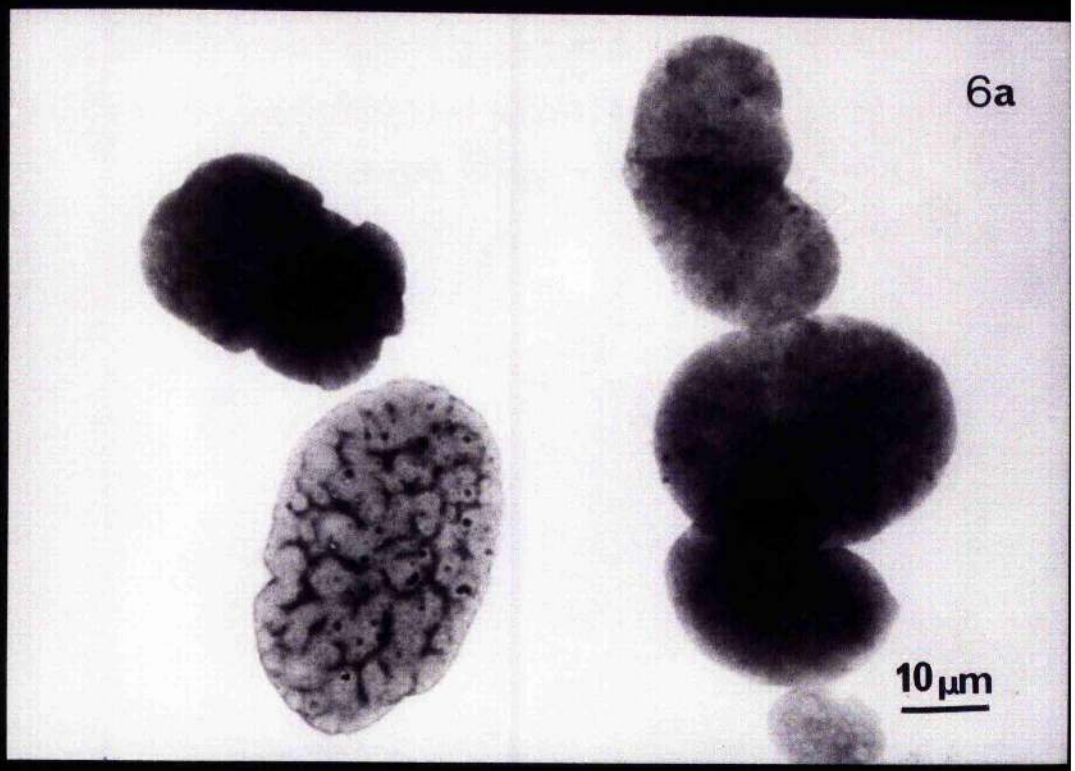
IV - 5b. As fig. IV - 2a. Some chromosomes of the same set show more exchanges (top chromosome) than others (bottom chromosome).

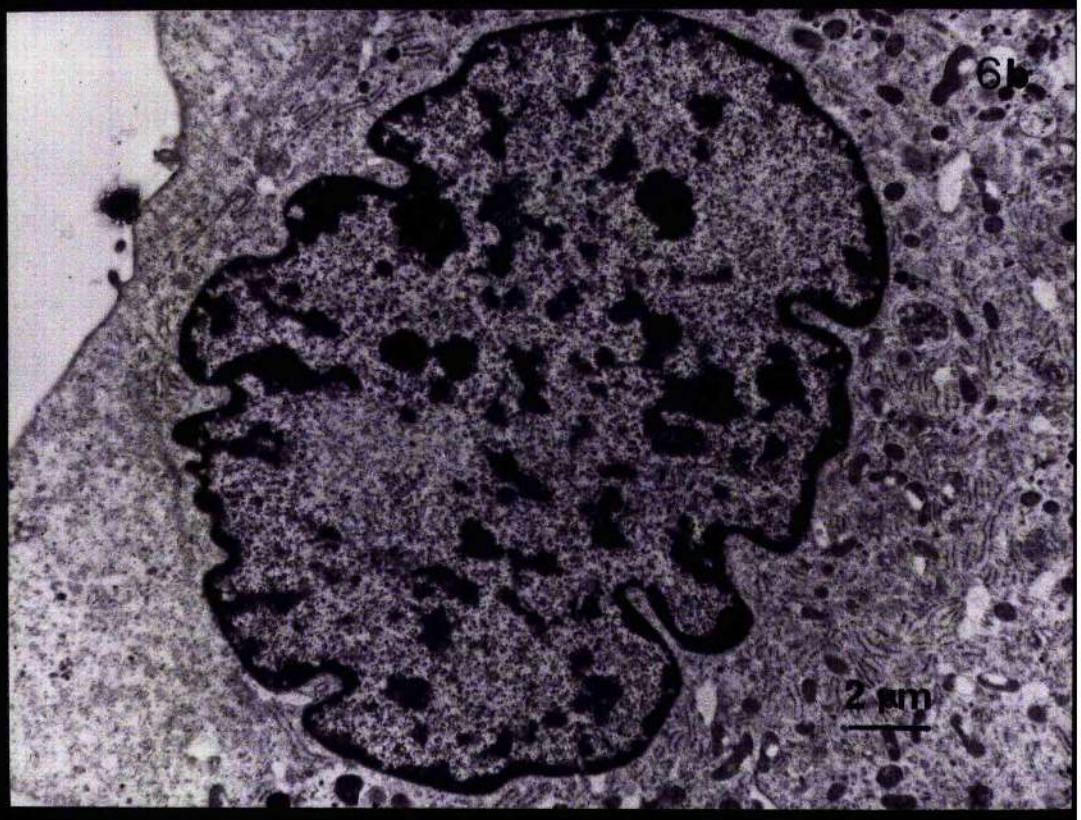
IV - 5c. As fig. IV - 2a. The end region of one chromosome shows "isostaining" (arrowed). For exaplnation see text.


5a
10 µm

IV - 6a. Interphase cells from T.c. carnifex tissue culture grown in medium containing BUdR (1 µg/ml.) for two periods of DNA synthesis and stained by the FPG technique of Perry & Wolff (1974).

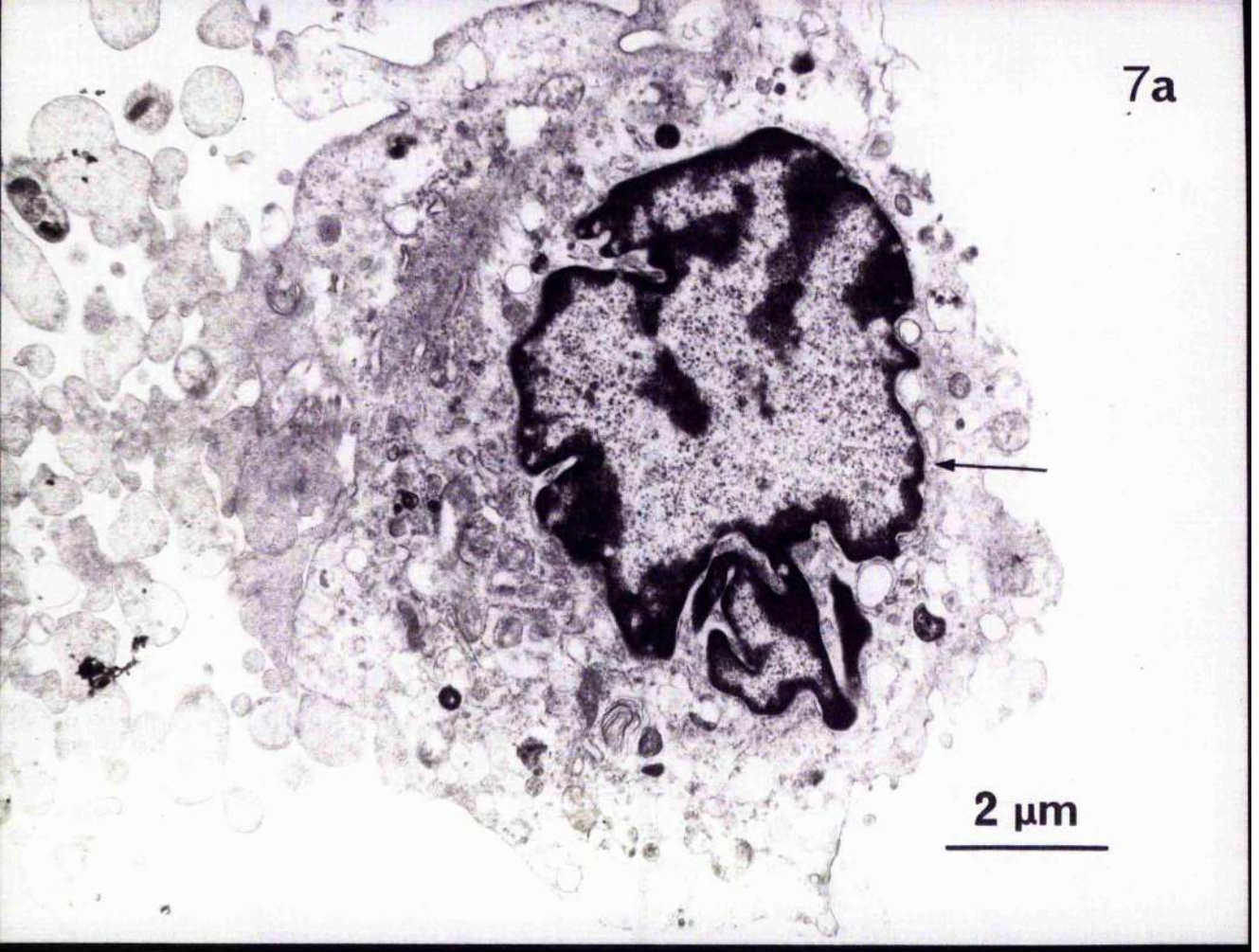
IV - 6b. Electron micrograph of a section through a T.c. carnifex tissue culture cell grown in medium containing BUdR (20 µg/ml.) for 8 days. Numerous mitochondria and endoplasmic reticulum with attached ribosomes can be seen in the cytoplasm. The nuclear chromatin is densely packed around the peripheral areas with other condensed regions in the nuclear interior.

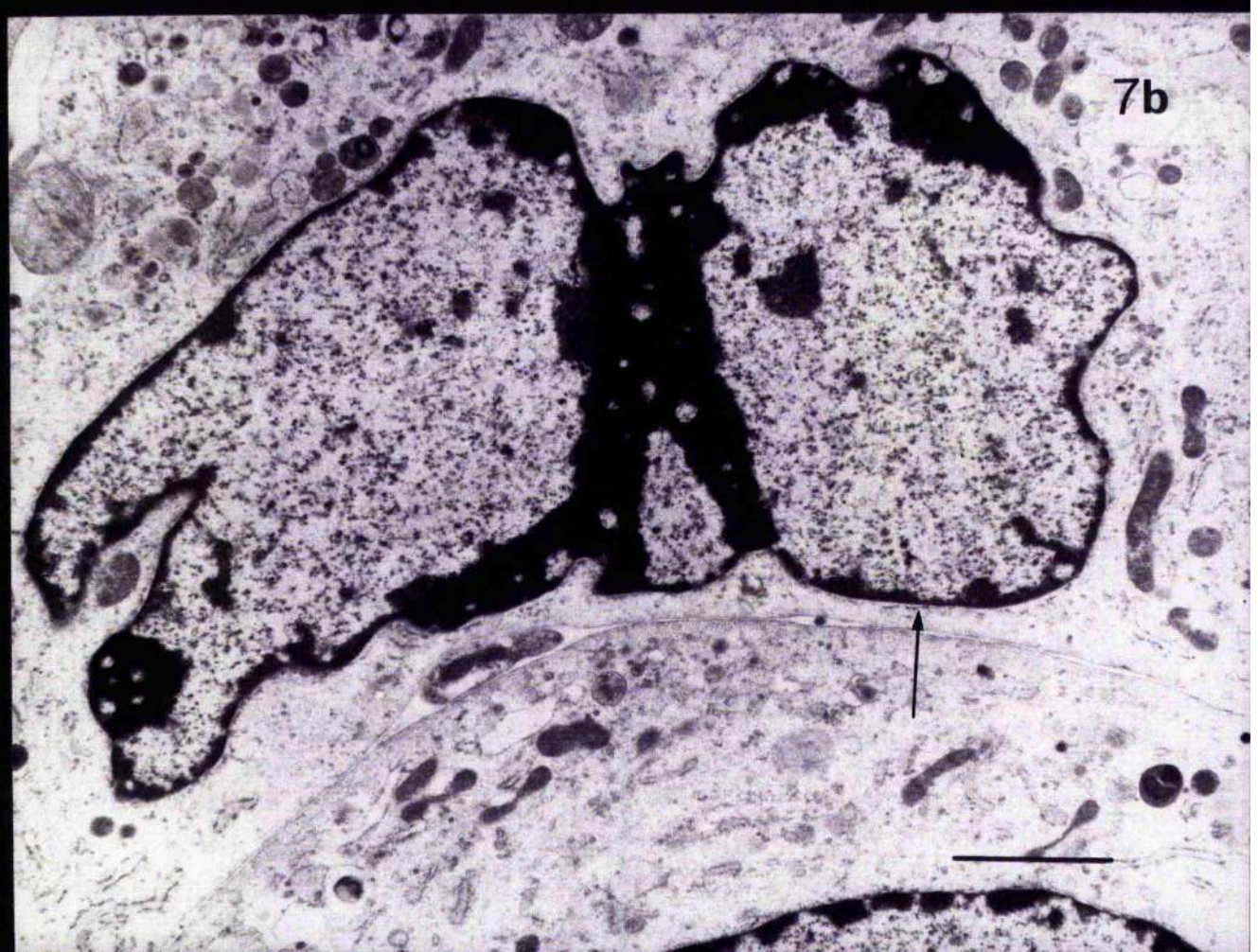

6a
10 µm


6b
2 µm

IV - 7a. Electron micrograph of a section of a T.c. carnifex tissue culture cell grown in normal medium for 8 days. n = nucleus.

IV - 7b. Electron micrograph of a section of a T.c. carnifex tissue culture cell grown in medium containing BUdR (20 µg/ml.) for 8 days. n = nucleus.


7a
2 µm

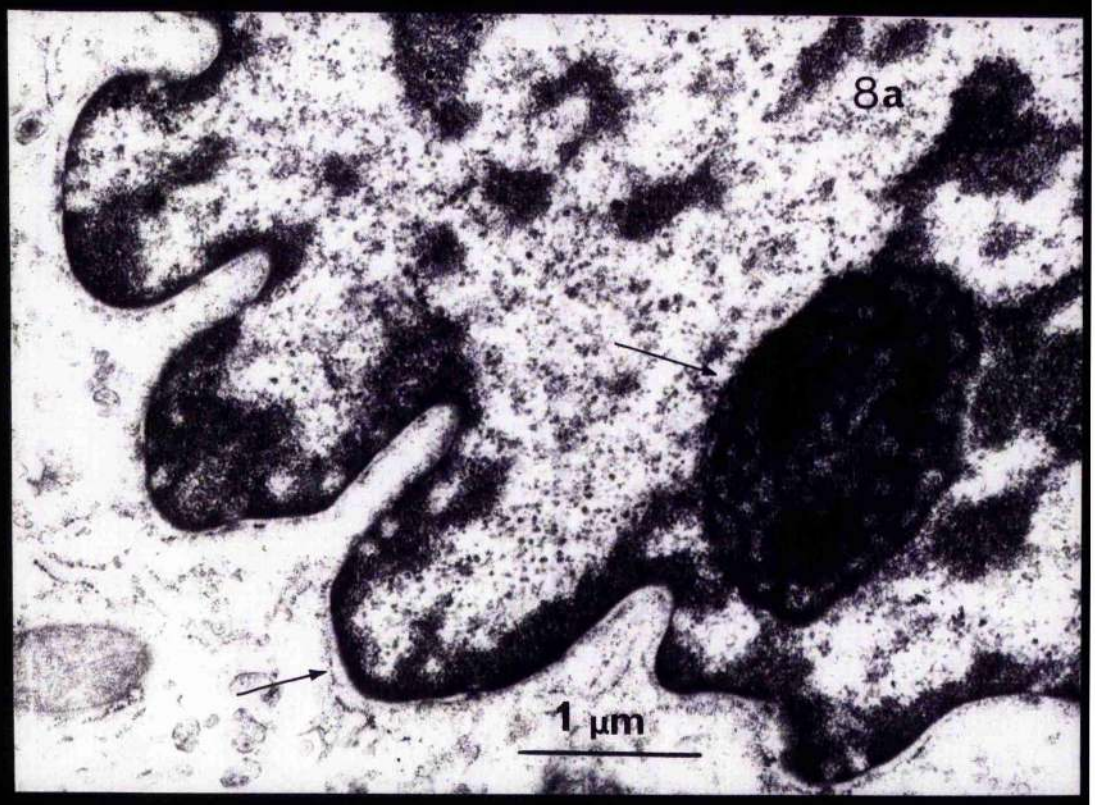

7b

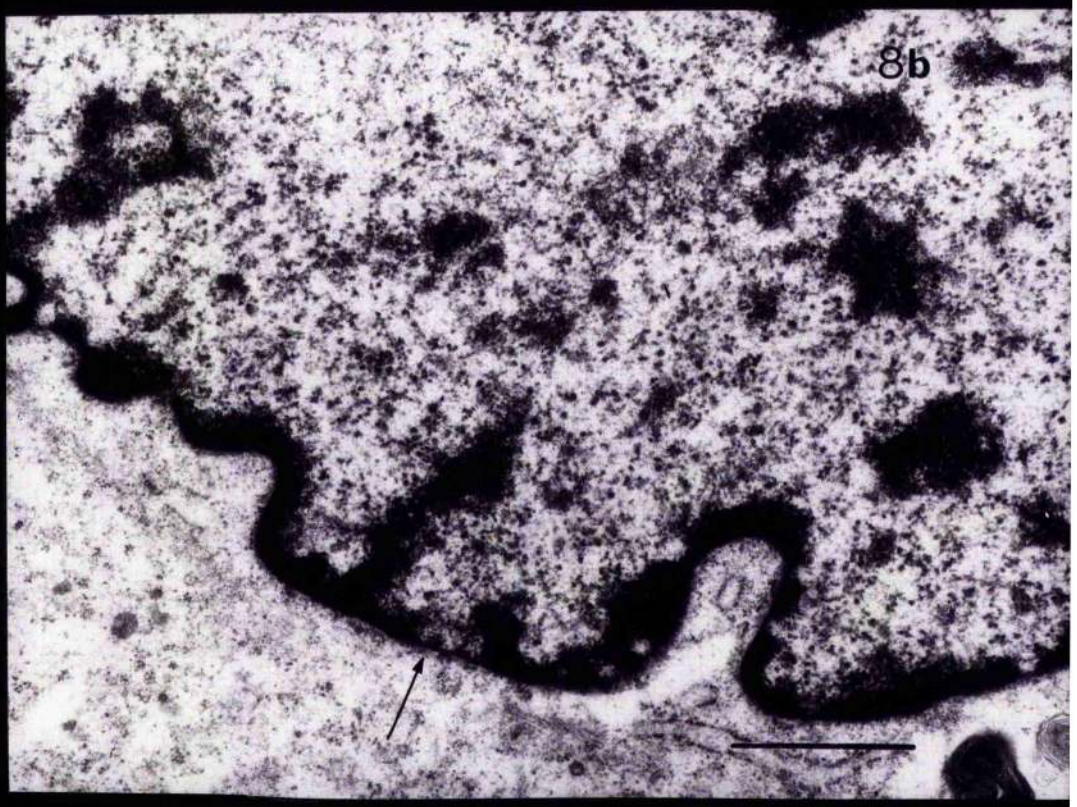
IV - 8a. As fig. IV - 7a but higher magnification.

N = nucleolus. nm = nuclear membrane.

IV - 8b. As fig. IV - 7b but higher magnification.

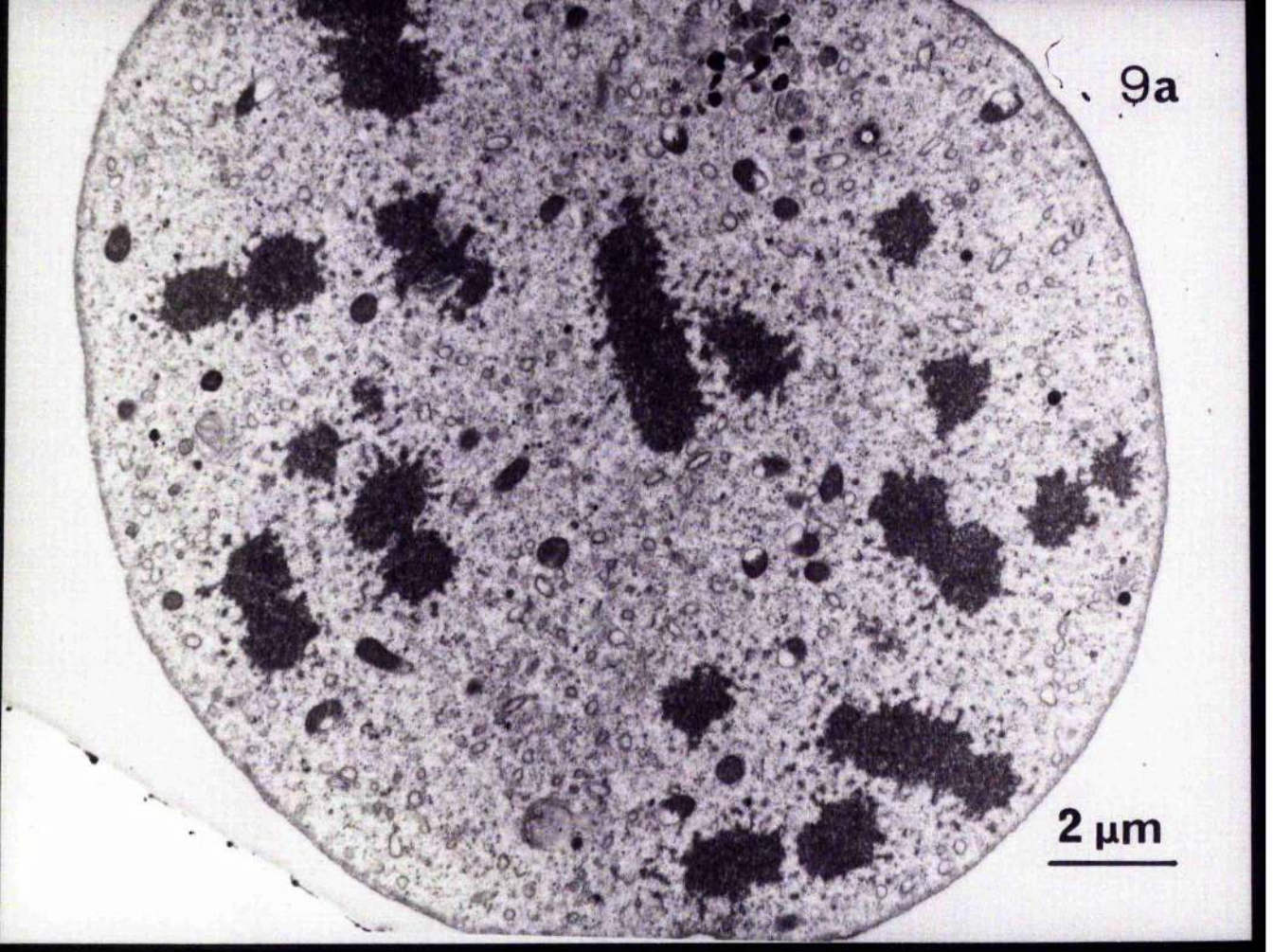
nm = nuclear membrane.

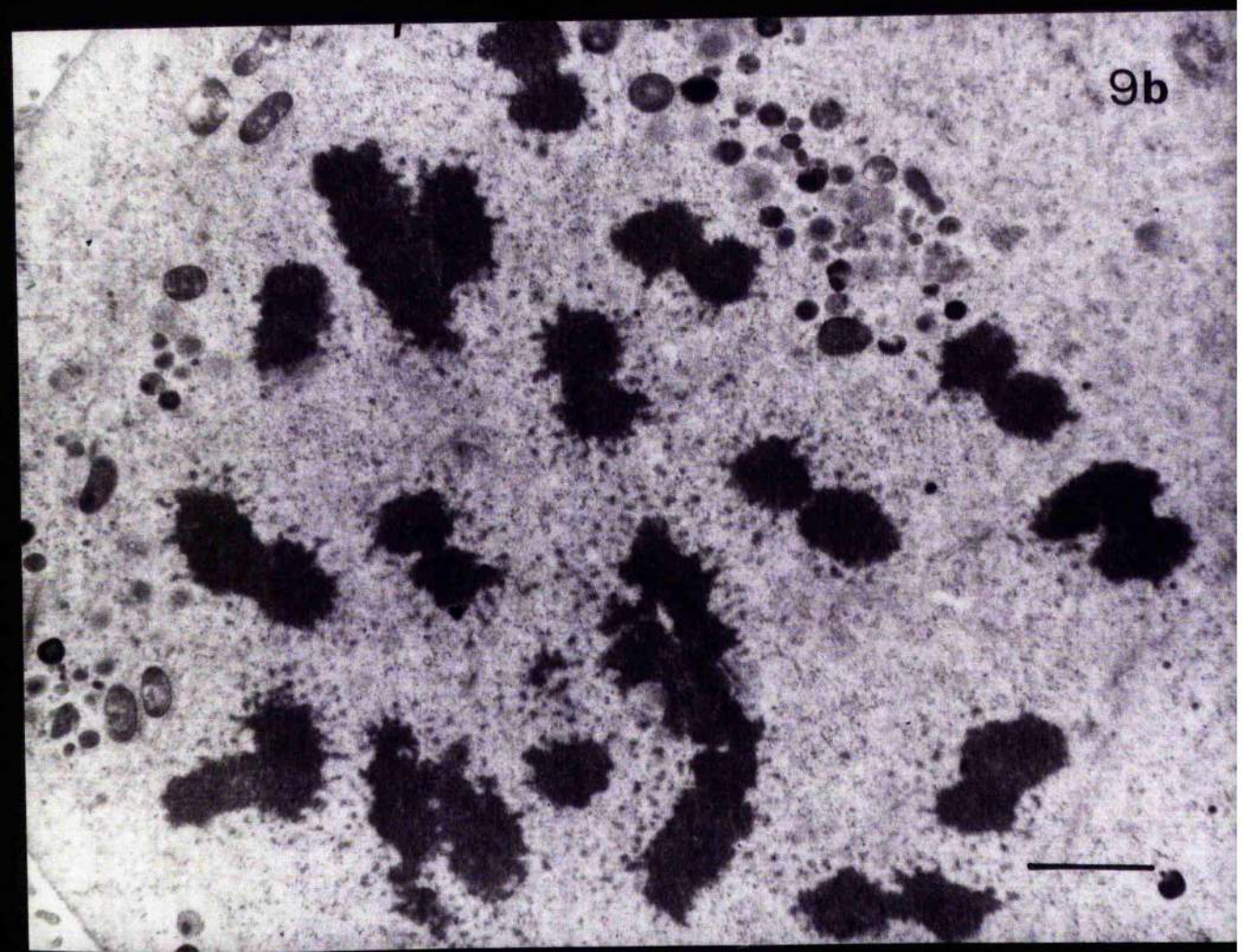

8a
1 µm


8b

IV - 9a. Electron micrograph of a section of a mitotic metaphase cell of T.c. carnifex tissue culture grown in normal medium for 8 days.

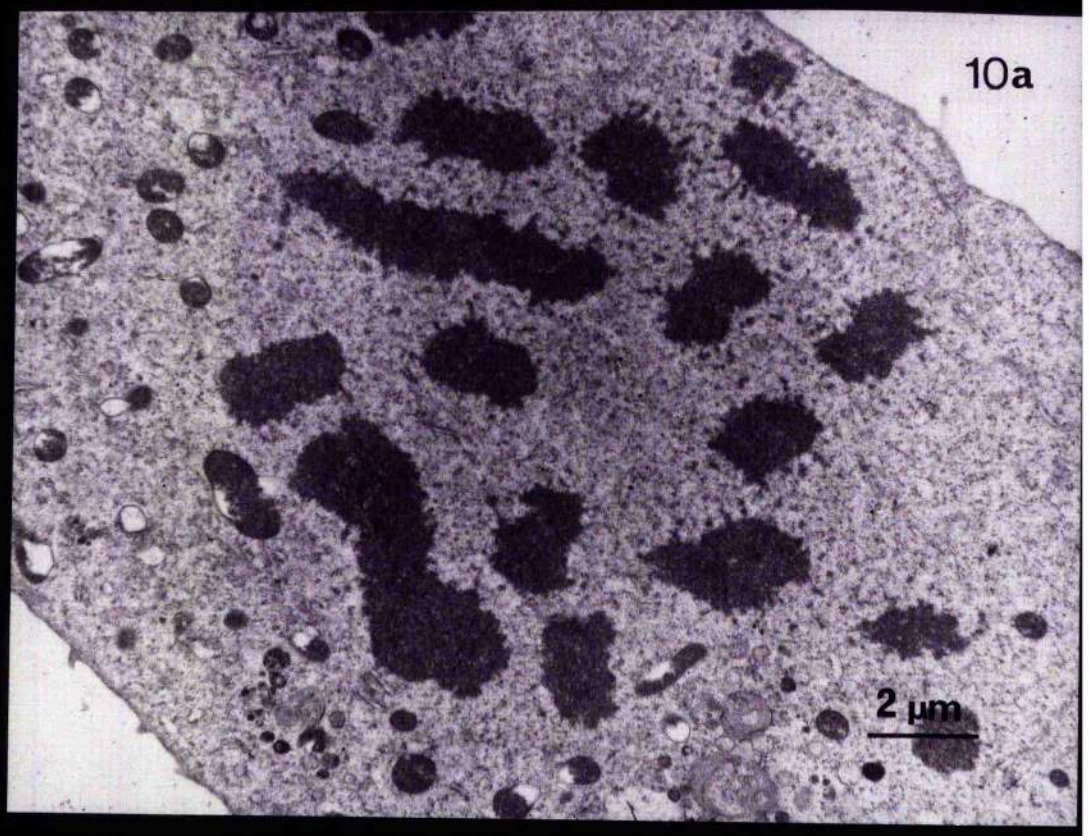
IV - 9b. Electron micrograph of a section of a mitotic metaphase cell of T.c. carnifex tissue culture grown in medium containing BUdR (20 µg/ml.) for 8 days.

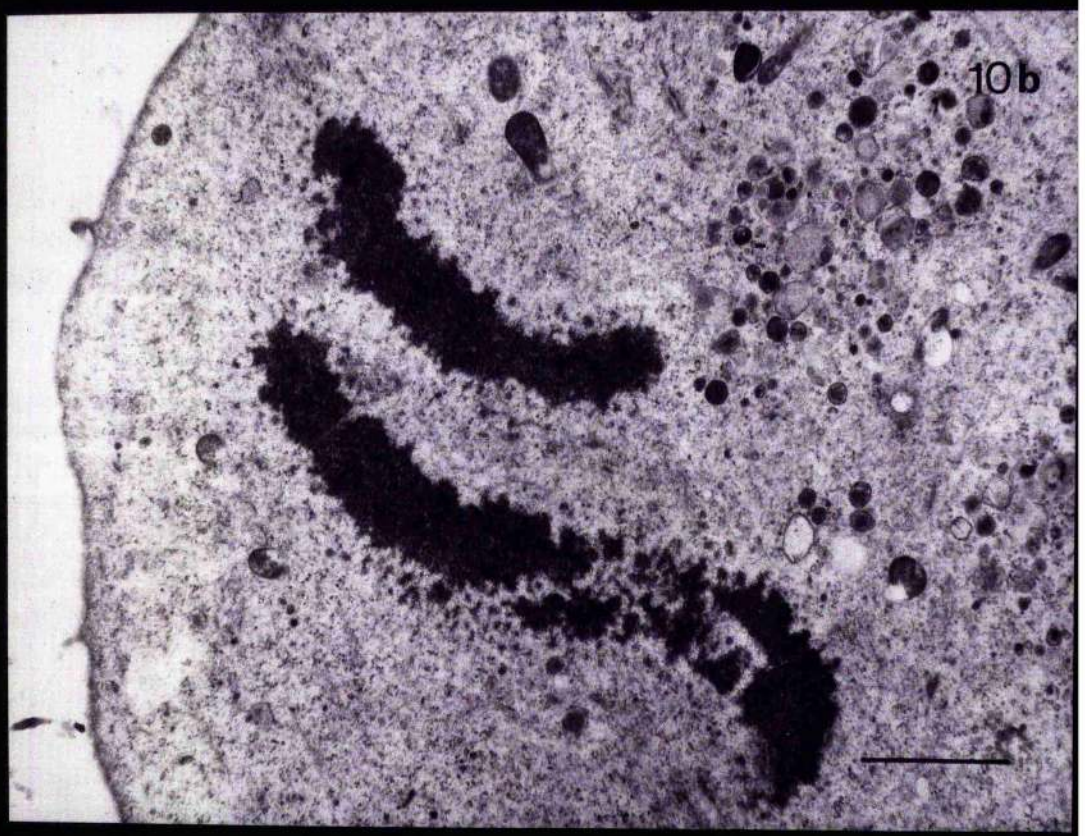

9a
2 µm


9b

IV - 10a. As fig. IV - 9a.

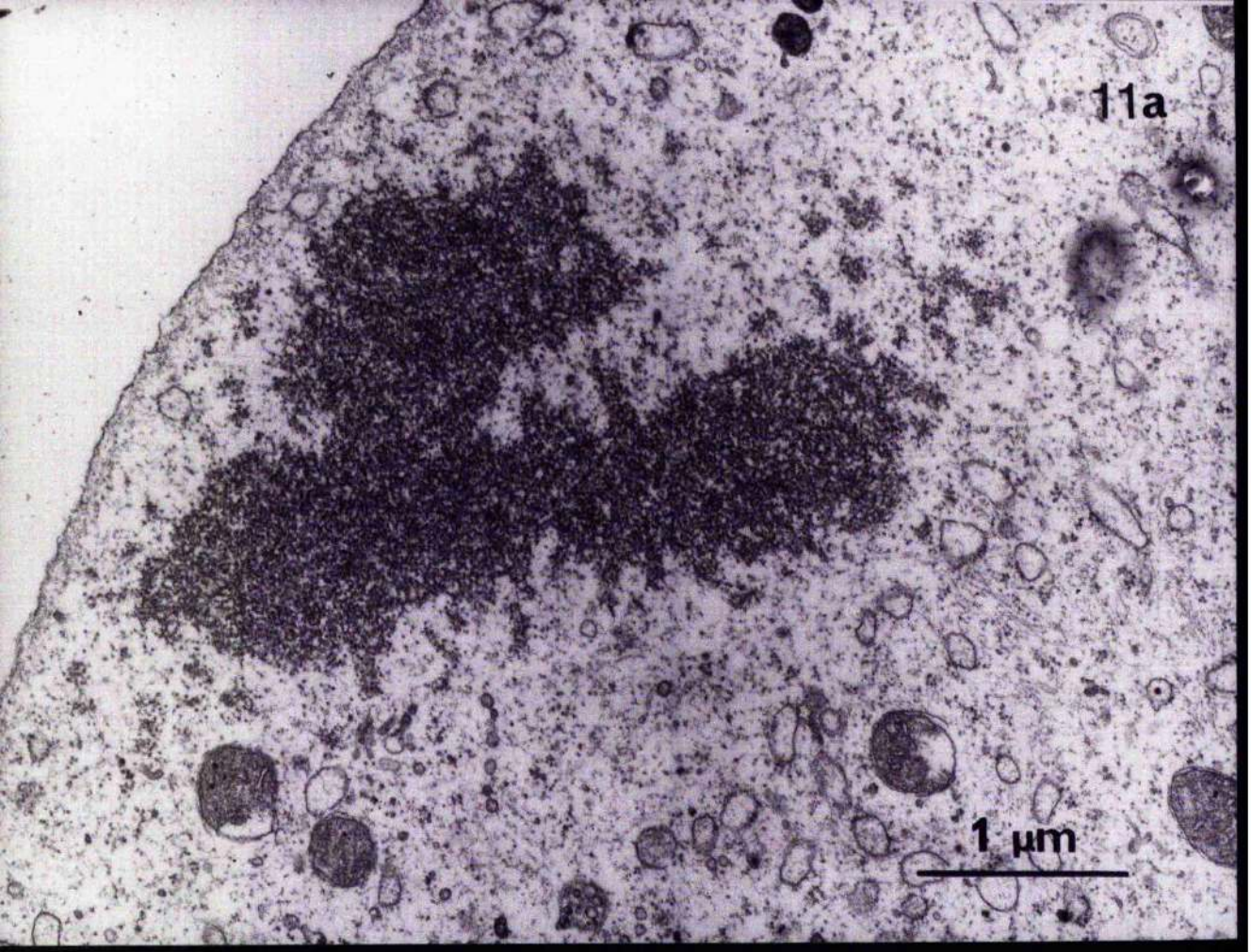
IV - 10b. As fig. IV - 9b. One region of one chromatid appears very "spotty" and undercondensed.

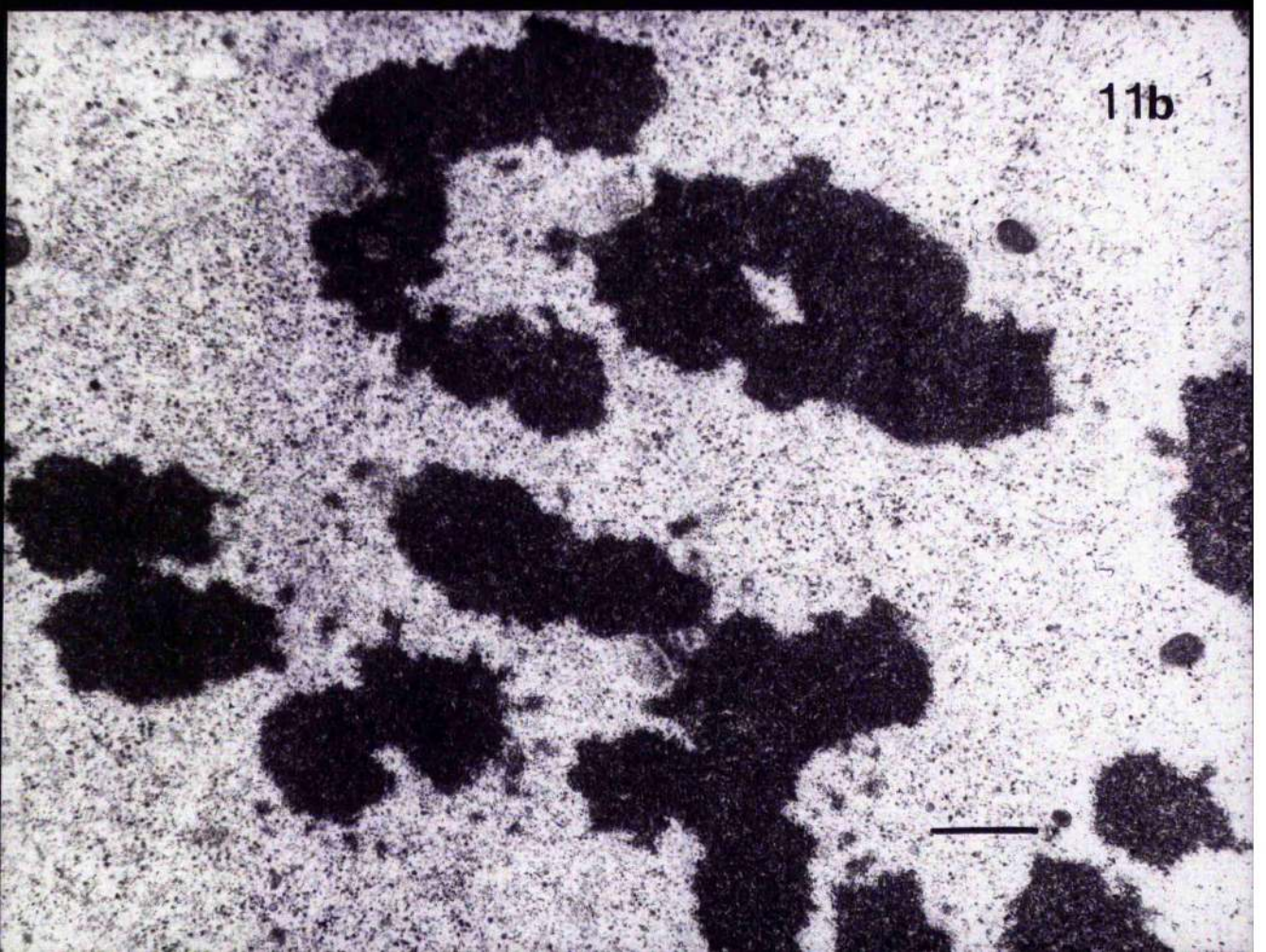

10a
2 µm


10b

IV - 11a. As fig. IV - 9a.

IV - 11b. As fig. IV - 9b.

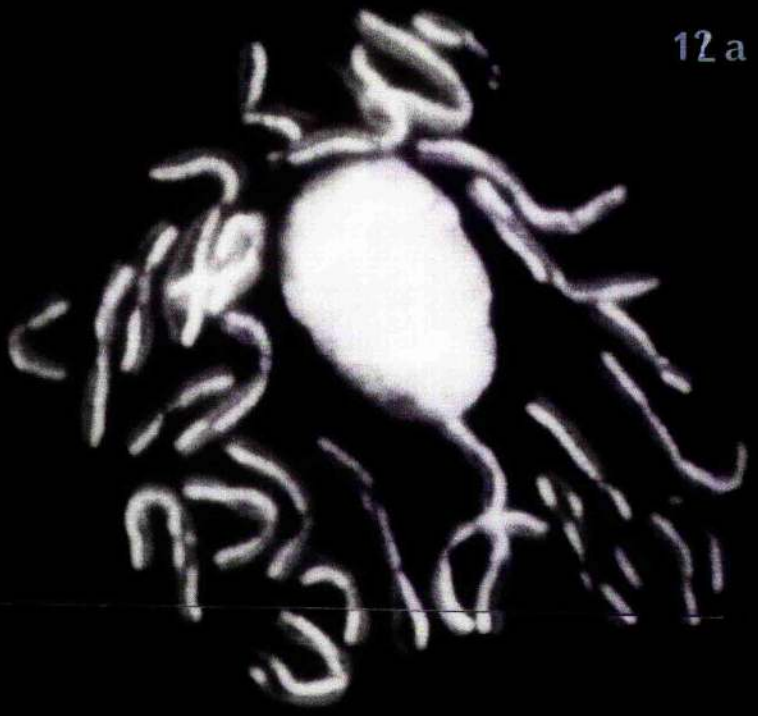

11a
1 µm


11b

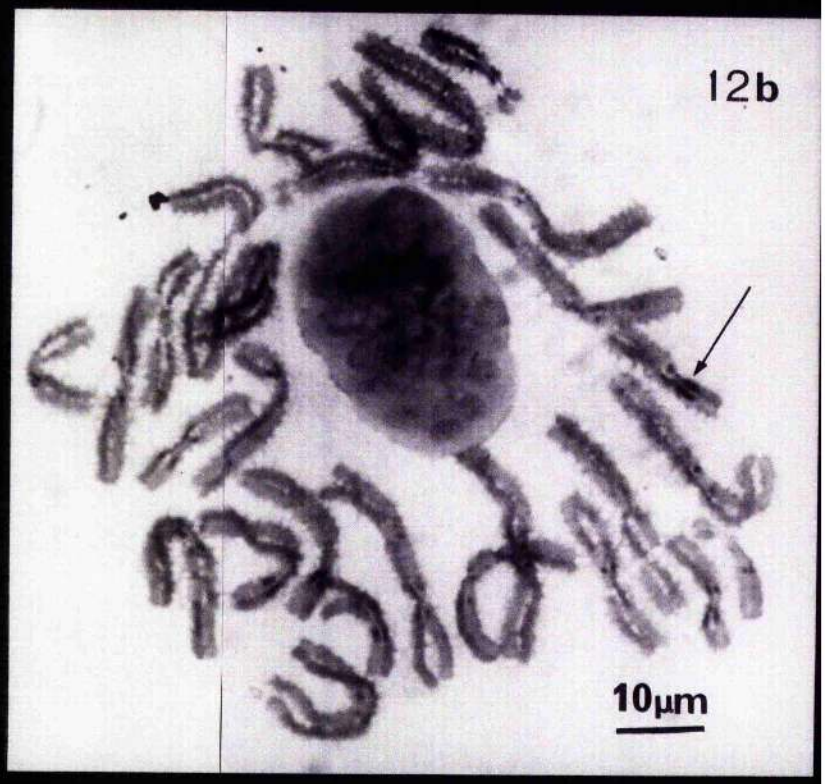
IV - 12a. A mitotic metaphase chromosome set of a T.c. carnifex tissue culture cell grown in medium containing BUdR (1 µg/ml.) for 8 days. The preparation was stained with 33258 Hoechst and examined by UV microscopy. Monofilarly substituted chromatids fluoresce brightly while bifilarly substituted chromatids are pale.

IV - 12b. The same cell as shown in fig. IV - 12a stained with Giemsa by the FPG technique. The regions which stain intensely with Giemsa after cold-treatment are deeply stained. Sister chromatids show only a faint differential staining. One exchange (arrowed) has occurred in a heterochromatic region.

The chromatids of this cell show the reverse of the staining behaviour usually seen in chromatids stained with Giemsa after culture in medium containing BUdR. When compared with the fluorescence photograph above, it can be seen that chromatids which fluoresce brightly with 33258 Hoechst (monofilarly substituted) stain palely (instead of deeply) with Giemsa.



12a



SUMMARY

CHAPTER I.

After a period of culture at low temperature or in a medium containing the thymidine analogue bromodeoxyuridine, mitotic metaphase chromosomes of Triturus cristatus show a characteristic pattern of secondary constrictions, most of which are pericentrically located. The chromatin in the cold-induced constrictions stains intensely with Giemsa. When mitotic chromosome preparations are stained according to a C-banding technique (Arrighi & Hsu, 1971), the centromeres of the chromosomes, and the interstitial regions which are differentially stained after a period of cold-treatment, stain intensely with Giemsa. Electron micrographs of sections through metaphase chromosomes in tail-fin cells of cold- and colchicine-treated larvae show that the chromatin fibres are more densely packed in the constricted regions than elsewhere. Hypotonically-treated spermatogonia or tissue-culture cells of T.c. carnifex show spiral structure throughout the metaphase chromatids. The mitotic chromosomes of T. vulgaris also have pericentrically-located regions which stain intensely with Giemsa.

CHAPTER II.

Skin cells of T.c. carnifex can be maintained in monolayer culture in a predominantly diploid state for more than 14 months. The cells will grow at 25°C in medium originally formulated for mammalian cells (Eagles' MEM) supplemented with 10% foetal calf serum and glutamine. The generation time of these cells is approximately 4 days. Preparations of mitotic chromosomes from colchicine-treated cultures can be made by a technique involving air-drying hypotonically-treated and fixed cells onto slides. This is the only diploid urodele cell line maintained in any laboratory.

CHAPTER III.

Purified and iodine-labelled ribosomal (28 + 18S) RNA extracted from T.c. carnifex ovaries hybridises in situ to a region 2/5 of the way down the long arms of both chromosomes X of mitotic chromosomes of T.c. carnifex tissue culture cells. When this RNA preparation is hybridised in situ to mitotic chromosomes of T. vulgaris larval brain cells, labelled regions are found (i) near the telomeres of both chromosomes II, (ii) halfway down the long arms and at the ends of the long arms of both chromosomes X, (iii) near the centromere of one large metacentric chromosome, and (iv) halfway down the long arm of a medium-sized submetacentric chromosome. There is variation in the labelling pattern shown by different T. vulgaris animals, and perhaps some cell to cell variation within the same animal. Unequal crossing-over between sister and homologous chromatids is suggested as the mechanism responsible for the variation between animals of the same species in the positions and sizes of the nucleolar organiser loci.

Mitotic metaphase chromosomes of T.c. carnifex spermatogonia hybridized in situ with iodine-labelled 5S RNA are labelled about halfway down the long arms of both chromosomes X.

The numbers of nucleoli in methyl green/pyronine or "silver stained" cells of T.c. carnifex tissue culture range from 1 - 12 with most cells containing 3 - 5 nucleoli; most T.c. carnifex spermatogonia contain 2 - 3 nucleoli; most T. vulgaris larval brain cells contain 3 - 5 nucleoli. In situ hybridization results show that there are only one or two clusters of 28 + 18S rRNA genes in non-dividing T.c. carnifex tissue culture cells, therefore the numbers of nucleoli seen after "silver staining" must be composed of fragmented material from the two original nucleoli.

CHAPTER IV.

The sister chromatid exchange (SCE) frequency of *T.c. carnifex* tissue culture cells grown for two periods of DNA synthesis in medium containing BUdR and stained by the FPG technique of Perry & Wolff (1974), increases from about 20 at 1 μg/ml. BUdR to about 50 at 100 μg/ml. BUdR. The spontaneous exchange frequency is about 10 - 14 exchanges per cell cycle per diploid cell.

An electron microscopic analysis of chromatin packing in 'normal' and BUdR-treated cells suggests that BUdR incorporated into DNA does not have any significant effects on chromatin packing.

ACKNOWLEDGEMENTS

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REFERENCES

AMALDI, F. & BUONGIORNO-NARDELLI, M. (1971). Molecular hybridization of Chinese hamster 5S, 4S and "pulse labelled" RNA in cytological Exp. Cell Res. 65, 329-334.

AUCLAIR, W. (1968). Cultivation of monolayer cultures of frog renal cells. Nature 192, 467-468.

BAILLY, S., GUILLEMIN, C., LABROUSSE, M. (1973). Comparaison du nombre et de la position des zones spécifiques révélées sur les chromosomes mitotiques de l'Amphibien Urodèle Pleurodeles waltlii Michah, par les techniques de coloration au colorant de Giemsa et à la moutarde de quinacrine. C.R. Acad. Sci. (Paris) 276, 1867-1869.

BOSTOCK, C.J. & CHRISTIE, S. (1976). Analysis of the frequency of sister chromatid exchange in different regions of chromosomes of the Kangaroo rat (Dipodomys ordii). Chromosoma (Berl.) 56, 275-287.

CALLAN, H.G. (1942). Heterochromatin in Triton. Proc. Roy. Soc. Lond. 130, 324-335.

CALLAN, H.G. (1966). Chromosomes and nucleoli of the Axolotl, Ambystoma mexicanum. J. Cell Sci. 1, 85-108.

CALLAN, H.G. (1972). Replication of DNA in the chromosomes of eukaryotes. Proc. R. Soc. Lond. B. 181, 19-41.

CALLAN, H.G. (1973). DNA replication in the chromosomes of eukaryotes. Cold Spring Harbor Symposiia on quantitative biology. XXXVIII, 195-203.

CARRANO, A.V. & WOLFF, S. (1973). Distribution of sister chromatid exchanges in the euchromatin and heterochromatin of the Indian Muntjac. Chromosoma (Berl.) 53, 361-369.

CAROLL, D. & BROWN, D.D. (1976a). Repeating units of Xenopus laevis oocyte-type 5S DNA are heterogeneous in length. Cell 7, 467-475.

CAROLL, D. & BROWN, D.D. (1976b). Adjacent repeating units of Xenopus laevis 5S DNA can be heterogeneous in length. Cell 7, 477-486.

CHAGANTI, R.S.K., SCHONBERG, S. & GERMAN, J. (1974). A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. Proc.nat. Acad. Sci. U.S. 71, 4508-4512.

CHANDLEY, A.C. & KOFMAN-ALFARO, S. (1971). "Unscheduled" DNA synthesis in human germ cells following UV irradiation. Exp. Cell Res. 69, 45-48.

COMMERFORD, S.L. (1971). Iodination of nucleic acids in vitro. Biochemistry 10, 1993-1999.

DENTON, T.E., HOWELL, W.M., BARRETT, J.V. (1976). Human nucleolar organiser chromosomes: Satellite associations. Chromosoma (Berl.) 55, 81-84.

DUTRILLAUX, B., FOSSE, A.M., PRIEUR, M., LEJEUNE, J. (1974). Analyse des échanges de chromatides dans les cellules somatiques humaines. Traitement au BUdR (5-bromodéoxyuridine) et fluorescence bicoloré par l'Acridine Orange. Chromosoma (Berl.) 48, 327-340.

EAGLE, H. (1959). Amino acid metabolism in mammalian cell cultures. Science 130, 432-437.

EGUCHI, G., ABE, S-I., WATANABE, K. (1974). Differentiation of lens-like structures from newt iris epithelial cells *in vitro*. Proc. nat. Acad. Sci. U.S. 71, 5052-5056.

FORD, P.J. & MATHIESON, T. (1976). Control of 5S RNA synthesis in *Xenopus laevis*. Nature 261, 433-435.

FREED, J.J. (1962). Continuous cultivation of cells derived from haploid *Rana pipiens* embryos. Exp. Cell Res. 26, 327-333.

FREED, J.J., MEZGER-FREED, L., SCHATZ, S.A. (1969). Characteristics of cell lines from haploid and diploid *Rana pipiens* embryos. In: *Recent results in cancer research*. Special suppl. *Biology of amphibian tumours*. (M. Mizell, ed.) p. 101-111. Springer Verlag.

FREED, J.J. & ROSENFELD, S. (1965). Frog renal adenocarcinoma: cytological studies *in situ* and *in vitro*. Ann. N.Y. Acad. Sci. 126, 99-114.

FUJIWARA, Y. (1972). Characteristics of DNA synthesis following ultraviolet light irradiation in mouse L cells. Exp. Cell Res. 75, 483-489.

GALL, J.G. & PARDUE, M.L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. nat. Acad. Sci. (Wash.) 63, 378-383.

GEARD, C.R. & PEACOCK, W.J. (1969) Sister chromatid exchanges in *Vicia faba*. Mutation Res. 7, 215-223.

GERMAN, J. (1969). Bloom's syndrome. I. Genetical and clinical observations in the first twenty-seven patients. Amer. J. Hum. Genet. 21, 196-227.

GERMAN, J., ARCHIBALD, R., & BLOOM, D. (1965). Chromosome breakage in a rare and probably genetically determined syndrome of man. Science 148, 506-507.

GERMAN, J., CRIPPA, L.P., BLOOM, D. (1974). Bloom's Syndrome. III. Analysis of the chromosome aberration characteristic of this disorder. Chromosoma (Berl.) 48, 361-366.

GIBSON, D.A. & PRESCOTT, D.M. (1972). Induction of sister chromatid exchanges in chromosomes of rat kangaroo cells by tritium incorporated in DNA. Exp. Cell Res. 74, 397-402.

GIBSON, D.A. & PRESCOTT, D.M. (1973). Sister chromatid exchanges in isolabelling. Exp. Cell Res. 83, 445-447.

GOODPASTURE, C. & BLOOM, S.E. (1975). Visualization of nucleolar organiser regions in mammalian chromosomes using silver staining. Chromosoma (Berl.) 53, 37-50.

GRIFFIN, C.S., SCOTT, D. & PAPWORTH, D.G. (1970). The influence of DNA content and nuclear volume on the frequency of radiation-induced chromosome aberrations in Bufo species. Chromosoma (Berl.) 30, 228-249.

HAMBURGER, V. (1966). A manual of experimental embryology. University of Chicago Press.

HAYFLICK, L. (1965). The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37, 614-636.

HENDERSON, A.S., EICHER, E.M. YU, M.T., ATWOOD, K.D. (1974). The chromosomal location of ribosomal DNA in the mouse. Chromosoma (Berl.) 49: 155-160.

HENNEN, S., MIZUNO, S. & MACGREGOR, H.C. (1975). *In situ* hybridization of ribosomal DNA labelled with ^{125}Iodine to metaphase and lampbrush chromosomes from newts. Chromosoma(Berl.) 50, 349-369.

HOLLIDAY, R. (1964). A mechanism for gene conversion in fungi. Genet. Res. 5, 282-304.

HOWELL, W.M., DENTON, T.E. & DIAMOND, J.R. (1974). Differential staining of the satellite regions of human acrocentric chromosomes. Experientia 31, 260-262.

HSU, T.C. & SOMERS, C.E. (1961). Effect of 5-bromodeoxyuridine on mammalian chromosomes. Proc. nat. Acad. Sci. U.S. 47, 396-403.

HUTCHISON, N. & PARDUE, M.L. (1975). The mitotic chromosomes of *Notophthalmus (= Triturus) viridescens*. Localization of C-banding regions and DNA sequences complementary to 18S, 28S, and 5S ribosomal RNA. Chromosoma (Berl.) 53, 51-59.

HUMPHREY, R.R. (1961). A chromosomal deletion in the Mexican axolotl (*Siredon mexicanum*) involving the nucleolar organizer and the gene for dark color. Am. Zool. 1, 361.

JAYLET, A. (1965). Technique de culture de leucotyes pour l'étude chromosomique d'Amphibiens Urodèles diploides et hétéroploides. C.R. Acad. Sc. Paris 260, 3160-3163.

JOHN, A.H., BIRNSTIEL, M.L. & JONES, K.W. (1969). RNA-DNA hybrids at the cytological level. Nature 223, 582-587.

JOHNSON, L.P., HENDERSON, A.S., ATWOOD, K.C. (1974). Location of the genes for 5S RNA in the human chromosome complement. IN: New Haven Conference (1973): First international workshop on human gene mapping. Birth Defects: Original articles series X : 3 (D. Bergsma, ed.) p. 103-105. New York: The National Foundation.

KABACK, M.M., SAKSELA, E. & MELLMAN, W.J. (1964). The effect of 5-Bromodeoxyuridine on human chromosomes. Exp. Cell Res. 34, 182-212.

KATO, H. (1973). Induction of sister chromatid exchanges by UV light and its inhibition by caffeine. Exp. Cell Res. 82, 383-390.

KATO, H. (1974a). Induction of sister chromatid exchanges by chemical mutagens and its possible relevance to DNA repair. Exp. Cell Res. 85, 239-247.

KATO, H. (1974b). Is isolabelling a false image? Exp. Cell Res.89, 416-421.

KATO, H. (1974c). Photoreactivation of sister chromatid exchanges induced by ultraviolet radiation. Nature (Lond.) 249, 552-553.

KATO, H. (1974d) Possible role of DNA synthesis in the formation of sister chromatid exchanges. Nature (Lond.) 252, 739-741.

KATO, H. (1974e). Spontaneous sister chromatid exchanges detected by a BUdR-labelling method. Nature (Lond.) 251, 70-72.

KELLY, R.B., COZZARELLI, N.R., DEUTSCHER, M.P., LEHMAN, I.R., KORNBERG, A. (19 Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. J. biol. Chem. 245, 39-45.

KEZER, J. & MACGREGOR, H.C. (1973). The nucleolar organizer of Plethodon cinereus cinereus (Green). II: The lampbrush nucleolar organizer. Chromosoma (Berl.) 42, 427-444.

KIHLMAN, B.A. (1975). Sister chromatid exchanges in Vicia faba. II. Effects of thiotepan, caffeine and 8-ethoxycaffeine on the frequency of SCEs. Chromosoma (Berl.) 51, 11-18.

KIHLMAN, B.A. & KRONBERG, D. (1975). Sister chromatid exchanges in Vicia faba. I. Demonstration by a modified fluorescent plus Giemsa (FPG) technique. Chromosoma (Berl.) 51, 1-10.

KOFMAN-ALFARO, S. & Chandley, A.C. (1971). Radiation-initiated DNA synthesis in spermatogenic cells of the mouse. Exp. Cell Res. 69, 33-44.

KORENBERG, J.R. & FREEDLENDER, E.F. (1974). Giemsa technique for the detection of SCEs. Chromosoma (Berl.) 48, 355-360.

KOTZIN, B.L. & BAKER, R.F. (1972). Selective inhibition of genetic transcription in sea urchin embryos. J. Cell Biol. 55, 74-81.

LATT, S.A. (1973). Microfluorimetric detection of deoxyribonucleic acid replication in human metaphase chromosomes. Proc. nat. Acad. Sci. U.S. 70, 3395-3399.

LATT, S.A. (1974a). Sister chromatid exchanges, indices of human chromosome damage and repair: detection by fluorescence and induction by mitomycin C. Proc. nat. Acad. Sci. U.S. 71, 3162-3166.

LATT, S.A. (1974b). Localization of sister chromatid exchanges in human chromosomes. Science 185, 74-76.

MACGREGOR, H.C. & KEZER, J. (1973). The nucleolar organizer of Plethodon cinereus cinereus (Green). I: Location of the nucleolar organizer by in situ nucleic acid hybridisation. Chromosoma (Berl.) 42, 415-426.

MACGREGOR, H.C., MIZUNO, S. (1976). In situ hybridization of "Nick-translated" ^{3}H-ribosomal DNA to chromosomes from salamanders. Chromosoma (Berl.) 54, 15-25.

MANCINO, G., NARDI, I., RAGGHIANTI, M. (1972). Lampbrush chromosomes from semi-albino crested newts, Triturus cristatus carnifex (Laurenti). Experientia (Basel) 28, 856-860.

MANCINO, G., RAGGHIANTI, M, INNOCENTI, S.B. (1973). I cariotipi di Triturus marmoratus e T. cristatus studiati con il "C-staining method". Atti Accad. naz. Lincei Rc. Ser. 8, 55, 559-564.

MARIN, G. & PRESCOTT, D.M. (1964). The frequency of sister chromatid exchanges following exposure to varying doses of ^{3}H-thymidine or X-rays. J. Cell Biol. 21, 159-167.

McFARLANE, P.W. & CALLAN, H.G. (1973). DNA replication in the chromosomes of the chicken, Gallus domesticus. J. Cell Sci. 13, 821-839.

MILLER, L. & BROWN, D.D. (1969). Variation in the activity of nucleolar organizers and their ribosomal gene content. Chromosoma 28, 430-444.

MILLER, .R.C., ARONSON, M.M. & NICHOLS, W.W. (1976). Effects of treatment on differential staining of BUdR labelled metaphase chromosomes: three way differentiation of M_3 chromosomes. Chromosoma (Berl.) 55, 1-11.

MOORE, P.D. & HOLLIDAY, R. (1976). Evidence for the formation of hybrid DNA during mitotic recombination in chinese hamster cells. Cell 8, 573-579.

NARDI, I., RACCHIANTI, M. & MANCINO, G. (1973). Banding patterns in newt chromosomes. Chromosoma 40, 321-331.

OHNUKI, Y. (1968). Structure of chromosomes. I. Morphological studies of the spiral structure of human somatic chromosomes. Chromosoma (Berl.) 25, 402-428.

PARDUE, M.L., BROWN, D.D. & BIRNSTIEL, M.L. (1973). Location of the genes for 5S ribosomal RNA in Xenopus laevis. Chromosoma (Berl.) 42, 191-203.

PEACOCK, W.J. (1963). Chromosome duplication and structure as determined by autoradiography. Proc. nat. Acad. Sci. U.S. 49, 793-801.

PERA, F. & MATTIAS, P. (1976). Labelling of DNA and differential sister chromatid staining after BrDU treatment in vivo. Chromosoma (Berl.) 57, 13-18 (1976).

PERRY, P. & EVANS, H.J. (1975). Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258, 121-125.

PERRY, P. & WOLFF, S. (1974). New Giemsa method for the differential staining of sister chromatids. Nature 251, 156-158.

PILONE, G.B., NARDI, I., BATISTONI, R., ANDRONICO, F. & BECCARI, E. (1974). Chromosome location of the genes for 28S, 18S and 5S rRNA in Triturus marmoratus (Amphibia, Urodela). Chromosoma (Berl.) 49, 135-153.

PRENSKY, W., STEFFENSEN, D.M. & HUGHES, W.L. (1973). The use of iodinated RNA for gene localization. Proc. nat. Acad. Sci. U.S. 70, 1860-1864.

PUKKILLA, P.J. (1975). Identification of the lampbrush chromosome loops which transcribe 5S ribosomal RNA in Notophthalmus (Triturus) viridescens. Chromosoma (Berl.) 53, 71-89.

RAFFERTY, K.A. Jr. (1969). Mass culture of amphibian cells: methods and observations concerning stability of cell type. In: Recent results in cancer research. Special Suppl. Biology of Amphibian Tumors (ed. M. Mizell). Springer-Verlag.

RAGGHIANTI, M..INNOCENTI, S. & MANCINO, G. (1973). Bandeggiature indotta da "C-, G- e Q-staining methods" e pattern di replicazione dei cromosomi di Triturus. Atti Accad. naz. Lincei Rc. Ser. 8, 55, 764-770.

REYNOLDS, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208-212.

RITOSSA, F., ATWOOD, K. & SPIEGELMAN, S. (1966). A molecular explanation of the bobbed mutants of Drosophila as partial deficiences of "ribosomal" DNA. Genetics 54, 819-834.

ROMMELAERE, J., CORNELIS, J.J., MILLER-FAURÈS, A & ERRERA, M. (1974). The influence of 5-bromodeoxyuridine on DNA repair in chinese hamster cells exposed to ultraviolet radiation. Biochim. Biophys. Acta. 340, 388-399.

ROMMELAERE, J. & ERRERA, M. (1972). The effect of caffeine on the survival of U.V.-irradiated diploid and tetrapolid chinese-hamster cells. Int. J. Radiat. Biol. 22, 285-291.

ROMMELAERE, J. & MILLER-FAURÈS, A. (1975). Detection by density equilibrium centrifugation of recombinant-like DNA molecules in somatic mammalian cells. J. Mol. Biol. 98., 195-218.

ROMMELAERE, J., SUSSKIND, M. & ERRERA, M. (1973). Chromosome and chromatid exchanges in chinese hamster cells. Chromosoma (Berl.) 41, 243-257.

ROSBASH, M., FORD, P.M., BISHOP, J.O. (1974). Analysis of the C-value paradox by molecular hybridization. Proc. nat. Acad. Sci. U.S. 3746-3750.

ROTHSTEIN, H., LANDER, J.M. & WEINSIEDER, A. (1965). In vitro culture of amphibian lenses. Nature 206, 1267-1269.

RUDAK, E. & CALLAN, H.G. (1976). Differential staining and chromatin packing of the mitotic chromosomes of the newt Triturus cristatus. Chromosoma (Berl.) 56, 349-362.

RUPP, W.C. & HOWARD-FLANDERS, P.J. (1968). Discontinuities in the DNA synthesized in an excision-defective strain of E. coli following ultra violet irradiation. J. Mol. Biol. 31, 291-304.

SCHMID, M. & KRONE, W. (1976). The relationship of a specific chromosomal region to the development of the acrosome. Chromosoma (Berl.) 56, 327-347.

SCHWARTZ, S.A. & KIRSTEN, W.H. (1974). Distribution of 5-Bromodeoxyuridine in the DNA of Rat embryo cells. Proc. nat. Acad. Sci. U.S. 71, 3570-3574.

SETO, T. (1964). Cultivation of tissues from cold-blooded amphibians with special reference to the culture methods in Amphibia. Japanese J. Genet. 39, 268-275.

SETO, T., POMERAT, C.M & KEZER, J. (1964). The chromosomes of Necturus maculosus as revealed in cultures of leukocytes. Am. Nat. XCVIII, No. 899, 71-78.

SETO, T. & ROUNDS, D.E. (1969). Cultivation of tissues and leukocytes from amphibians. In: Methods in Cell Physiology 3, (75-94). (ed. D.M. Prescott). Academic Press.

SINCLAIR, J.H., CARROLL, C.R. & HUMPHREY, R.R. (1974). Variation in rDNA redundancy level and nucleolar organizer length in normal and variant lines of the Mexican axolotl. J. Cell Sci. 15, 239-257.

STEFFENSEN, D., DUFFEY, P. & PRENSKY, W. (1973). Localisation of the 5S ribosomal RNA genes in the human genome. In: New Haven conference (1973): First international workshop on human gene mapping. Birth defects; Original article series X : 3. (D. Bergsma, ed.) P. 153-254. New York: The National Foundation. (1974).

STERN, C. (1927). Ein genetischer und zytologischer Beweis füs Veverbung in Y-chromosome von Drosophila melanogaster. Z.i.A.V. 44, 187-231.

TAYLOR, J.H. (1958). Sister chromatid exchanges in tritium-labelled chromosomes. Genetics 43, 515-529.

TAYLOR, J.H., WOODS, P.S. & HUGHES, W.L. (1957). The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labelled thymidine. Proc. nat. acad. Sci. U.S. 43, 122-128.

VOLPE, E.P. & GEBHARDT, B.M. (1966). Evidence from cultured leucocytes of blood cell chimerism in ex-parabiotic frogs. Science 154, 1197-1198.

WALEN, K.H. (1965). Spatial relationships in the replication of chromosomal DNA. Genetics 51, 915-929.

WALLACE, H. & BIRNSTIEL, M.L. (1966). Ribosomal cistrons and the nucleolar organizer. Biochim. biophys. Acta (Amst.) 114, 296-310.

WELLAUER, P.K. DAWID, I.B. & BROWN, D.D. & REEDER, R.H. (1976). The molecular basis for length heterogeneity in ribosomal DNA from Xenopus laevis. J. mol. Biol. 105, 461-486.

WEN, W., LEON, P. & HAGUE, D. (1974). Multiple gene sites for 5S and 18S + 28S RNA on chromosomes of Glyptotendipes barbipes (Staeger). J. Cell Biol. 61, 132-144.

WHITEHOUSE, H.L.K. (1963) A theory of crossing-over by means of hybrid deoxyribonucleic acid. Nature (Lond.) 199, 1034-1040.

WIMBER, D.E., DUFFEY, P., STEFFENSEN, D.M. & PRENSKY, W. (1974). Localizatio of the 5S RNA genes in Zea mays by RNA-DNA hybridization in situ. Chromosoma (Berl.) 47, 353-359.

WIMBER, D.E. & STEFFENSEN, D.M. (1970). Localization of 5S RNA genes on Drosophila chromosomes by RNA-DNA hybridization. Science 170, 639-641.

WOLFF, S. (1964). Are sister chromatid exchanges sister strand crossovers or radiation-induced exchanges? Mutation Res. 1, 337-343.

WOLFF, S. & PERRY, P. (1974). Differential Giemsa staining of sister chromatids and the study of sister chromatid exchange without autoradiography. Chromosoma (Berl.) 48, 341-353.

WOLF, K. & QUIMBY, M.C. (1964). Amphibian cell culture: Permanent cell line from the Bullfrog (Rana catesbeiania). Science 144, 1578-1580.

ZAKHAROV, A.F. & EGOLINA, N.A. (1972). Differential spiralization along mammalian mitotic chromosomes. I. BUdR-revealed differentiation in Chinese hamster chromosomes. Chromosoma (Berl.) 38, 341-365.

Chromosoma (Berl.) 56, 349–362 (1976)

CHROMOSOMA

Differential Staining and Chromatin Packing of the Mitotic Chromosomes of the Newt *Triturus cristatus*

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Abstract. Mitotic metaphase chromosomes of cold-treated *Triturus cristatus* show a characteristic pattern of constrictions, most of which lie close, though not immediately adjacent, to the centromeres. The chromatin in these cold-induced constrictions stains intensely with Giemsa. Cold-treated spermatogonia show spiral structure throughout the metaphase chromatids; the packing of chromatin fibrils is much tighter in the constricted regions than elsewhere, and the gyres in the constricted regions are narrower and of shorter pitch.

Introduction

It has been known for many years that the morphology of the chromosomes in the dividing cells of certain eukaryotes can be affected by culture at low temperature (Flovik, 1938; Geitler, 1940; Darlington and La Cour, 1940, 1941; Callan, 1942; Wilson and Boothroyd, 1944). Cold treatment causes a differential condensation of the chromatin, which results in the appearance of secondary constrictions, or segments of reduced width, at specific places along the metaphase chromosomes. The cold-induced constrictions are extra to those secondary constrictions which mark the positions of nucleolar organizers, the latter being generally visible without special treatment. Unlike the nucleolar organizers, the functional significance of the regions of the chromosomes whose morphology is differentially affected by low temperature is not yet known. Cold-induced secondary constrictions can be valuable aids in karyotype analysis, and they have been particularly useful in studies on Amphibia Urodela (*Ambystoma*, Signoret, 1965; Callan, 1966; *Pleurodeles*, Aimar, 1967; Labrousse, 1969, 1970).

More recently, other techniques have been devised which assist in chromosome identification. These techniques, primarily developed for mammalian material (Caspersson et al., 1968, 1970; Summer et al., 1971; Arrighi and Hsu, 1971), involve pre- or post-fixation treatments which modify the structure of the chromosomes and render specific regions differentially reactive towards

certain dyes, notably Giemsa. As a result of the successful application of these techniques, the chromosomes display characteristic patterns of transverse, intensely stained bands interspersed with less heavily stained regions. Despite extensive studies, the chemical basis for the differential staining reactions remains largely undetermined (Sumner, Evans and Buckland, 1973; Sumner and Evans, 1973; Sumner, 1973; Lober et al., 1973; Latt et al., 1974; Comings, 1975; Comings et al., 1975a, b). There are, however, some lines of evidence which suggest that differential chromatin condensation along the chromosomes may be the factor primarily responsible for differential staining (Golomb and Bahr, 1974b; Okada and Comings, 1974). We therefore decided to examine the effects of low temperatures on the mitotic chromosomes of the newt *Triturus cristatus*, and to investigate possible relationships between secondary constrictions, chromosome spiralization, "Giemsa bands" and chromatin packing.

Materials and Methods

Specimens of the Italian great crested newt *Triturus cristatus carnifex* were purchased from Gerrard and Haig, Newdigate, Surrey, or from the Zoological Station, Naples. Specimens of the British race *Triturus cristatus cristatus* were kindly supplied by Dr. T. Halliday, who collected them in the neighbourhood of Oxford. The newts were kept in tanks of water in the laboratory at 18° C.

Our first preparations of newt mitotic chromosomes were obtained from regenerating livers of adults. Each newt was anaesthetized with MS222, its abdominal cavity opened, and about 50 mm^3 of liver removed. After stitching the body-wall with catgut, the animal was placed in a tank of water at 18° C, and was fed for 14 days. Sixteen h before fixation the newt was injected intraperitoneally with 0.25 ml of a 0.5% solution of colchicine in water. Each newt was finally killed, its liver removed, cut into small pieces and the pieces placed in distilled water for 10 min. The liver fragments were fixed in a freshly prepared 3:1 mixture of absolute ethanol and glacial acetic acid, and were stored at 4° C.

Squash preparations were made from these fragments of liver. To make each preparation a small portion was placed in a drop of 45% acetic acid on a siliconised coverslip. The cells were disaggregated by tapping out the tissue with a nylon rod, larger pieces of connective tissue and other débris being removed by forceps. A subbed slide (Gall and Pardue, 1971) was now lowered over the cell suspension on the siliconised coverslip, with the latter resting on a filter paper pad, the preparation pressed gently, then inverted, and finally squashed firmly between folds of filter paper. After removal of coverslips by the dry-ice procedure, the preparations were dehydrated and hardened in 96% ethanol for 1 day and in absolute alcohol for 2 days. Thereafter they were air dried and stored in dust-proof boxes at room temperature until required for further processing.

Preparations of mitotic chromosomes were also obtained from newt testes. The newts from which these preparations were made started off inbreeding condition, with their testes (and vasa deferentia) full of spermatozoa and their spermatogonial cells quiescent. Spermatogonial mitoses were induced by transferring the animals to a room held constant at 25° C. The newts were kept at this temperature for 28 days, were fed regularly, and were given a change to clean water each day. These newts were now transferred to a cooled incubator at 2° C, where they remained for 4 days. 18 h before fixation each newt was injected intraperitoneally with 0.25 ml of a 0.5% aqueous solution of colchicine. The testes were thereafter removed, each was cut into about 8 pieces, and the pieces fixed in a freshly prepared 3:1 mixture of absolute ethanol and glacial acetic acid. A few fixations were made of testis fragments previously soaked for 15 min in distilled water; these provided chromosomes displaying spiral structure. Squash preparations were made as described earlier.

Squash preparations of mitotic chromosomes were also obtained from cold-treated newt larval tissues. Pairs of newts in breeding condition were kept at 18° C in large glass aquaria containing

plentiful growths of Canadian pondweed, *Elodea*. Eggs laid on the pondweed were collected daily, and any that were not developing normally were discarded. When the larvae were 24 days old they were placed in small tanks in a cooled incubator at 2° C. Larvae of this age have not yet begun to feed, and yolk is still evident in the gut. Cold treatment lasted for 4 days, the larvae spending the final 18 to 20 h in 0.5% colchicine in 1/10th Steinberg solution. Larvae were fixed whole in 3:1, and squashes of brain, tailtip, gut and liver were made in 45% acetic acid in the manner already described for adult liver fragments.

A few samples of cold-treated spermatogonial cells were stained for 5 min in 0.5% orcein (G.T. Gurr synthetic) in 45% acetic acid prior to squashing. However, for the most part we have stained our preparations with Giemsa. Hardened, air-dried squash preparations were placed in a Coplin jar containing 50 ml of 0.05 M phosphate buffer, pH 7.1 to 7.2, and 4 ml of Giemsa stain (B.D.H.) added and mixed. After staining for 10 min the stain was flushed out of the Coplin jar with a jet of tap water, the slides removed, rinsed in distilled water and air dried at room temperature.

Some squash preparations of cold-treated spermatogonia were treated according to the "C-banding" technique of Arrighi and Hsu (1971). This involved digestion for one hour at 37° C in pancreatic RNase (100 µg/ml) dissolved in double concentration standard sodium citrate, 2 × SSC (0.3 M NaCl, 0.03 M Na citrate), two rinses in 2 × SSC, rinses in 70% and 96% ethanol, followed by air drying; then 2 min in 0.07 N NaOH at room temperature, 2 rinses in 2 × SSC, rinses in 70% and 96% ethanol followed by air drying; then 18 h in 2 × SSC at 65° C, rinses in 70% and 96% ethanol, again followed by air drying. These preparations were finally stained in Giemsa as already described.

The squash preparations of regenerating liver of *T.c. carnifex*, and a few samples of cold-treated larval tissues of *T.c. cristatus*, were handled according to a "G-banding" technique. Hardened, air-dried squash preparations were digested for 6 min in 0.025% trypsin (Difco Bacto-Trypsin) in Earle's balanced salt solution lacking Ca and Mg, rinsed in distilled water, and then stained in Giemsa.

All Giemsa-stained preparations were left uncovered, and examined under Zeiss immersion oil. Photographs were taken on Ilford Pan F film using a Zeiss × 100 planapochromatic oil objective.

Some cold-treated larvae, which had spent their final 18 to 20 h in 0.5% colchicine in 1/10th Steinberg solution, were fixed for electron microscopy. Each larva was placed in 2.5% glutaraldehyde in phosphate buffer, pH 7.8, and its tail fins detached. The tail fins were left to fix for one hour, the rest of the body being discarded. Tail fins were post-fixed for 10 min in buffered osmium, then dehydrated, and embedded in Spurr. 500 Å-thick sections were cut with a glass knife on an LKB ultratome III, the sectioning plane shaving the flat epithelial cells. The sections were mounted on copper grids and examined in a Siemens Elmiskop I operating at 60 kV.

Observations

When the mitotic chromosomes of various tissues of cold- and colchicine-treated larvae are stained with Giemsa, the outcome is one of two alternatives. Either the chromosomes are uniformly stained and show constrictions (Fig. 2) or the chromosomes are in general lightly stained but with their cold-induced constrictions stained intensely (Figs. 3 and 4). In a given set of mitotic chromosomes it is usual for all to be stained in similar fashion, but different chromosome sets on one and the same slide may be stained in either of the two ways. The mode of staining evidently has nothing to do with the tissue of origin, for squash preparations of gut, liver, brain and tail tip epithelium all may show the two alternative staining behaviours side by side; indeed very occasionally both may be included in a single chromosome complement.

The cold-induced constrictions, just like nucleolar organizer constrictions, can be readily distinguished from centromeres in that both chromatids are

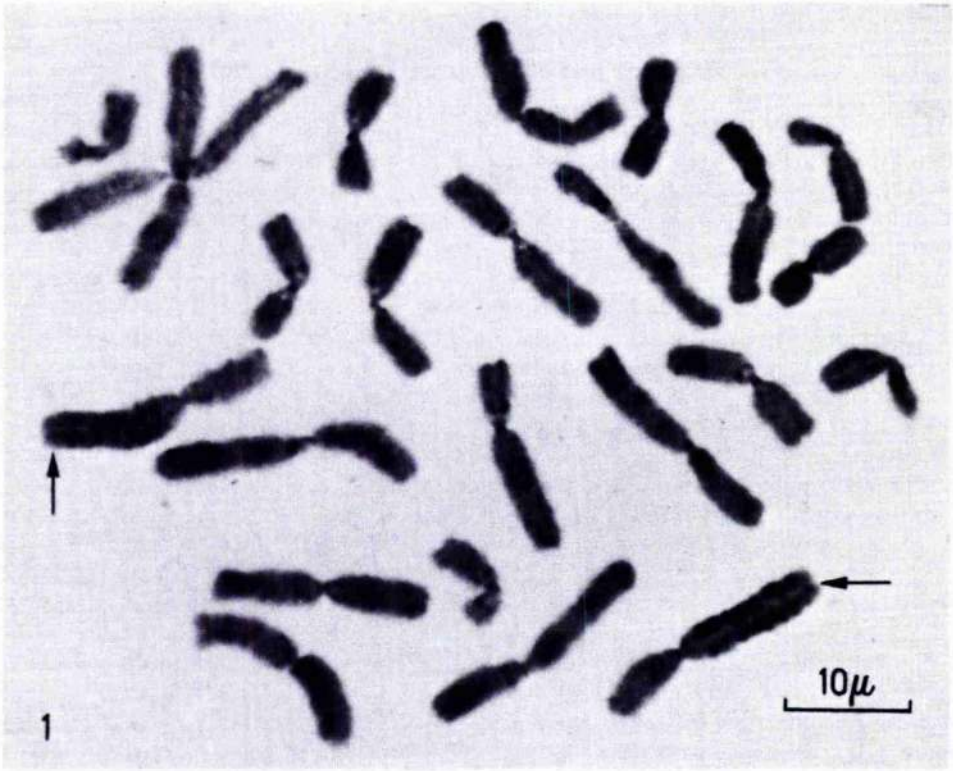


Fig. 1. Mitotic chromosomes of a spermatogonium of *Triturus cristatus carnifex*. The newt spent 4 days at 2° C, colchicine being injected 16 h before fixation. The preparation was stained according to the C-banding technique. The longer arms of chromosomes I are indicated by arrows

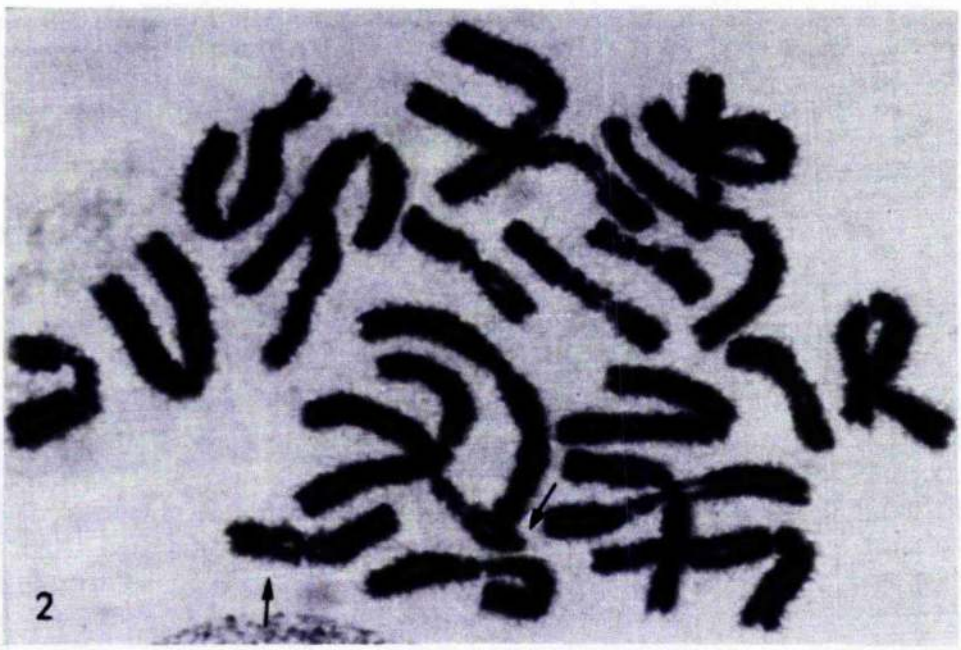
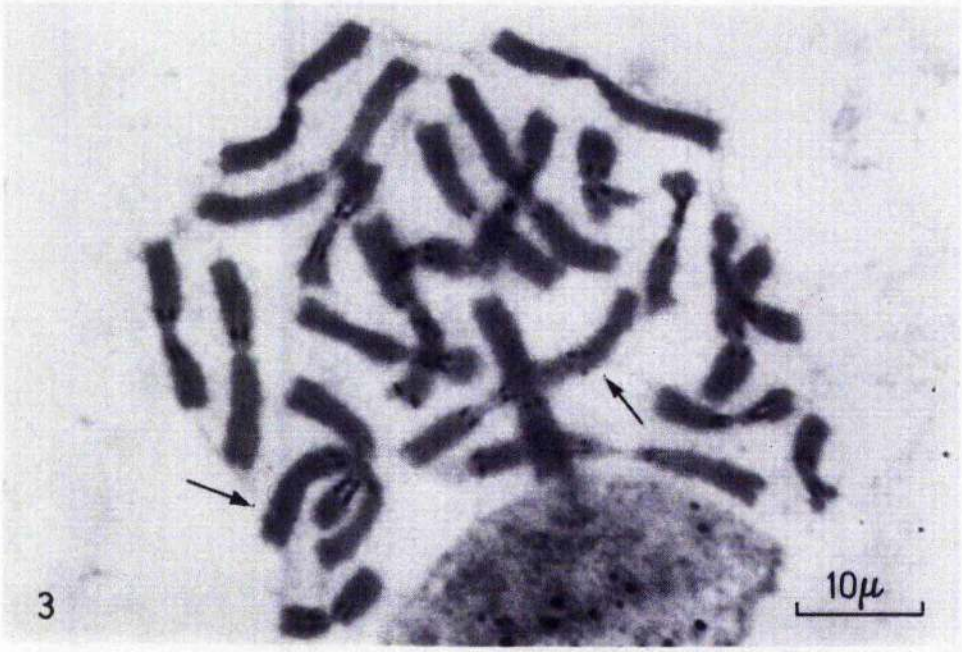
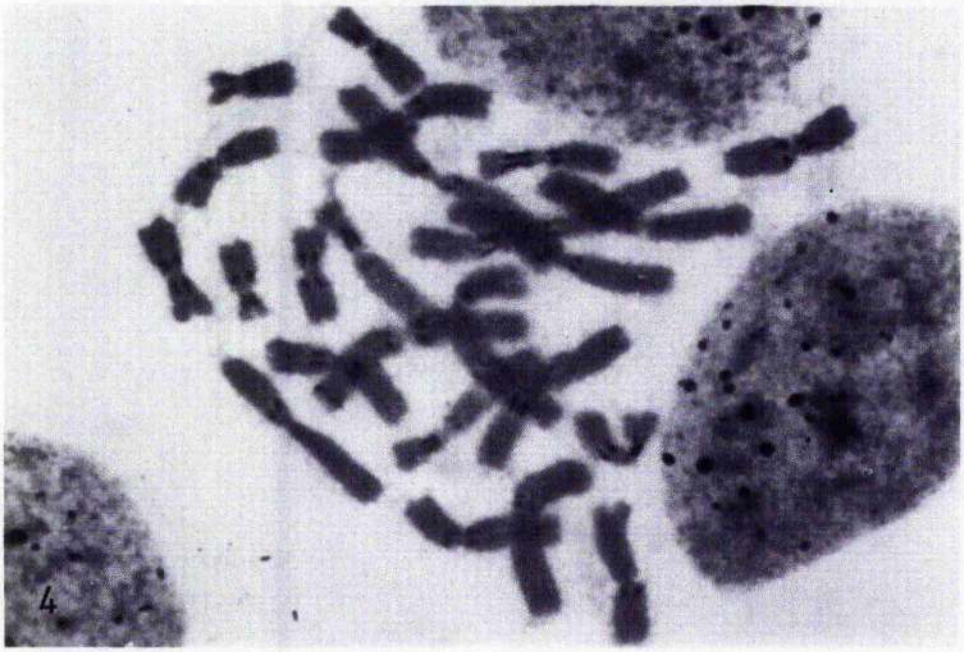


Fig. 2. Giemsa-stained mitotic chromosomes of a gut cell of *T.c. carnifex*. The larval newt spent 4 days at 2° C, and was in colchicine solution for the last 18 h before fixation. The nucleolar organizer constrictions are visible on one each of chromosomes VI and IX, and these are indicated by arrows. Magnification as in Figure 1





Figs. 3 and 4. Two examples of Giemsa-stained mitotic chromosome complements of brain cells of *T.c. carnifex*. The larval newt spent 4 days at 2° C, and was in colchicine for the last 18 h before fixation. These chromosomes show deeply stained constrictions. The longer arms of chromosomes I are indicated by arrows in Figure 3

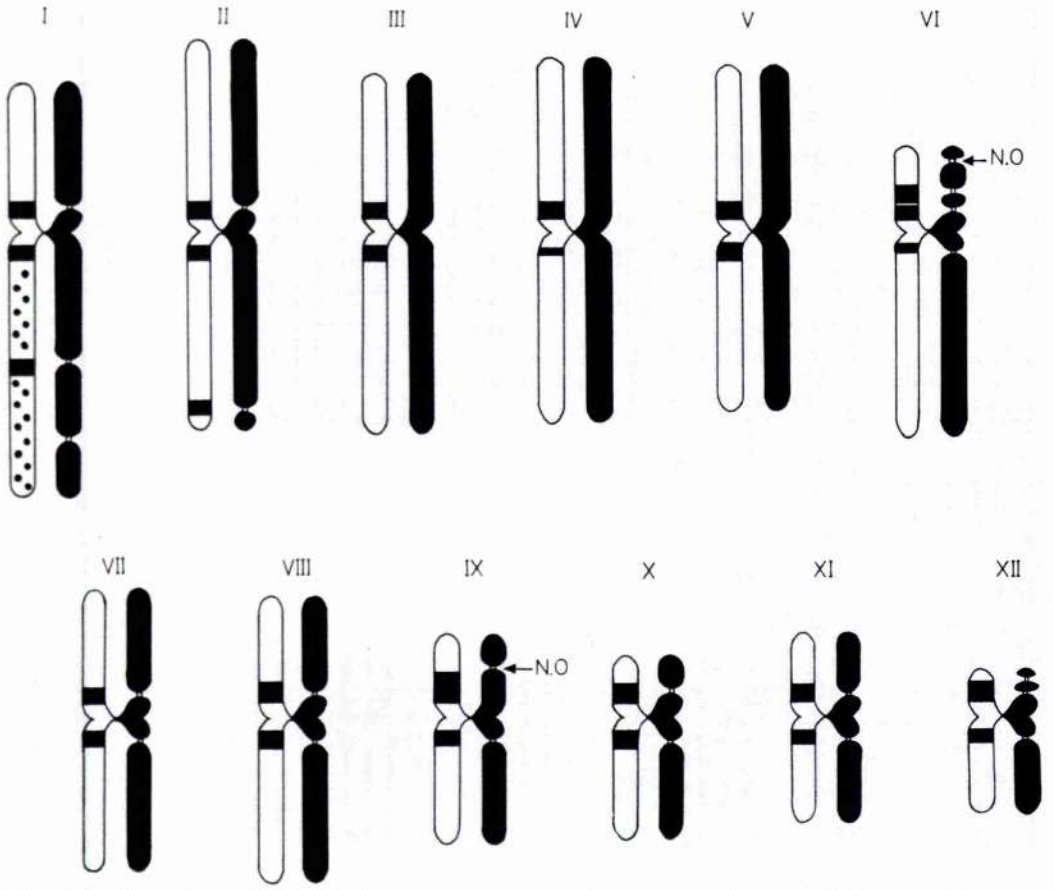


Fig. 5. A diagram of the haploid chromosome complement of cold-treated *T.c. carnifex*. In each chromosome the chromatid to the left is drawn so as to indicate the regions which stain intensely with Giemsa; the chromatid to the right is drawn so as to indicate the positions of secondary constrictions. *N.O.* nucleolar organizer constrictions on chromosomes VI and IX

independently constricted. As can be seen in Figure 5, most of the cold-induced constrictions, lie close to the centromeres, in one or both chromosome arms. Chromosomes III, IV and V do not show constrictions. Chromosome I has two intercalary constrictions in its longer arm, and chromosome II has a subterminal constriction in one arm. The shorter arm of chromosome VI has two cold-induced constrictions, and a subterminal nucleolar organizer constriction (as originally identified in lampbrush chromosomes by Mancino et al., 1972), making three in all. Chromosome IX carries another nucleolar constriction about half way along its shorter arm (see also Mancino et al., 1972); chromosome XII has two cold-induced constrictions in its shorter arm. Figure 5 shows the entirety of cold-inducible constrictions that we have observed, but it should be appreciated that not all chromosome complements show every constriction.

It will be apparent from Figure 5 that the chromosome regions which stain intensely with Giemsa following cold treatment show a general correspondence to the pattern of constrictions. There are, however, some discrepancies. Thus, for example, chromosomes III, IV and V show intensely staining regions on both arms close to the centromeres but as has already been mentioned, no

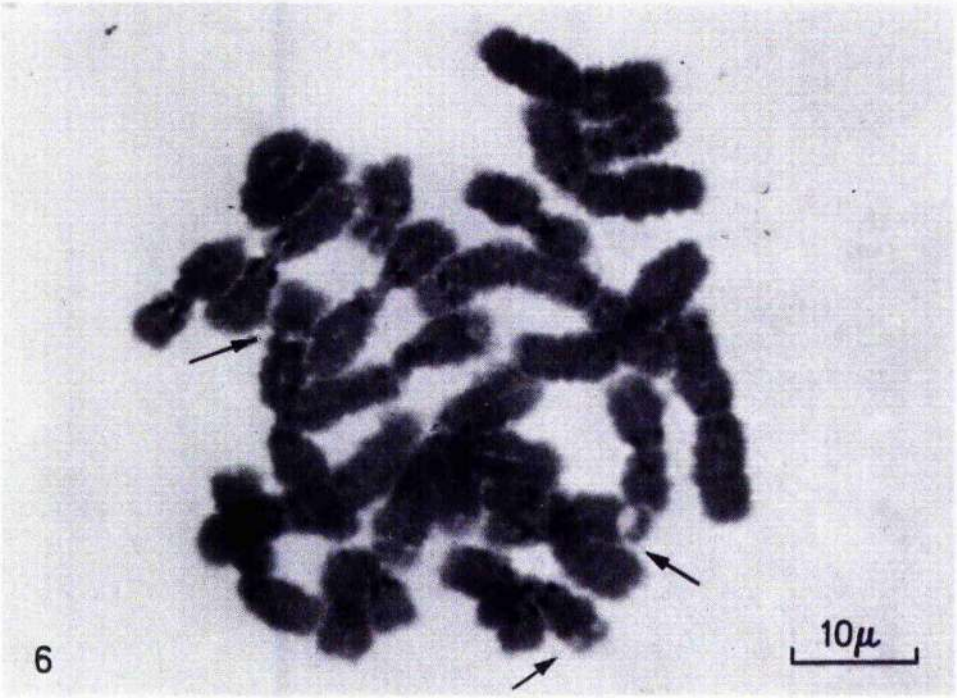


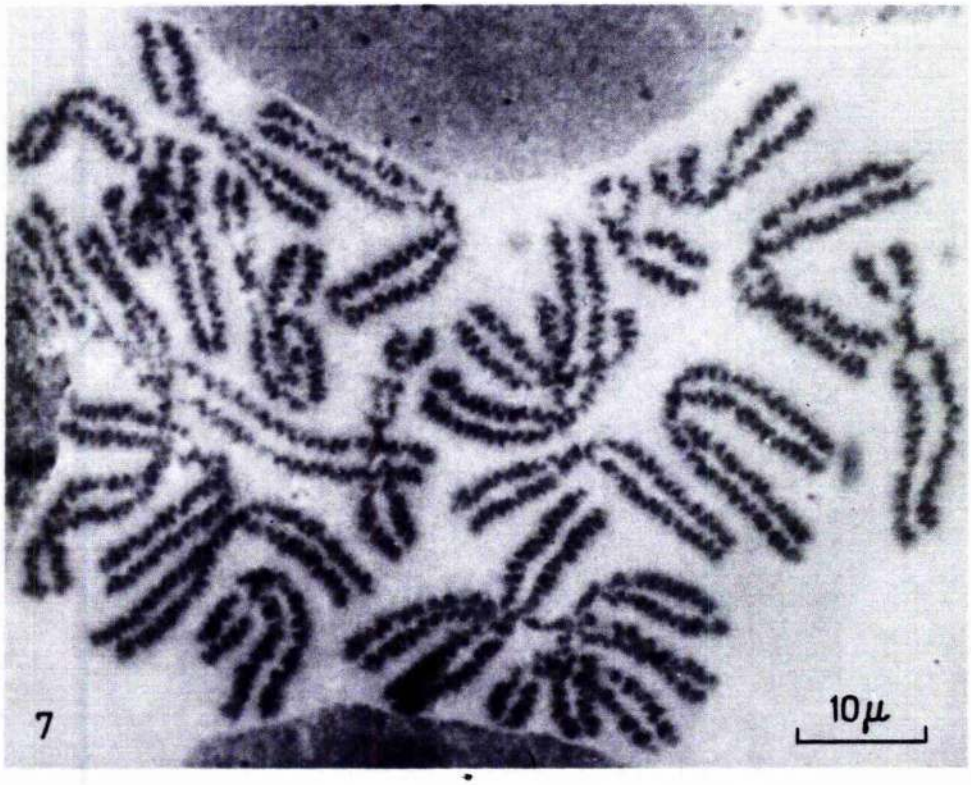
Fig. 6. Giemsa-stained mitotic chromosomes of a brain cell of *T.c. cristatus*. The larval newt spent 4 days at 4° C and was in colchicine for the last 18 h before fixation. The preparation was digested by trypsin before staining. 3 presumed nucleolar organizer constrictions are indicated by arrows

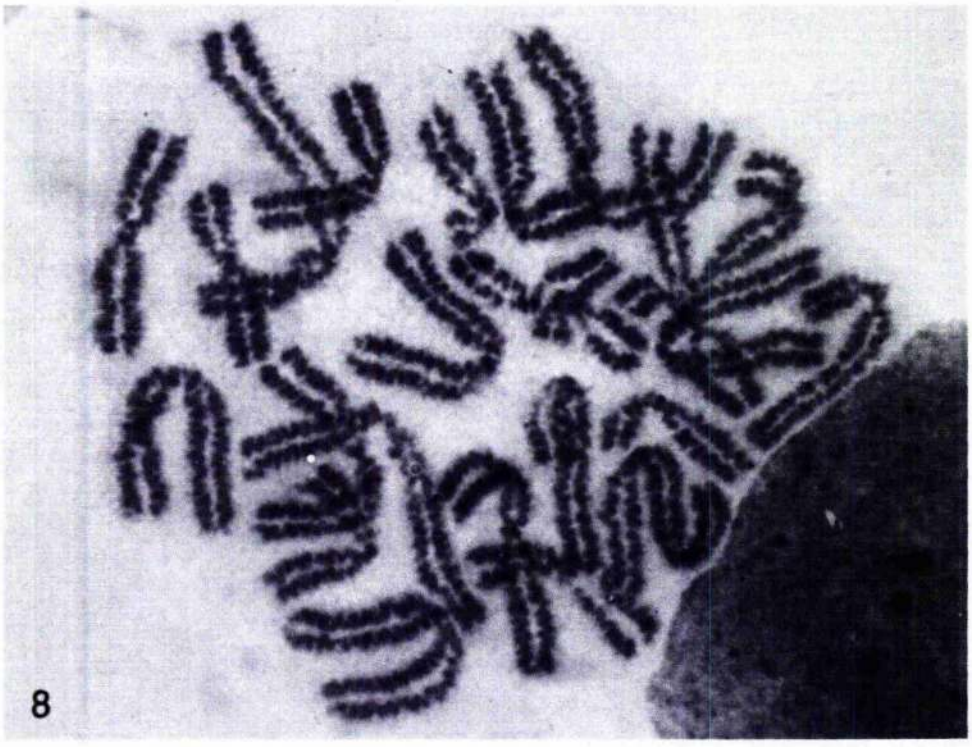
constrictions. If one compares the constrictions visible in Figure 2 with the intensely stained regions to be seen in Figures 3 and 4, the latter appear decidedly larger than their constricted counterparts. This may be illusory, for in *Triturus cristatus* (unlike *Ambystoma mexicanum*) the limits of the constrictions are not clearly defined.

The nucleolar organizer constrictions, unlike the cold-induced constrictions, do not stain intensely with Giemsa. This is especially noteworthy in the case of chromosome IX, where in the short arm there is a particularly large deeply staining segment not associated with a constriction, and a nucleolar organizer constriction that has no intensely staining counterpart. Another feature of interest is the long arm of chromosome I, which in Giemsa-stained preparations appears mottled.

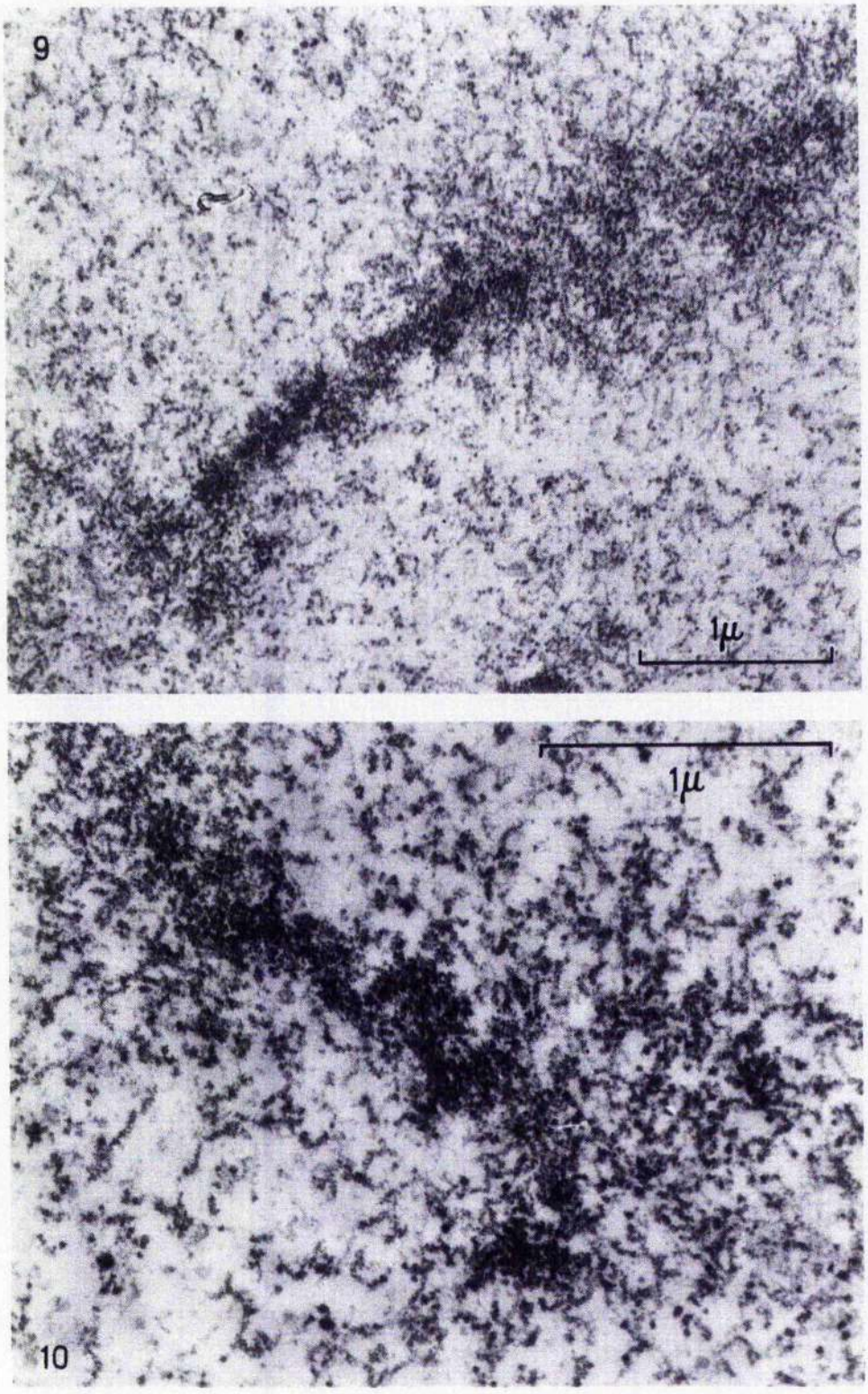
Giemsa-stained, cold- and colchicine-treated spermatogonial mitoses similarly show constrictions and/or differentially stained segments whose distribution is just like that found in larval somatic chromosome complements. However, a feature peculiar to spermatogonia is that the chromosomes frequently display spiral structure. It is not always possible to identify constricted regions in chromosomes showing spirals, but it is our impression that they are generally present, and distributed as in somatic chromosomes.

Preparations of spermatogonial mitoses treated according to the C-banding technique of Arrighi and Hsu (1971) also show chromosomes with regions differentially stained by Giemsa (Fig. 1). The intensely staining regions correspond in position to those found in Giemsa-stained somatic mitoses of cold-





Figs. 7 and 8. Two examples of orcein-stained mitotic chromosome complements of spermatogonia of *T.c. carnifex* showing spiral structure. The adult newt spent 4 days at 3° C, colchicine being injected 14 h before fixation. Immediately prior to fixation testis fragments were placed in distilled water for 15 min



Figs. 9 and 10. Ultrathin sections of parts of mitotic chromosomes of *T.c. carnifex* in epithelial cells of a larval tail fin. The sections were cut in the plane of the tail fin. The larva spent 4 days at 2° C prior to fixation, the last 18 h being in the presence of colchine. Both figures include constricted regions, where the chromatin fibres are more densely packed than elsewhere

treated newts, though they are smaller and less conspicuous; in addition, the centromeres show up as tiny deeply stained spots.

The preparations of hepatocyte mitoses from adult newts which had not been cold-treated prior to fixation were put through a G-banding technique involving digestion with trypsin before Giemsa staining. These preparations showed the same distribution of intensely stained regions in the chromosome complements as was later found in preparations of cold-treated tissues that had not been subjected to a G-banding technique. However the former preparations were of inferior quality, and we have therefore concentrated most of our attention on the cold-treated material.

Figure 6 shows a Giemsa-stained chromosome set from a brain cell of a larva of *T.c. cristatus* which had been cold- and colchicine-treated in the usual way, but the squash preparation was trypsin-digested before staining. It shows the intensely staining regions particularly clearly, and their distribution appears to be identical to that found in *T.c. carnifex*. There are also constrictions which do not have associated intensely staining material, and three such are evident in Figure 6. If these are nucleolar organizer constrictions, and this is likely, their positions differ from those of *T.c. carnifex*.

When examined in the electron microscope, favourable thin sections through epithelial cells of cold- and colchicine-treated larvae of *T.c. carnifex* show that the packing of the chromatin fibres is much tighter in the constricted regions than elsewhere (Figs. 9 and 10). Furthermore there is clear evidence of spiral organization in the constricted regions, the gyres having a pitch of about 0.4 μ. In cold- and colchicine-treated spermatogonial metaphase chromosomes, where the ordinary unconstricted parts manifest spiral structure (Figs. 7 and 8), the gyres are considerably wider and their pitch greater, of the order 0.8 μ.

Discussion

In Giemsa-stained metaphase chromosomes of cold-treated *Triturus cristatus carnifex* the pattern of intensely stained regions is identical to that produced by the C-banding technique except that the centromeres are not stained and, because they are constricted and elongate, the intensely stained regions are more distinct. In view of their appearance in the light microscope we have avoided applying the term "band" to the intensely stained regions, though they clearly correspond to the C-bands in *T.c. carnifex* described by Mancino et al. (1973).

We have not seen a chromosome complement of a cold-treated *T.c. carnifex* which shows secondary constrictions at all of the intensely staining regions, though the fact that all constrictions (apart from nucleolar organizers) coincide with intensely staining regions suggests that the density of chromatin packing in these regions is at least one factor responsible for their differential staining by Giemsa. By this statement we mean to imply that all the intensely staining regions are likely to be regions of especially dense chromatin packing, and hence potentially capable of constricting. Just as in *Ambystoma mexicanum* (Callan, 1966) some constrictions are visible in the great majority of chromosome

sets of cold-treated *T.c. carnifex*, whereas others are seen less frequently. The most comprehensive pattern of secondary constrictions is to be found in cells that were fixed at prometaphase, though the fuzzy chromosome outlines at this stage of mitosis undoubtedly mask some of the less pronounced constrictions. Moreover, we have frequently noticed that secondary constrictions which were originally visible in phase-contrast, before removal of the coverslip, are no longer evident after Giemsa staining, even when the chromosomes are dyed intensely and uniformly.

The pattern of intensely stained regions in the metaphase chromosomes of cold-treated newt tissues is also identical to that produced by the G-banding technique when applied to the chromosomes of newt tissues which had not been cold-treated before fixation. In these latter, however, the intensely stained regions generally extend slantwise across or partially across the chromatids, instead of lengthwise along constricted portions. We may therefore infer that whereas, when cultured at ordinary temperatures, the intensely staining regions are incorporated in the uniform spiral organization of the chromatids without disturbing that organization, culture at low temperature results in the rejection of the intensely staining regions from the standard spiral, whereupon they become constrictions with their own and different spiral organization, having narrower gyres of shorter pitch.

La Cour and Wells (1974) have studied the ultrastructure of the chromosomes of *Fritillaria lanceolata* where, as in newt chromosomes, segments of reduced width are induced by culture at low temperature. In this plant, however, the constricted regions are *less* opaque to electrons than are neighbouring unconstricted segments, the chromatin in these regions being more diffusely packed. This condition is the reverse of that obtaining in newt chromosomes, and we may therefore infer that the relationship between gyre width and pitch and the degree of chromatin compaction is more complex than might otherwise have been assumed.

Mancino et al. (1973) first described the predominantly pericentric distribution of the intensely staining regions in *T.c. carnifex* chromosomes, and noticed the unusual staining properties of the long arm of chromosome I, which on evidence from lampbrush chromosomes (Callan and Lloyd, 1960) is thought to be the sex-differentiating "heteromorphic" segment of this animal's karyotype. Ragghianti et al. (1973) also drew attention to the general correspondence between intensely staining regions, cold-inducible constrictions and the pattern of late DNA replication in this and other newt species (see also Callan, 1966; Callan and Taylor, 1968).

The simplicity of the pattern of intensely staining regions along the chromosomes of *T.c. carnifex* is surprising. There are only 27 such regions in a newt's haploid complement of 12 chromosomes, with a C-value of 29 pgm. The haploid human complement of 23 chromosomes, with a C-value of 3.5 pgm includes, by contrast, some 130 G-bands (Paris Conference, 1971). We had expected to encounter a pattern far more complex in newts, and we had anticipated that many of the intensely staining regions, if they were similar in size to the generality of the G-bands of the human genome, would occupy only parts of gyres of the spiralized chromatids. It is only in the long arm of chromosome I, where

the irregularly-staining region consists of a scatter of dark spots, none of which span the full width of the chromatid, that this expectation has been borne out.

It is hazardous to compare the newt and human genomes on the basis of the distribution of C- and G-bands because there is still so much uncertainty as to the factor or factors responsible for differential staining with Giemsa; however, let us suppose that differential density of chromatin packing is the prime factor. One point is then manifest: newt chromosomes (apart from chromosome I) do not have a multiplicity of small, differentially packed regions; it is as though some nondescript chromatin were liberally distributed throughout the lengths of newt chromosomes (except in the pericentric constricting regions) "diluting out" those component parts which, were they contiguous, would pack differentially from their neighbours.

It would be satisfying if one could make some correlation between the differentially staining regions of newt mitotic chromosomes and significant features of newt lampbrush chromosomes. Callan (1966) was unable to relate the cold-inducible secondary constrictions in the mitotic chromosomes of *Ambystoma mexicanum* to lampbrush characteristics of this organism. Similarly Hutchison and Pardue (1975), who were able to correlate the pattern of C-bands in *Notophthalmus viridescens* with the distribution of cold-inducible constrictions in the mitotic chromosomes of this newt, were unable of relate any of the pericentric C-bands to discernible lampbrush features, though they speculated tentatively about lampbrush correlates of subterminal C-bands on two chromosomes. Nardi et al. (1973) were also unable to correlate any of the pericentric C-bands in *Triturus vulgaris meriodionalis* with specific lampbrush features, though subterminal bands on two chromosomes were tentatively correlated with "sphere" loci.

The "spotty" staining behaviour of the long arm of chromosome I of *T.c. carnifex* possibly provides a clue as to lampbrush correlates of the regions of mitotic chromosomes which stain intensely with Giemsa. The heteromorphic arm of lampbrush chromosome I is distinguished by a few especially long lateral loops, and also by many axial granules. These latter are particularly large chromomeres, or aggregates of chromomeres, with or without associated lateral loops, and the number of these granules is of the same order as the number of intensely staining spots on the long arm of mitotic chromosome I.

Seven out of the 12 lampbrush chromosomes of *T.c. carnifex* have pericentrically located axial granules that are striking enough to have been recorded as landmark features. However, taken alone, each one of these axial granules represents a far shorter length of a lampbrush chromosome's axis than does any one of the pericentric intensely staining regions in a mitotic chromosome; furthermore there are a few outlying axial granules without intensely staining counterparts, while 5 of the chromosomes of the lampbrush complement lack recognizable axial granules in the appropriate pericentric locations. Whilst one may reasonably doubt that the "sphere" loci of *T.c. carnifex* are related to intensely staining regions (and the same is true of the "sphere" loci in *Ambystoma mexicanum*), this is a negative statement only, and it must be admitted

that the present study has failed to establish with any kind of assurance the identity of the lampbrush chromosome counterparts of regions which are so striking a feature of newt mitotic chromosomes.

References

Aimar, C.: Étude de la structure des chromosomes de la blastula, après traitement thermique, chez Pleurodeles waltlii Michah. C.R. Acad. Sci. (Paris) **264**, 1453–1456 (1967)

Arrighi, F.E., Hsu, T.C.: Localization of heterochromatin in human chromosomes. Cytogenetics **10**, 81–86 (1971)

Callan, H.G.: Heterochromatin in Triton. Proc. roy. Soc. Lond. **130**, 324–335 (1942)

Callan, H.G.: Chromosomes and nucleoli of the Axolotl, Ambystoma mexicanum. J. Cell Sci. **1**, 85–108 (1966)

Callan, H.G., Lloyd, L.: Lampbrush chromosomes of crested newts Triturus cristatus (Laurenti). Phil. Trans. B **243**, 135–219 (1960)

Callan, H.G., Taylor, J.H.: A radioautographic study of the time course of male meiosis in the newt Triturus vulgaris. J. Cell Sci. **3**, 615–626 (1968)

Caspersson, T., Farber, S., Foley, G.E., Kudynowski, J., Modest, E.J., Simonsson, E., Wagh, U., Zech, L.: Chemical differentiation along metaphase chromosomes. Exp. Cell Res. **49**, 219–222 (1968)

Caspersson, T., Zech, L., Johansson, C.: Differential binding of alkylating fluorochromes in human chromosomes. Exp. Cell Res. **60**, 315–319 (1970)

Comings, D.E.: Mechanisms of chromosome banding. IV. Optical properties of the Giemsa dyes. Chromosoma (Berl.) **50**, 89–110 (1975)

Comings, D.E., Avelino, E.: Mechanisms of chromosome banding. VII. Interaction of methylene blue with DNA and chromatin. Chromosoma (Berl.) **51**, 365–379 (1975)

Comings, D.E., Kovacs, B.W., Avelino, E., Harris, D.C.: Mechanisms of chromosome banding. V. Quinacrine banding. Chromosoma (Berl.) **50**, 111–145 (1975)

Darlington, C.D., La Cour, L.F.: Nucleic acid starvation of chromosomes in Trillium. J. Genet. **40**, 185–213 (1940)

Darlington, C.D., L. Cour, L.F.: The detection of inert genes. J. Hered. **32**, 115–121 (1941)

Flovik, K.: Cytological studies of arctic grasses. Hereditas (Lund) **24**, 265–376 (1938)

Gall, J.G., Pardue, M.L.: Nucleic acid hybridization in cytological preparations. In: Methods in enzymology, **21 D**, 470–480. New York-London: Academic Press 1971

Geitler, L.: Temperaturbedingte Ausbildung von Spezialsegmenten an Chromosomenenden. Chromosoma (Berl.) **1**, 554–561 (1940)

Golomb, H.M., Bahr, G.F.: Human chromatin from interphase to metaphase. A scanning electron microscopic study. Exp. Cell Res. **84**, 79–87 (1974a)

Golomb, H.M., Bahr, G.F.: Correlation of the fluorescent banding pattern and ultrastructure of a human chromosome. Exp. Cell Res. **84**, 121–126 (1974b)

Hutchison, N., Pardue, M.L.: The mitotic chromosomes of Notophthalmus (=Triturus) viridescens: localization of C-banding regions and DNA sequences complementary to 18S, 28S and 5S ribosomal RNA. Chromosoma (Berl.) **53**, 51–69 (1975)

Labrousse, M.: Aberrations chromosomiques induites et différenciation embryonnaire chez les amphibiens. Annls Embryol. Morph., Suppl. **1**, 199–210 (1969)

Labrousse, M.: Topographie des chromosomes mitotiques larvaires chez deux espèces d'Urodeles. C.R. Acad. Sci. (Paris) **271**, 2134–2136 (1970)

La Cour, L.F., Wells, B.: Fine structure and staining behaviour of heterochromatic segments in two plants. J. Cell Sci. **14**, 505–521 (1974)

Latt, S.A., Brodie, S., Munroe, S.H.: Optical studies of complexes of quinacrine with DNA and chromatin: implications for the fluorescence of cytological chromosome preparations. Chromosoma (Berl.) **49**, 17–40 (1974)

Lober, G., Zimmer, C., Sarfert, E., Dobel, P., Reigler, R.: Methylene blue nucleic acid complexes and the differential Giemsa staining. Studia Biophys. (Berl.) **40**, 141–150 (1973)

Mancino, G., Nardi, I., Ragghianti, M.: Lampbrush chromosomes from semi-albino crested newts, Triturus cristatus carnifex (Laurenti). Experientia (Basel) **28**, 856–860 (1972)

Mancino, G., Ragghianti, M., Innocenti, S.B.: I cariotipi di Triturus marmoratus e T. cristatus studiati con il "C-staining method". Atti Accad. naz. Lincei Rc. Ser. 8, **55**, 559–564 (1973)

Nardi, I., Ragghianti, M., Mancino, G.: Banding patterns in newt chromosomes. Chromosoma (Berl.) **40**, 321–331 (1973)

Okada, T.A., Comings, D.E.: Mechanisms of chromosome banding. III. Similarity between G-bands of mitotic chromosomes and chromomeres of meiotic chromosomes. Chromosoma (Berl.) **48**, 65–71 (1974)

Paris Conference (1971): Standardization in human cytogenetics. Cytogenetics **11**, 313–362 (1972)

Ragghianti, M., Innocenti, S.B., Mancino, G.: Bandeggiatura indotta da "C-, G- e Q-staining methods" e pattern di replicazione dei cromosomi di Triturus. Atti Accad. naz. Lincei Rc. Ser. 8, **55**, 764–770 (1973)

Schweizer, D.: Differential staining of plant chromosomes with Giemsa. Chromosoma (Berl.) **40**, 307–320 (1973)

Signoret, J.: Étude des chromosomes de la blastula chez l'Axolotl. Chromosoma (Berl.) **17**, 328–335 (1965)

Sumner, A.T.: Involvement of protein disulphides and sulphydryls in chromosome banding. Exp. Cell Res. **83**, 438–442 (1973)

Sumner, A.T., Evans, H.J.: Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa. II. The interaction of dyes with the chromosomal components. Exp. Cell Res. **81**, 223–236 (1973)

Sumner, A.T., Evans, H.J., Buckland, R.A.: New technique for distinguishing between human chromosomes. Nature (Lond.) New Biol. **232**, 31–32 (1971)

Sumner, A.T., Evans, H.J., Buckland, R.A.: Mechanisms involved in the banding of chromosomes with quinacrine and Giemsa. I. The effects of fixation in methanol-acetic acid. Exp. Cell Res. **81**, 214–222 (1973)

Wilson, G.B., Boothroyd, E.R.: Temperature-induced differential contraction in the somatic chromosomes of Trillium erectum L. Canad. J. Res. C **22**, 105–119 (1944)

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ABSTRACT

THE STRUCTRUAL ORGANIZATION OF NEWT MITOTIC CHROMOSOMES

Summary of thesis submitted for the degree of Doctor of Philosophy, October 1976, by Edwina-Anne Rudak, Department of Zoology, University of St. Andrews.

Chapter I.

After a period of culture at low temperature, mitotic chromosomes of the newt species Triturus cristatus and T. vulgaris show a characteristic pattern of predominantly pericentrically located secondary constrictions. The chromatin in these constricted regions stains intensely with Giemsa. When mitotic chromosome preparations are stained according to a C-banding technique, the centromeres and the interstitial regions which stain differentially after cold-treatment, stain intensely with Giemsa. Electron micrographs of sections through metaphase chromosomes in tail-fin cells of cold- and colchicine-treated larvae show that the chromatin fibres are more densely packed in the constricted regions than elsewhere. Hypotonically treated spermatogonia or tissue-culture cells of T.c. carnifex show spiral structure throughout the metaphase chromatids.

Chapter II.

T.c. carnifex skin fibroblasts can be maintained in monolayer culture in a predominantly diploid state for more than 14 months. The cells grow at 25°C in Eagles' MEM supplemented with 10% foetal calf serum and glutamine. The cell generation time is approximately 4 days. This is the only diploid urodele cell line maintained in any laboratory.

Chapter III.

Purified and iodine-labelled ribosomal RNA extracted from T.c. carnifex ovaries hybridises *in situ* to a region $^{2}/_{5}$ of the way down the long arms of both chromosomes X of T.c. carnifex tissue culture cells. When this RNA preparation is hybridised *in situ* to mitotic chromosomes of T.c. carnifex larval brain cells, labelled regions are found (i) near the telomeres of both chromosomes II, (ii) halfway down the long arms and at the ends of the long arms of both chromosomes X, (iii) near the centromere of one large metacentric chromosome, and (iv) halfway down the long arm of a medium-sized submetacentric chromosome. There is a variation in the labelling pattern shown by different T. vulgaris animals, and perhaps some cell to cell variation within the same animal.

Mitotic metaphase chromosomes of T.c. carnifex spermatogonia hybridised in situ with iodine-labelled 5S RNA are labelled about halfway down the long arms of both chromosomes X.

The numbers of nucleoli in methyl green/pyronine or "silver stained" cells of T.c. carnifex tissue culture or spermatogonia and T. vulgaris larval brain, are greater (up to 6-fold) than expected from in situ hybridization results. The apparent increase in nucleolar number is probably due to fragmentation of material from the original nucleoli.

Chapter IV.

The sister chromatid exchange frequency of T.c. carnifex tissue culture cells analysed by the FPG technique increases from about 20 at 1 µg/ml BUdR to about 50 at 100 µg/ml BUdR.