STUDIES ON THE PROTEINS OF THE OOCYTE NUCLEUS OF THE CRESTED NEWT, TRITURUS CRISTATUS CARNIFEX

Kinsey George Maundrell

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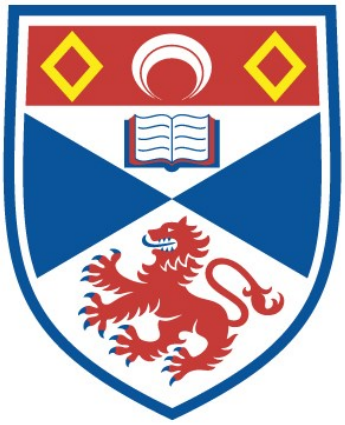
at the
University of St Andrews



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STUDIES ON THE PROTEINS
OF THE OOCYTE NUCLEUS OF THE CRESTED NEWT
Triturus cristatus carnifex

by

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A thesis submitted for the Degree of Doctor of Philosophy

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DECLARATION

I hereby declare that this thesis is my own composition, and that except where otherwise stated, the experimental work was performed by me alone. Some experiments performed in collaboration with Professor H.G. Callan, F.R.S., and Dr. R.J. Hill have been included in Chapter III, and a preliminary report of this work has been published in Nature New Biology, 242, 20-22 (1973).

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

2.4.73. K.G. MAUNDRELL.

CERTIFICATE

I certify that Mr. Kinsey George Maundrell has spent 12 terms at research work on the proteins of the oocyte nucleus of crested newts, that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

2.4.73. H.G. CALLAN.

UNIVERSITY CAREER

I began my University career in the School of Biological Sciences at the University of East Anglia in October 1966, and graduated with an upper second class Honours B.Sc. in June 1969. I entered the Zoology Department of the University of St. Andrews in October 1969, and spent three years doing post-graduate research into the proteins of the oocyte nucleus of the crested newt, *T.c. carnifex*. Part of my post-graduate work was carried out during a period of 6 months at the Max-Planck-Institut, Tübingen. The results of my researches are presented here for the degree of Doctor of Philosophy.

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CHAPTER I.

GENERAL INTRODUCTION

Development of the amphibian oocyte is protracted. In natural populations of Triturus cristatus, the great crested newt, this process may take several years to complete. For most of this time the course of meiosis is arrested at diplotene, and the oocyte undergoes a period of intense synthetic activity and growth during which it changes from a translucent cell of some 200μ diameter to a mature, yolk-laden and opaque oocyte with a diameter of over 1.5 mm. (Fig. 1). Throughout this period of development the chromosomes assume the characteristic 'lampbrush' appearance shown in Figure 6(a), in which paired lateral loops arise from regions of densely packed chromatin, the chromomeres, situated at intervals along the length of the chromosome (Callan, 1955; Gall, 1954, 1956). Enzymatic digestion experiments indicate that each lateral loop consists of a central axis of extended DNA supporting a ribonucleoprotein (RNP) matrix (Macgregor & Callan, 1962). The polarised distribution of RNP matrix that is evident in many of the loops, suggests that matrix accumulates with time, and this is borne out by autoradiographic evidence that newly synthesized RNA and protein become rapidly associated with the loop structure (Gall & Callan, 1962).

One of the most significant features of this period of growth is the enormous increase in the total amount of cellular RNA (Osawa & Hayashi, 1953; Davidson et al., 1964; Gall, 1966). Biochemical fractionation shows that most of this RNA is ribosomal, and indeed in the case of Xenopus oocytes, Davidson & Mirsky (1965) estimate that this fraction constitutes about 98% of the RNA synthesized during the lampbrush stage of oogenesis (stages 4 and 5 according to Duryee, 1950). In addition, they show that

a considerable quantity (47 ng) of template active RNA is synthesised by the oocyte, (Davidson et al., 1966) and that this represents activity of at least 10^4 genes (Davidson & Hough, 1969). It seems that much of this informational RNA remains inactive during oogenesis, and is inherited by the embryo at fertilization. There is now an impressive body of evidence to suggest that the maternal mRNA becomes derepressed during development, and is responsible for controlling the early stages of embryogenesis (for a review of this topic, see Spirin, 1966).

The capacity to store mRNA in a repressed form heralds an innovation of great consequence in eukaryotic physiology, namely the ability to regulate not only the transcription of RNA, but also to exert translational control over its ultimate expression. In the developing embryo, the flexibility inherent in such an arrangement is fully exploited. In this case it seems probable that information required to carry out the early stages of development cannot be effectively dispensed by transcription from the chromosomes of the embryo, for during this period the chromosomes are almost totally involved in the process of replication, and furthermore the duration of interphase is very much curtailed by recurrent mitoses. Instead, therefore, the information required for this process is synthesised months beforehand, during oogenesis, and is stored in a repressed form in the cytoplasm of the mature egg to await fertilization. When fertilization occurs, the egg undergoes a dramatic increase in the rate of protein synthesis (Hultin, 1950; Mano & Nagano, 1966), which is maintained throughout early development, even though very little synthesis of RNA on the embryonic chromosomes (other than histone mRNA, (Kedes & Gross, 1969)) can be detected until gastrulation (Bachvarova et al., 1966; Crippa et al., 1967). Early development of the embryo is therefore directed by the maternal mRNA inherited through the egg cytoplasm.

An understanding of the mechanism by which derepression of specific messengers occurs according to the developmental schedule seems a formidable task. What appears certain is that each mRNA must have a unique identity recognisable by the cellular machinery, and I think it likely that such individuality is somehow conferred on the mRNA molecule soon after its synthesis during oogenesis. However, the idea that "embryogenesis begins at oogenesis" is not a new one(Wilson, 1896).

In a provocative essay on a possible mechanism by which translational control could operate, Stent (1964) proposed that in bacteria there is an obligatory coupling between transcription and translation. The discussion was based largely on Beckwith's (1963) observations on the so-called operator negative mutation of E. coli, in which synthesis of RNA from the lac operon (Jacob & Monod, 1961) had become non-inducible as a result of a mutation at the operator region, the acceptor site for RNA-polymerase. Jacob & Monod had considered that this condition resulted from the polymerase being no longer able to recognise this sequence of DNA. However, Beckwith found that the mutation was suppressible, that is, RNA could be produced normally from the lac operon, if a second mutation was introduced into another part of the genome coding for a tRNA molecule. On the basis of these observations, Stent postulated that the original mutation in the operator region had produced a nonsense codon for which there was no tRNA; that the attachment and operation of RNA polymerase had been unaffected; but that since the nonsense codon was untranslatable, ribosomes were unable to travel past this point on the nascent messenger, except in those strains in which the second, suppressor mutation in the tRNA anticodon had been sustained. From these conclusions, he argued that immediate translation of the nascent messenger RNA was necessary to release it from contact with the DNA template,

and thus allow subsequent RNA polymerase molecules to proceed along the DNA. In other words, genetic activity could be controlled by regulating the process of translation. This idea is supported by the later observations of Jones et al. (1968), that RNA transcribed in vitro from T7 DNA remains bound in an RNA-DNA-RNA polymerase complex, and further, that addition of purified 70S ribosomes causes dissociation of this complex and release of the messenger in the form of an RNA-ribosome complex. Essentially the same results were obtained by Bremer & Konrad (1964) and by Shinn & Moldave (1966). Stent then took his argument one stage further, and proposed that as a result of the degeneracy in the genetic code, adjusting the population of tRNA molecules in circulation could, in theory at least, permit the expression of certain regions of the DNA by allowing its nascent mRNA to be translated, but repress others in which the nascent RNA contained codons not matched by anticodons in the tRNA pool. To what extent this scheme of control could operate in eukaryotic organisms is another matter. On the face of it, the fact that animal cells have the capacity to produce mRNA's, which are not translated immediately, argues against a direct application of Stent's proposal to eukaryotic cells, as indeed does the fact that transcription and translation become separated by the nuclear membrane. Nevertheless, a limited amount of protein synthesis is still a possibility in animal cell nuclei (Allfrey et al., 1964) and it may be that newly synthesised RNA, even that destined for long term storage, is translated once to free it from the DNA. Moreover, this is particularly germane to the situation in oocytes, where a high turnover of radioactive amino-acids in the loop matrix has been observed (Gall & Callan, 1962). I think the possibility of nuclear synthesis of protein is not excluded by existing data.

In the next chapter I have investigated the site of origin of the protein in the oocyte nucleus, after which the relevance of these ideas to eukaryotic physiology will be assessed in more detail. In the third chapter, I investigate some of the properties of the proteins associated with oocyte nuclear RNA.

Experimental material

All the work in this thesis has been performed on oocytes of the Italian race of the crested newt, Triturus cristatus carnifex (Laurenti). As experimental material in a study of nuclear proteins, ovarian tissue from urodele amphibia has two notable advantages. First, in an appropriate saline, oocytes can be incubated for up to 20 hours without significant reduction in synthesising ability, which has allowed the in vitro incorporation of radio-active precursors into RNA and protein to be achieved with convenience; and secondly, nuclei from mid-oogenesis oocytes are sufficiently large to allow manual isolation and collection to be undertaken as a routine procedure, and this has proved particularly useful in the second section of the work in which it was important to obtain nuclear protein free from cytoplasmic contamination.

The techniques for handling this material have been set out in detail previously, (Callan & Lloyd, 1960), and with the exception of some of the salines used, these procedures have been adhered to throughout.

CHAPTER II.

AN AUTORADIOGRAPHIC STUDY OF PROTEIN MOVEMENT WITHIN THE OOCYTE

Introduction

Evidence for protein synthesis within the cell nucleus has been sought from many eukaryotic systems in the past, though no decisive results have emerged in support of it (Allfrey et al., 1964; Reid & Cole, 1964; Reid et al., 1968). On the other hand, despite a wealth of circumstantial evidence, direct attempts to disprove nuclear synthesis of proteins has been equally inconclusive. For example, the autoradiographic studies in which oocytes are given a short pulse of ^{3}H-amino-acids, result in a uniform distribution of label in both nucleus and cytoplasm. Clearly, transport of protein during such an experiment can affect the final distribution, and no firm conclusions regarding the actual site of synthesis can be drawn from these data. By reducing the length of the pulse to 2 minutes, about the time required to synthesise a protein molecule, I hoped it might be possible to eliminate the complication arising from transport of labelled protein, and that in this situation the presence or absence of protein synthesis in the nucleus could be inferred from the pattern of incorporation. But the low level of incorporation above background, and the uncertainty over the extent of penetration of precursors inherent in such a short-term experiment, precluded any conclusions from being drawn.

In the experiments described below, I have investigated the origin of nuclear protein by adopting a different procedure. The experimental protocol in this case was to allow oocytes to incorporate ^{3}H-amino-acids

for a given length of time, and then to inhibit further synthesis of protein by addition of cycloheximide. If nuclear proteins are indeed synthesised in the cytoplasm, incubation of oocytes for increasing periods of time after suppression of protein synthesis should lead to transport of previously labelled proteins across the nuclear membrane, and hence to progressive accumulation of radioactive material in the nucleus. The results of the following experiment are expressed as a series of ratios (each being grain counts over a 50μ square of nucleus/grain counts over a 50μ square of cytoplasm), obtained from oocytes fixed at various times after addition of cycloheximide. The results support the idea that proteins present in the oocyte nucleus are synthesised in the cytoplasm.

Materials and methods

In vitro incorporation of ^{3}H-amino-acids into ovary fragments

Ovaries were removed from the newt through a ventral incision in the body wall, as described in detail elsewhere (Callan & Lloyd, 1960). Freshly isolated ovarian material was placed in frog Ringer solution (NaCl 0.65%, KCl 0.014%, $CaCl_2$ 0.012%, NaH_2PO_4 0.001%), and all mature oocytes were removed. This step was included because the high concentration of dense yolk characteristic of the later stages of oogenesis, prevents adequate penetration of wax necessary to achieve successful embedding and sectioning. The remainder of the ovary was divided into fragments each containing about 12 oocytes within the size range 0.4 mm to 0.7 mm, and these were used for the experimental incubation with ^{3}H-amino-acids.

Incubations were carried out in covered solid watch glasses (embryo-cups) containing 0.2 mls frog Ringer, with bovine serum albumen present at a concentration of 30 mgm/ml. The action of BSA here is twofold; first it helps to produce an osmotically acceptable environment for the oocytes, and secondly it has the effect of chelating trace amounts of contaminating heavy metal ions which could be toxic to the oocyte metabolism.

Tritiated amino acids were obtained from the Radiochemical Centre, Amersham. The mixture of radioactive precursors was made up of equal volumes of the following amino-acids:- L-tryptophane (500 mCi/mM), L-S-phenylalanine (750 mCi/mM), L-leucine (500 mCi/ mM), and L-lysine (256 mCi/mM). The final activity of the solution was 1 mCi/ml, and 10 µl aliquots were used for each incubation.

Preparation for autoradiography

After experimental incubations, oocyte fragments were rinsed briefly, and fixed overnight in San Felice's fixative made up as follows:-

Solution A.	2% chromic acid	100 ccs
	20% acetic acid	60 ccs
Solution B.	Distilled water	60 ccs
	40% formaldehyde	100 ccs

Solutions A and B were mixed in the ratio 1 : 1 immediately prior to use.

Fixation was followed by prolonged washing in running tap water to remove all traces of chromium salts which could otherwise produce 'chemical' exposure of the autoradiographic emulsion. Dehydration, and

embedding in 55°C melting-point wax, was carried out according to standard procedure, and 5μ sections were cut and dried on to slides coated with a thin layer of albumen. The wax was removed with xylene, and the slides then taken through a series of alcohols to water, and again washed overnight in running tap water. AR10 stripping film (Kodak Ltd.) was applied, and after thorough drying in a stream of air, the slides were stored in a light tight box and left at 4°C to expose.

Processing and analysis of autoradiographs

Autoradiographs were developed after 16 days exposure. The use of AR10 on unsubbed slides is complicated by the fact that when wet, the film tends to lift up from the slide, and shift with respect to the section. This can be prevented by hardening the film in 2% formalin for 10 minutes as soon as possible after fixation. Treated in this way, the film adheres to the slide, and the subsequent stages in the processing can be carried out with safety. The slides were washed thoroughly, after formalin treatment, and while the film was supple, the flaps covering the back of the slide were carefully trimmed away with a razor blade. Sections were stained for 10 minutes in toluidine blue (0.004% in M/1000 phosphate buffer, pH 6), rinsed in distilled water, and left to dry in racks above a hotplate. Quantitative autoradiography directly from the slide proved difficult and inaccurate, so I decided to photograph the sections, and then measure grain densities from the enlarged prints. Differential contraction of the nucleus and cytoplasm when the sections were dried on to slides, produced preparations in which grains over the nucleus were at a lower focal plane than those over the cytoplasm, so for any given oocyte section,

TABLE 1.

cycloheximide concentration (μg/ml)	specimen weight (mg)	counts per minute (adjusted for mean background of 38 cpm)	cpm/mgm of tissue
0	1.2	1294	1080
	1.9	4256	2240
2.5	1.5	71	47
5	2.6	78	30
	0.8	102	128
10	2.2	44	20
	1.8	47	26
20	2.2	51	23
	2.7	88	33
200	3.1	62	20
	1.8	50	28

the nucleus and cytoplasm were photographed separately. Grains were counted over unit areas, arbitrarily 50μ squares, of which four were routinely counted in each photograph.

Results and discussion

(A) To determine the concentration of cycloheximide necessary to inhibit protein synthesis in vitro

Cycloheximide, an antibiotic which has been isolated from Streptomyces griseus (Whiffen et al., 1946), has been shown to be an effective inhibitor of protein synthesis in many eukaryotic systems. A study of its mode of action shows that it blocks the transfer of amino-acids from the amino-acyl-tRNA complex to the growing polypeptide chain bound to the ribosome (Siegel & Sisler, 1965), and that in cell-free extracts this occurs at very low concentrations. Using intact cells however, the effective concentration of inhibitor shows considerable variation depending on the system used, and for this reason, the empirical determination of levels of cycloheximide necessary to inhibit protein synthesis in the oocyte is an essential prerequisite for this work.

Ovary fragments were prepared according to the standardised procedure, and duplicate samples were incubated in the following concentrations of cycloheximide: 0 μg/ml (control), 2.5 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 200 μg/ml. After 1 hour in contact with the drug, radioactive amino-acids were added, and the samples left to incubate for a further 4 hours. At the end of this time, incorporation was stopped by fixing the oocytes in cold 0.5% TCA, where they were left overnight to allow unbound amino-acids to diffuse out. The samples were then dried to

constant weight on pre-weighed coverslips, so that in each case the dry weight of material could be calculated. The dried oocytes were then transferred to scintillation vials, digested in Nuclear Chicago Solvent (obtained from G.D. Searle & Co.) and counted in 5 mls of a 0.4% PPO - 0.005% POPOP - toluene cocktail. From these data, a value for the c.p.m. per milligramme of tissue was calculated for each sample, and the effectiveness of different concentrations of inhibitor compared (Table 1). Taking the figures for 0 µg/ml cycloheximide as 100% incorporation it is clear from the results that a drug concentration of 10 µg/ml produces 98% inhibition of protein synthesis, and further, that concentrations in excess of this produce no enhancement of the inhibitory performance. 10 µg/ml thus appears to be the lowest effective concentration of cycloheximide to use with this system. This concentration was used in all the following experiments in which inhibition of protein synthesis was involved.

(B) An autoradiographic study of the origin of nuclear protein

In the experiment described below, I present evidence for the cytoplasmic origin of nuclear protein by showing transport of previously labelled protein from the cytoplasm into the nucleus during conditions in which further synthesis of protein was inhibited. Pre-labelling of oocytes was carried out for either 1 hour or 2 hours before addition of cycloheximide. The incubations were as follows:

TABLE 2.

Oocyte size	Experimental details: Number	Period of labelling before addition of cycloheximide (hr.)	Period of incubation after addition of cycloheximide (hr.)	Mean grain counts per 50μ square of nucleus	Mean grain counts per 50μ square of cytoplasm	Ratio of N:C grain counts
0.4 mm						
	1(a)	1	0	52 (7.7*/12**)	97 (15.1*/12**)	0.54
	1(b)	1	1	86 (7.3/8)	76 (10.0/8)	1.13
	1(c)	1	2	156 (14.4/16)	128 (10.1/16)	1.22
	2(a)	2	0	177 (26.1/16)	257 (24.1/16)	0.68
	2(b)	2	1	208 (20.1/12)	211 (24.5/12)	0.99
	2(c)	2	2	412 (15.6/12)	368 (26.7/12)	1.12
	3			49 (6.0/8)	60 (4.5/6)	0.82
0.7 mm						
	1(a)	1	0	63 (13.7/14)	104 (12.9/16)	0.61
	1(b)	1	1	134 (11.9/16)	147 (16.1/16)	0.91
	1(c)	1	2	95 (15.8/16)	86 (11.1/13)	1.10
	2(a)	2	0	69 (7.7/15)	99 (10.4/15)	0.70
	2(b)	2	1	137 (11.0/16)	158 (10.8/16)	0.87
	2(c)	2	2	164 (16.6/12)	160 (9.8/12)	1.01
	3			41 (6.1/8)	39 (10.8/6)	1.05

Figures in brackets show standard deviation (*) and number of sections examined (**).

1 (a) 1 hour with ^{3}H-amino-acids.

(b) 1 hour with ^{3}H-amino-acids, followed by 1 hour with cycloheximide.

(c) 1 hour with ^{3}H-amino-acids, followed by 2 hours with cycloheximide.

2 (a) 2 hours with ^{3}H-amino-acids.

(b) 2 hours with ^{3}H-amino-acids, followed by 1 hour with cycloheximide.

(c) 2 hours with ^{3}H-amino-acids, followed by 2 hours with cycloheximide.

3 Cycloheximide control to ensure that protein synthesis was inhibited in the experimental conditions used:-

1 hour with cycloheximide, followed by a further 2 hours after addition of ^{3}H-amino-acids.

At the end of experimental incubations the oocytes were removed from the medium, rinsed briefly and processed for autoradiography as described under Materials and Methods.

The results of grain counting over 50μ squares of nucleus and cytoplasm from sections of oocytes fixed at various times after the addition of cycloheximide are shown in Table 2. For each set of results, a mean value for the ratio : grain count over 50μ square of nucleus/grain count over 50μ square of cytoplasm was calculated and plotted against time after addition of cycloheximide. Data were collected for two sizes of oocytes, 0.4 mm diam and 0.7 mm diam. The results are presented in figs 3(a) and 3(b). The behaviour of newly synthesized protein within the oocyte can be inferred from the changing distribution of radioactive material. In all 4 cases, there is an increase in the grain count ratio with time after addition of cycloheximide, and I interpret this as showing movement of newly synthesized protein from the cytoplasm and into the nucleus.

Essentially the same conclusion has been reached by several other people for other biological systems, (Zetterberg, 1966; Robbins & Borun, 1967; Arms, 1968; Merriam, 1969), and it certainly seems that migration of proteins from their site of synthesis in the cytoplasm, into the nucleus is a general feature of eukaryotic cells.

A quantitative analysis of this experiment is more difficult. It is not known, for instance, to what extent the labelled amino-acids dilute the pre-existing precursor pool within the 2 hours of the experimental incorporation. Nor is it known what proportion of the newly synthesized protein is made up of the 4 radioactive amino-acids used in the experiment. Consequently, even knowing the specific activities of the radioactive precursors, and the amount of radioactivity incorporated, any attempt to derive the absolute quantity of protein synthesized would be unrealistic. In spite of this difficulty, I include the following section because I think it yields profitable, even if approximate, information.

From Figure 3, it is apparent that in all 4 cases the rate of entry of radioactive protein into the nucleus falls off during the second hour of incubation with cycloheximide. It might be argued that this reflects an overall reduction in metabolic activity during the incubation. However, I have shown (Fig. 4) that under similar <u>in vitro</u> conditions, incorporation of ^{3}H-amino-acids into TCA-precipitable material is constant for at least 12 hours of incubation, which suggests that for the few hours duration of this experiment, any general reduction in metabolic activity is minimal. I think there is an alternative explanation of the results in Fig. 3. Implicit in the demonstration that entry of protein into the nucleus occurs independently of protein synthesis, is the fact that newly synthesized protein destined for the nucleus first forms a pool in the cytoplasm.

During the extended incubation with cycloheximide, migration of protein from the cytoplasm into the nucleus, causes a gradual depletion of cytoplasmic pool, which in the absence of further protein synthesis cannot be restored. The rate of entry of labelled protein into the nucleus therefore decreases during the later stages of incubation, as shown in Fig. 3.

If this latter explanation is correct, that is, if migration of protein into the nucleus during incubation with cycloheximide occurs at the expense of protein in a cytoplasmic pool, it should be possible to estimate the magnitude of this mobile protein fraction. For the reasons given above, to do this in absolute terms would be unwise, so I propose here to express the mobile protein as a proportion of the total newly synthesized protein in the cytoplasm. The calculation depends upon the following assumptions:

(a) given a sufficiently long incubation in cycloheximide, all the nuclear protein in the cytoplasmic pool would eventually migrate into the nucleus. Extrapolating from the curves in Fig. 3, I suggest that an approximate value for the grain count ratio at this point would be around 1.2.

(b) no significant movement of labelled protein from the nucleus to the cytoplasm occurs during the incubation.

For convenience I have taken as unit quantity of protein, that quantity which under the present conditions of incubation produces 1 grain in the autoradiographic emulsion after 16 days exposure. I have assumed that grains have only been produced by disintegrations within the top micron of the section, and therefore that the grain count over a 50μ square is an arbitrary measure of the amount of protein in a volume of

50 x 50 x 1 cu. μ. In 0.7 mm oocytes, the nuclear diameter is about 300μ. Using the data in table 2 for 0.7 mm oocytes in experiment 1 (a):-

Total quantity of newly synthesised protein in the cytoplasm

= (grain count/cu. μ) x (volume of cytoplasm in cu. μ)

$$= \frac{104}{50 \times 50 \times 1} \times \frac{4\pi}{3} \times (350^3 - 150^3)$$

$$= \underline{6 \times 10^6 \text{ units}}$$

Quantity of mobile protein in the cytoplasmic pool

= ((postulated max grain count/cu. μ in the nucleus at the end of cycloheximide incubation) - (grain count/cu.μ at the beginning of cycloheximide incubation)) x (volume of nucleus in cu. μ)

$$= \frac{(104 \times 1.2) - 63}{50 \times 50 \times 1} \times \frac{4\pi}{3} \times 150^3$$

$$= \underline{3 \times 10^5 \text{ units}}$$

Thus the magnitude of the cytoplasmic pool of nuclear protein

$$= \frac{3 \times 10^5}{6 \times 10^6} \times 100 = 5\%$$ of newly synthesised protein in the cytoplasm.

It is somewhat arbitrary to express the pool size as a proportion of total protein synthesised within 1 hour; perhaps a more informative calculation based on these results would be to determine for any given moment in time what proportion of total protein synthesised is protein destined to enter the nucleus. Making the same assumptions as before, I have expressed the amount of radioactive isotope incorporated into nuclear protein within 1 hour, as a proportion of radioactive incorporation

into cytoplasmic protein during the same period. Again using the data from 0.7 mm oocytes from experiment 1,

Total nuclear protein synthesized within 1 hour,

= (postiated max. grain count/cu. μ in the nucleus) x (volume of nucleus)

$$= \frac{1.2 \times 10^4}{50 \times 50 \times 1} \times \frac{4\pi}{3} \times 150^3$$

$$= \underline{7 \times 10^5 \text{ units}}$$

As above, total cytoplasmic protein synthesised within 1 hour

$$= \underline{6 \times 10^6 \text{ units}}$$

∴ the proportion of nuclear protein to total protein synthesised within 1 hour

$$= \frac{7 \times 10^5}{(6 \times 10^6 + (7 \times 10^5)} = 0.1$$

In other words 10% of protein synthesis occurring in 0.7 mm oocytes concerns protein destined to move into the nucleus.

Finally, using these values, it is possible to deduce the rate of flow of nuclear protein through the cytoplasmic pool. Making the two assumptions stated above, I have shown that 10% of protein synthesised within 1 hour is destined for the nucleus, and furthermore 5% of the protein synthesised within 1 hour is nuclear protein present in a cytoplasmic pool. Clearly, at any given moment the cytoplasmic pool contains 5/10ths of the nuclear protein synthesised within 1 hour, from which it follows, assuming a constant pool size, that protein remains in the cytoplasmic pool for an average period of 5/10 hours, i.e. about 30 minutes, before being transported into the nucleus.

Such a lengthy treatment of these results may be objectionable, in view of the speculative nature of the assumptions on which it is based. I include it without comment, except that I think in the absence of anything more precise, the approximate information afforded by these results is worthwhile.

Incidental observations on the labelling characteristics of the nucleoli in the present study deserves mention. A comprehensive account of this subject is not possible because, as will be recalled from the materials and methods section, older yolky oocytes were removed before incubation to facilitate sectioning, and observations are therefore restricted to oocytes up to about 0.7 mm diameter. It is clear from these, however, that in all except the very youngest oocytes, considerable accumulation of newly synthesised protein occurs in the nucleoli. The significance of this observation is not understood at present. Whether or not it is related to synthesis of ribosomal RNA, which starts soon after the beginning of oogenesis, is an open question.

Consideration of the function of rapidly labelled protein entering the nucleus

From an autoradiographic study of this nature only a very limited insight into the intra-nuclear localisation of the newly synthesised protein is possible. In a previous study, (Gall & Callan, 1962), short term incorporation of ^{3}H-amino-acids followed by autoradiography of isolated chromosome preparations has shown that extensive accumulation of newly synthesised protein occurs on the loop matrix. Although the possibility that this protein is synthesised locally is difficult to eliminate, the present demonstration that protein migrates from the cytoplasm to the

TABLE 3.

Oocyte size	Experimental details			Mean grain counts per 50μ square of nucleus	Mean grain counts per 50μ square of cytoplasm	Ratio of N:C grain counts
	Experiment	Period of incubation with Act-D. (hr.)	Period of incubation with radioactive amino-acids (hr.)			
0.7 mm						
	Act-D.	1	2	72 (15.1*/16**)	99 (19.2*/15**)	0.73
	control	0	2	69 (7.7/15)	99 (10.4/15)	0.70

Figures in brackets show standard deviation (*), and number of sections examined (**).

nucleus, and the wealth of circumstantial evidence that translation is a cytoplasmic phenomenon, argues against this idea, and supports the more likely alternative that all nuclear protein is synthesised in the cytoplasm, and that upon entering the nucleus, a proportion of it becomes associated with the loop matrix. Furthermore, in view of the demonstrable synthesis of RNA on the loops, it seems likely that this protein is involved in some aspect of the production or processing of nascent RNA. In the following experiment, I have investigated the effect of inhibiting RNA synthesis on the transport of newly synthesized protein from the cytoplasm into the nucleus.

Oocytes were prepared routinely, were incubated for 1 hour with 10 µg/ml actinomycin D, a concentration competent to suppress RNA synthesis in vitro (Snow & Callan, 1969), and then for a further two hours with ^{3}H-amino-acids. At the end of this time, the oocytes were removed from the medium, and processed for autoradiography as described above. The grain count ratio was again taken as a convenient parameter to study the intracellular distribution of newly synthesized protein, and the results are shown in table 3. The data from oocytes labelled for 2 hours with ^{3}H-amino-acids but in which prior actinomycin D treatment was omitted, is included in the table as a control. Evidently, the grain count ratios from the two experiments are indistinguishable, from which it follows that suppression of RNA synthesis has no detectable effect on the short-term distribution of newly synthesized protein within the oocyte.

The effect of actinomycin D on lampbrush chromosomes has been studied in detail (Snow & Callan, 1969). Apart from causing an abrupt termination of RNA synthesis, exposure of oocytes in vitro to a drug

concentration of 10 µg/ml for 2 hours produces in all but a few exceptional cases, loss of matrix from the lateral loop axis, and collapse of loop structure. Under these conditions, it seems unlikely that protein could continue to accumulate on the loops, and yet the evidence from this experiment indicates that protein entry into the nucleus continues apparently undisturbed. (This point will be taken up again later).

In considering the function of protein on the loops it is apposite to mention certain features of the giant granular loop on chromosome XII of T. cristatus cristatus. This loop is unusual in several respects. As shown by Snow & Callan, it is one of the few loops to retain its gross morphological features following exposure to actinomycin D, and this reaction is almost certainly related to another peculiarity, namely that transcription is limited to a short region of the loop axis close to the thin insertion end. It is noteworthy that in spite of this restricted transcription, newly synthesised protein accumulates throughout the entire length of the loop (Gall & Callan, 1962) and although the significance of this observation is unknown it follows that in this, albeit unusual loop, as well as protein associated directly with newly synthesised RNA, secondary addition of protein to the matrix also occurs.

If, as the experiments in this section indicate, nuclear protein is synthesised in the cytoplasm, and is subsequently transferred to the nucleus by a process which is independent of RNA synthesis, then some reappraisal of the idea discussed in the introduction that transcription and translation are linked, seems required for eukaryotic organisms. In Stent's original proposal based on studies with bacteria, two aspects of this situation are discussed. In the first place, translation of nascent RNA is necessary to free the newly formed molecule from its contact with

the DNA template, and so allow transcription to continue. Secondly, as an implicit extension of this scheme, direct control of genetic expression is possible through regulating the process of translation.

I suggest that in eukaryotic organisms, both these aspects may be equally applicable, but in the situation where stability of the intracellular environment is guaranteed, such instantaneous control over transcription is no longer necessary, and this has allowed the two events to become separated in time. I propose that in a eukaryotic system, the release of RNA from the DNA template is performed not by the ribosomes, but by a component of the newly synthesised protein which enters the nucleus and which becomes associated with the RNA; and that the ultimate expression of genetic information in the resulting RNA/protein complex is deferred, and regulated in the cytoplasm, by control of translation. Participation of tRNA in this latter process, perhaps along the lines proposed by Stent (see p. 4) presents an interesting possibility. Certainly changes in tRNA have been observed in a variety of differentiated tissues (Suoeka & Kano Suoeka, 1970), though whether these changes are a cause or a result of differentiation is unknown. The discovery that removal of the terminal trinucleotide sequence, -pCpCpA, from tRNA destroys its amino-acid accepting ability (Preiss *et al.*, 1959), and the fact that enzymes capable of restoring this sequence have been isolated and purified (Furth *et al.*, 1961), implies the competence of tRNA to exert both qualitative and quantitative control over RNA translation (Ames & Hartman, 1963). Moreover, such a mechanism could be especially important as a controlling influence in early development, during which, in sea urchins at any rate, considerable turnover of nucleotides in the -pCpCpA terminal of tRNA has been demonstrated (Glisin & Glisin, 1964; Gross *et al.*, 1965). In a process as complex as translation, however, several levels of control could, and most probably do, operate.

C H A P T E R III.

ANALYSIS OF OOCYTE NUCLEAR PROTEINS BY GEL ELECTROPHORESIS

Introduction

Protein constitutes about 98% of the material present in the lateral loop matrix of lampbrush chromosomes (J. Sommerville, unpublished data). A consideration of lampbrush chromosome structure, and in particular the morphological diversity apparent at different loci raises the question which originally prompted this section of the work, namely to what extent are the matrix proteins 'loop specific', or conversely, to what extent does the common function of RNA production involve proteins common to all loops?

In approaching this problem I decided to use the technique of gel electrophoresis to analyse the nature of the chromosomal proteins. At first, solubilising the protein on the loops proved a difficult problem, though eventually two methods were developed by which this could be achieved. It is a pleasure to acknowledge the help of Dr. R.J. Hill and Professor H.G. Callan, in several aspects of this work.

Materials and experimental methods

(A) Isolation and collection of nuclei

In all experiments described below, oocytes were removed from the animal immediately prior to isolation of nuclei. Cytological evidence indicates that oocytes can be stored at $4^{\circ}C$ for a period of at least a day without deterioration; but in the present study it was felt preferable only to use freshly isolated material, so as to reduce the possibility of

sub-microscopical changes arising from endogenous enzymic activity. From such a freshly isolated ovary, a small fragment was taken, and placed in a saline solution consisting of 0.075 M KCl and 0.025 M NaCl, an ionic ratio and concentration closely resembling that in the oocyte (Riemann, Muir & Macgregor, 1969). The procedure for manually extracting handling nuclei has been described before, (Callan & Lloyd, 1960) and this procedure has been employed throughout. Briefly, it is as follows: the follicle membrane at the base of a selected oocyte is held with forceps, while the follicule membrane investing the oocyte is punctured using a sharpened mounted needle. By applying gentle pressure to the oocyte with the needle the cell contents can be extruded through the hole; in most cases the nucleus is easily distinguishable. It can be removed by pipetting, and any yolk and other cytoplasmic material adhering to the nuclear membrane can be dislodged by 'bouncing' the nucleus several times against the bottom of the dish. The nucleus is then transferred to a second dish containing a suitable saline (see below) and washed again. Routinely, nuclei isolated and cleaned in this way are massed in an ice-cold siliconised dimple dish. Care is taken in this final transfer to pass across as little fluid as possible and thus maintain a small sample volume, which is an important consideration for the subsequent electrophoresis. The whole process is carried out under a dissecting microscope at 16x magnification. It is necessary that this operation be performed with as little delay as possible, because once they have been isolated into saline, nuclei begin to swell up; the evidence from interference microscopy carried out on such isolated nuclei indicates that after about 2 minutes, though not before, this swelling is accompanied by loss of protein through the membrane (Macgregor, 1962). In most cases however, 2 minutes is a generous deadline for this operation.

Most of the experiments described have been in some way concerned in characterising the protein on the lateral loops, and for this reason, in all cases unless otherwise stated, nuclei have been obtained from oocytes of about 0.7 mm diameter, the oldest stage in which the loop extension is at a maximum.

For cytological observations, chromosomes were isolated into Tris buffered saline (TBS) pH 7.3 made up as follows:-

KCl/NaCl 3 : 1	0.1 M
Tris buffer, adjusted to pH 7.3	0.01 M
$MgCl_2$	0.001 M
$CaCl_2$	0.5×10^{-4} M

Calcium ions are included to encourage the dispersal of nuclear sap, hence freeing the chromosomes.

Preparations were examined in an observation chamber consisting of a bored microscope slide with a coverslip attached by paraffin wax to the lower surface. The chamber so formed can be filled with saline, and chromosomes isolated into the chamber settle on the bottom and can be examined through the lower coverslip using an inverted phase contrast microscope (Zeiss plankton).

For some experiments, nuclei were isolated into an unbuffered saline containing 0.075 M KCl, 0.025 M NaCl. This solution is referred to as "unbuffered 3 : 1".

(B) Electrophoresis

Separation of acid proteins on polyacrylamide gels was performed using the alkaline discontinuous buffer system of Ornstein & Davis (see Davis, 1964). According to this method, the proteins stack at pH 8.9, and separate at pH 9.5. The value of a stacking system is that it allows large samples to be concentrated into thin starting zones before electrophoresis. Briefly, the theory of stacking is based upon an early observation of Kohlrausch that ions moving in an electric field are subject to a 'regulating function' which causes them to stack and migrate in the order of their mobilities. By performing this operation in a large-pore matrix to prevent local distortion of the zones, it is theoretically possible to concentrate an infinitely large sample providing the distance through which stacking occurs is equally large. In the system of Ornstein and Davis the proteins stack at pH 8.5, under which conditions they assemble and migrate between the leading chloride ion, and the trailing glycine ion. The stacked proteins thus cross the pH discontinuity and enter the separating gel as a sharp zone. At the higher pH and with the smaller pore size of the separating gel, the regulating conditions break down, and the glycine ion runs ahead of the proteins leaving them to electrophorese in a uniform environment at about pH 9.5, in which mobility is determined by net charge and molecular size.

The use of a stacking system in these experiments, where collection of nuclei necessarily entails a relatively large sample volume, is virtually obligatory for successful separation.

Stock solutions

(A)	1N HCl	48 ml
	Tris	36.3 gm
	Temed**	0.23 ml
	H_2O to make (pH 8.8 - 9.0)	100 ml
(B)	1 N HCl	48 ml*
	Tris	5.98 gm
	Temed	0.46 ml
	H_2O to make (pH 6.6 - 6.8)	100 ml
(C)	Acrylamide	28.0 gm
	Bis***	0.735 gm
	H_2O to make	100 ml
(D)	Acrylamide	10 gm
	Bis	2.5 gm
	H_2O to make	100 ml
(E)	Riboflavine per 100 ml H_2O	4.0 mg

Catalyst: Ammonium persulphate 0.14%

Reservoir buffer (10x as used)

Tris	3.0 gm
glycine	14.4 gm
H_2O to make	1 litre

Temed, Acrylamide and Bis acrylamide were obtained from Eastman Chemical Company.

Riboflavine was obtained from Sigma Chemical Co.

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* pH adjusted by titration with 1 N HCl.

** Temed = N, N, N^1, N^1 - tetramethylethylene diamine

*** Bis = N, N^1 - methylene-bis acrylamide.

Working solutions mixed fresh daily

Separating gel solution	Stacking gel solution
1 part (A)	1 part (B)
2 parts (C)	2 parts (D)
1 part H_2O	1 part (E)
(to form gel combine 1:1 with catalyst)	4 parts H_2O
	(to form gel expose to fluorescent light)

Gels were polymerised in siliconised glass tubes held vertically in a rack. The polymerisation reaction is inhibited by oxygen, and for this reason the working solutions were de-gassed immediately before use. As a further precaution, atmospheric oxygen was excluded during the polymerisation by careful layering of water on to the surface of the monomer solution with a fine bore pipette. This step also has the advantage of producing a flat surface to the gel so that the zonation in the sample created during stacking remains undisturbed as it migrates across the interface between upper and lower gels.

Preparation of the sample

In most cases nuclei were collected in unbuffered 0.1 M 3 : 1 saline. Such a high ionic strength reduces the voltage gradient through the sample during electrophoresis and seems to disrupt the stacking process, producing a 'leading' effect on the migrating proteins. This difficulty can be overcome by diluting the sample to a final ionic strength of about 0.05 M before electrophoresis. One tenth of the sample volume of stock solution B (upper gel buffer) is added to the sample to promote the Kohlrausch stacking conditions, and also 5 µl of 0.1% bromophenol blue is included to track the buffer front.

Two gel sizes have been used; 0.2 x 4.0 cms, and 0.5 x 5.0 cms depending upon the amount of material available. Optimum running conditions were as follows:

0.2 x 4.0 100 v until the protein moves into the gel, then 250v.

0.5 x 5.0 1 mA per tube until protein is in the gel, then 2.5 mA per tube. Samples were run until the bromophenol blue reached a measured mark on the tube.

Removal of the gel after electrophoresis was most easily accomplished by first of all breaking the contact between the gel and the inside of the tube using a long syringe needle, after which the gels slipped out either spontaneously, or else with slight pressure from a rubber bulb. Gels were stained in 0.25% Coomassie Brilliant Blue made up in methanol (25 parts) : acetic acid (7 parts) : water (68 parts). They were destained in the same methanol/HAc/H_2O solution.

(C) Photography of cytological preparations.

During the course of this work, the effect of various reagents on the integrity of the lampbrush loops has been investigated. Cytological preparations were made in an observation chamber described in Materials and Methods, and examined by phase contrast microscopy, using the Zeiss plankton microscope. Preparations of interest were photographed with the same optical system, but to overcome the problem of Brownian movement, the microscope light source was replaced by a Ukatron UN 60 flash unit, supplying flashes of 30 watt secs. A fine grain film, pan F was used, and suitable neutral density filters were included, depending on the magnification.

(D) In vitro incorporation of radioactive precursors into oocytes

Radioactive precursors of either RNA, or protein, were transferred to a solid watchglass and the solution dried down in a stream of warm air. The radioactive precipitate was then dissolved in 0.5 mls of Steinberg's medium (0.34% NaCl, 0.005% KCl, 0.008% $Ca(NO_3)_2.4H_2O$, 0.02% $MgSO_4.7H_2O$, 4.7 mM Tris, 1.00 N HCl to pH 7.4) which was found to be the most suitable for *in vitro* incubation of oocytes. Freshly excised pieces of ovary from which large yolky oocytes had been removed were then transferred to the radioactive medium, and the chamber sealed to prevent evaporation. With this arrangement incubation can be continued for at least 12 hrs at room temperature without significant reduction in synthesising capacity (see Fig. 4).

(E) Detection of radioactivity in polyacrylamide gels

The following procedure was used for detecting the association of rapidly labelled RNA with nuclear protein. After incubation of oocytes with ^{3}H-uridine for 6 hrs, protein was prepared from nuclei and electrophoresed in the normal way. After electrophoresis, the gel was stained for protein, and scanned in a Joyce Loebl densitometer. The gel was then frozen, and sliced transversely into 1 mm thick discs using a Mickle gel slicer. Each slice was transferred to a scintillation vial, and incubated with 2 mls H_2O_2 at 50°C for two days. At the end of this time, the gel matrix was completely dissolved, and 20 mls freshly prepared Bray's scintillant (naphthalene 6%, PPO 0.4%, POPOP 0.02%, methanol 10%, ethylene glycol 2% in 1,4 dioxane) was added to each vial. Radioactivity was recorded for each slice, and plotted against the densitometer trace.

(F) Electron microscopy

(i) Positively stained preparations

Fixation and embedding

Chromosomes were isolated into Tris buffered saline in an observation chamber made from a perspex disc 20 mm in diameter. In such a chamber, chromosomes can be centrifuged in the swinging bucket head of a Sorvall bench centrifuge. After centrifugation, chromosomes adhere firmly to the glass coverslip, and can be removed with the coverslip providing no air-water interface is allowed to pass across the preparation. Chromosomes were fixed for 5 minutes in 10% glutaraldehyde made up in 0.04 M phosphate buffer (pH 7.4) and post-fixed for a further 60 minutes in 1% osmium tetroxide in veronal acetate buffer pH 7.4. Dehydration and embedding were carried out as follows:

70% ethanol	30 min.
95% ethanol	1 hr.
abs. ethanol	1 hr.
abs. ethanol/impregnation mixture (1 : 1)	2 hr.
impregnation mixture	4 hr.
embedding mixture	4 hr.

Impregnation mixture

Epoxy resin (Araldite CY 212)	50 ml.
Dodecenylsuccinic anhydride (DDSA)	50 ml.
Dibutyl phthalate	2 ml.

For embedding the same mixture was used after addition of 2 ml 2,4,6,-tri(dimethylaminomethyl)phenol (DMP 30). Reagents were obtained from TAAB laboratories, Reading.

The specimen was transferred to fresh embedding medium, and a gelatin capsule containing araldite was inverted over the chromosomal preparation and left to polymerise at 60°C for 48 hours. After polymerisation, the block was chilled on solid CO_2 to facilitate removal of the coverslip. In most cases the coverslip came away easily leaving the chromosomes embedded in the face of the block.

Sectioning and staining

Under a dissecting microscope, chromosomes were just visible in the araldite. The block was pared away with a razor blade to a final size of about 1 mm square. Sections were cut using a Huxley ultramicrotome, picked up on copper E.M. grids coated with formvar, and dried in air. The staining procedure was as follows:

2% aqueous uranyl acetate	5 min. Drain.
double distilled water	1 min. Drain and dry.
0.3% lead citrate	2 min
0.02 N NaOH	2 min. Drain.
double distilled water	1 min. Drain and dry.

Sections were examined in a Siemens Elmiskop I electron microscope.

(ii) Negatively stained preparations

Chromosomes were centrifuged at 3000 rpm for 10 minutes on to carbon coated formvar films supported on a copper EM grid. The grid was removed, rinsed briefly in double distilled water, and negatively stained for 1 minute in filtered 1% PTA pH 6.5. Grids were dried in air and examined in a Siemens Elmiskop I electron microscope. In cases where chromosomes were treated first with RNase, titanium grids were used instead of copper.

(G) Molecular weight determination by electrophoresis in SDS-acrylamide gels

The procedure used for molecular weight determination of proteins was essentially that described by Shapiro et al., (1967), in which the electrophoretic mobility of an unknown protein in SDS gels is compared to the electrophoretic mobility of marker proteins of known molecular weight run under the same conditions. The presence of SDS causes denaturation of proteins, and the formation of highly negatively charged micelles. This effectively eliminates charge differences between proteins and in this situation comparison of electrophoretic mobilities reflects differences in molecular size only.

Preparation of the unknown sample

Details of collection and solubilisation of proteins will be described later. Once obtained, the protein was incubated in 0.1% SDS, 5% 2-mercaptoethanol for 3 hours. Glycerol was added to give a final concentration of 10%, and a small volume of bromophenol blue was included to mark the buffer front during electrophoresis.

Preparation of the marker proteins

The following proteins were obtained from the Sigma Chemical Co. and used to calibrate electrophoretic mobility:- phosphorylase-A (95,000), bovine serum albumin,BSA, (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), myoglobin (17,000), ribonuclease-A (13,500).

To make the stock solutions, 10 mg of each protein was dissolved in 1 ml of 0.01 M sodium phosphate buffer pH 7.1, 1% SDS, 1% 2-mercaptoethanol, and incubated for 3 hours at 37°C. Each protein was then dialysed overnight against 0.01 M sodium phosphate, 0.1% SDS, 0.1% 2-mercaptoethanol, and stored at - 20°C. In addition to the standard proteins prepared in this way, the unreduced BSA dimer (136,000) dissolved in 0.01 M sodium phosphate, 0.1% SDS but without 2-mercaptoethanol, was also included as a molecular weight marker. Each standard protein was then prepared for electrophoresis as follows:

Protein stock solution	10 pts ($(BSA)_2$ 15 pts)
Glycerol	10 pts
2-mercaptoethanol	5 pts (not with $(BSA)_2$)
0.1% bromophenol blue	2 pts
dialysis buffer	to make 100 pts

5 lambdas were taken from each sample for electrophoresis. Two proteins were run on each gel, with the exception of unreduced $(BSA)_2$ which was run alone.

Preparation of SDS-acrylamide gels

Stock solutions	A Acrylamide
	B 0.2 M phosphate
	C 5% SDS
	D Bis acrylamide
	E 0.085 gm ammonium persulphate in 8 mls H_2O
Electrode buffer	0.1 M sodium phosphate pH 7.1, 0.1% SDS.

To prepare the working solution, stocks were mixed in the following proportions:- 1A : 2.5B : 0.1C : 1D : 0.4E. Gels were polymerized in siliconized glass tubes measuring 0.2 x 4 cms.

Protein was layered carefully on to the gel surface under the electrode buffer, and electrophoresed at 1 mA per tube for 10 minutes, then at 3 mA per tube until the bromophenol blue front had travelled a measured distance through the gel. Gels were removed and stained for protein as described earlier.

In comparing mobilities of proteins in different gels, correction was made for the slightly differing distances travelled by the bromophenol blue buffer front during electrophoresis. Since the gel contracts on fixation, the first step involved measuring the gel length both before and after fixation, and reducing the bromophenol blue mobilities accordingly. Protein mobilities were then normalised against the corrected buffer front mobilities.

(H) Estimation of the absolute quantity of protein per ocoris muscle using the Lowry procedure

In this method for estimation of proteins, (Lowry et al., 1951) the final colour is obtained in two distinct steps (a) reaction of the protein with copper in alkali, and (b) reduction of the phosphomolybdic-phosphotungstic reagent by the copper-treated protein.

Reagents

A 2% Na_2Co_3 in 0.1 N NaOH
B 0.5% $CuSO_3$ in 1% solution of sodium tartrate
C Alkaline $CuSO_4$ produced by mixing 50 mls of reagent A with 1 ml of reagent B.
D Folin Ciocalteu reagent.

Procedure

20 nuclei from 0.7 mm oocytes, were collected and hydrolysed for 30 minutes in 0.5 mls of 0.1 N NaOH. To this sample, 2 ml of alkaline $CuSO_4$ (reagent C) was added, and the solution was allowed to stand at room temperature for about 10 minutes. 0.05 mls of Folin Ciocalteu reagent was then added, shaken immediately, and left to stand for a further 30 mins to allow the colour to develop. Absorption was read at 650 mμ and the amount of protein calculated from a standard dilution curve for bovine serum albumin.

Results and Discussion

(a) Analysis of the soluble proteins of the nuclear sap throughout development of the oocyte

For much of the year, the newt ovary contains oocytes in all stages of development. Two external features of the oocyte serve to indicate its developmental progress, namely, the diameter and the extent of yolk deposition. Oocyte diameter increases from about 0.2 mm in the very youngest cells to about 1.5 mm at maturity. Deposition of yolk starts in oocytes of about 0.6 mm diameter; the yolk at this stage appears white in colour, and it turns green later when the cell has reached a diameter of about 1 mm.

Identifiable nuclear changes also accompany the development of the oocyte. In the first place, the nucleus undergoes a considerable increase in size, although this is not directly related to its synthetic activity, as can be seen from an examination of the chromosomes at different stages of development. Well extended lateral loops are present in the smallest nuclei it is possible to examine. The loops increase in length reaching a maximum size, and transcribing capacity, in oocytes of about 0.5 - 0.8 mm diameter, but then from this point onwards, retraction of the loops into the chromomeres is progressive, and by maturity, when the nuclear volume is at its maximum, chromosomes are completely condensed in preparation for the first meiotic division. Increase in nuclear size is therefore unrelated to synthetic activity, but rather appears to be a result of accumulation of protein (and RNA) as the oocyte develops.

In some cases it is possible to follow the development of individual loci throughout oogenesis. The giant granular loop on chromosome XII of T.s. gristatum, for example, shows the general pattern of loop behaviour, increasing in size as oocytes increase in size to about 0.6 mm diameter, then remaining stable, and decreasing in size from about mid-oogenesis onwards. On the other hand, the giant fusing loop on chromosome XII behaves atypically, continuing to increase in size right up until maturation. This behaviour is also shown by the sphere loci. Changes in the distribution, synthetic activity and appearance of nucleoli have also been correlated with development of the oocyte. During the period of most active ribosomal RNA synthesis, they are firmly attached to the inner surface of the nuclear membrane, but finally, as maturation approaches, they detach from the membrane, and migrate into the centre of the nucleus.

In conducting the present experiment, I expected that the morphological and physiological changes discussed above would be accompanied by changes in the protein composition of the nuclear sap, and that such changes might be revealed by electrophoretic analysis. For this reason, and also to serve as a background for future experiments, a systematic study of nuclear proteins at different stages of oogenesis was undertaken.

Oocytes of T.s. carnifex can be divided into four classes, with at least superficial physiological justification. The number of nuclei isolated in each case was determined from consideration of nuclear volumes at different stages.

Oocyte stage		No. of nuclei
0.5 - 0.6 mm	previtellogenesis	50
0.6 - 0.7 mm	early yolk deposition	25
0.7 - 0.9 mm	lateral loop extension maximal	20
0.1 - 1.5 mm	extensive yolk deposition) chromosomes condensed	10

Nuclei were collected in unbuffered 3 : 1. After centrifugation, the low speed supernatant from each pool, containing the soluble proteins of the nuclear sap, was withdrawn, and run without further treatment on polyacrylamide gels. A comparison of acidic nuclear proteins from such an experiment is shown in Fig. 5. The features to emerge from this experiment are (a) the relatively uncomplicated banding pattern obtained from an electrophoretic analysis of the acidic proteins of the nuclear sap, and (b) the high degree to which this characteristic pattern is maintained throughout oogenesis. The presence of additional proteins (0.14, 0.49) in the nucleus as the oocytes reach maturity has been observed on some occasions, though this is not invariably the case.

In view of the physiological changes discussed above, which accompany the development of the oocyte, this constancy in the proteins composition of the nucleus is remarkable. However, in addition to the 8 or so major components, there is a considerable quantity of stained protein present in the background, and this presumably represents a large number of minor species present in amounts which are too low to form detectable bands. Regulatory proteins seem generally to be present in small amounts, and it is probable that the proteins involved in directing the changes during oogenesis would come into this category; they would certainly contribute to the background protein. It is also possible that

interaction between proteins during electrophoresis could be partly responsible for the continuous distribution of stainable material observed in this experiment.

The gradual decrease in the proportion of nuclear protein in band 0.56 in the later stages of oogenesis is, I think, related to the progressive retraction of the lateral loops into the chromomeres which occurs from about mid-oogenesis onwards. The reasons for coming to this conclusion will become apparent in section D of this chapter.

(B) Estimation of the protein content of an oocyte nucleus using the Lowry procedure.

The data in the previous section shows that development of the oocyte is accompanied by a marked increase in both size and protein content of the nucleus. From the electropherograms in Fig. 5 it appears that no new major species of protein enter the nucleus during oogenesis, but rather, that the increase in total protein is through further accumulation of those species already present. To gain some idea of the absolute quantities of protein involved, the present estimation was undertaken.

Most of the experimental work in this thesis has involved nuclei from 0.7 mm oocytes, the stage at which maximal extension of lateral loops has been attained. 20 such nuclei were hydrolysed in 0.1 N NaOH for 30 minutes. The Lowry procedure was then carried out on this sample as described in Materials and Methods, and the absorbancy at the end of the reaction read at 650 mμ. To determine the absolute quantity of protein from the absorbancy value so obtained, reference was made to a calibration curve in which the absorbance values of standard dilutions

of bovine serum albumin treated in the same way as the sample, were plotted against the amount of protein in each reaction (Fig. 19). The Folin-Ciocalteu reagent reacts strongly with tyrosine residues, and to a lesser extent with peptide bonds. Since proteins can differ widely in their tyrosine content, the use of bovine serum albumin for calibrating the absorbancy has an obvious limitation. However bearing this limitation in mind, the data in Figure 19 indicate that 20 nuclei from 0.7 mm oocytes contain approximately 24 µg of protein; in other words the protein content of a single nucleus at this stage of oogenesis is about 1.2 µg.

(C) Solubilisation of proteins from the loop matrix

The chemistry of interactions between RNA and protein is not well understood, and probably varies from one biological system to another. Techniques for dissociating RNA-protein complexes must to a large degree be developed empirically.

An advantage of using oocytes as experimental material is that chromosomes can be isolated and examined in an unfixed state, and the effects of a variety of treatments can be assessed by direct microscopic observation. The results of two such treatments for solubilising the protein component of the lampbrush loop matrix are discussed below.

(1) RNase/urea

Chromosomes isolated into dilute solutions of pancreatic RNase (2 µg/ml) show two cytologically distinguishable reactions. First, there is an immediate, and virtually total, loss of matrix from the lateral loops, and a concomitant accumulation of granular material in

the nucleoplasm. Second, after incubation at room temperature for about 10 minutes, the granules begin to link up to form fibres, and during the next 30 minutes this network of fibres thickens and gradually invades the entire chromosome preparation. Digestion of the preparation at this stage with pan-protease demonstrates the protein nature of the fibres. The stages in this reaction are documented in Fig. 6.

The molecular events underlying these observations, however, are not completely clear. In electron micrographs of negatively stained preparations (Fig. 7) the fibres can be seen as aggregates of a fairly uniform particle approximately 800 A in diameter. In most cases, the ultrastructure of this polymerising unit is obscure, but in occasional examples numerous smaller subunits can be distinguished, each measuring approximately 200 A in diameter. Electron micrographs of sectioned, positively stained chromosomes (Fig. 8) show that the loop matrix is also particulate in nature, with particle sizes in this case ranging from 200 A to 400 A in diameter. The following is suggested as the most plausible explanation of these data.

It is generally acknowledged that RNA is transcribed from the DNA template in the lateral loops (Miller, 1965). Addition of protein to the nascent RNA appears to be tightly coupled with the process of transcription, though it seems likely that a certain time interval may elapse during which the youngest region of the RNA molecule is unprotected. In any event, it seems reasonable to suppose that the growing end of the polynucleotide chain is the most vulnerable, and it is therefore not surprising that it becomes preferentially digested on exposure to RNase, thereby producing the observed stripping effect, and simultaneous accumulation of particles, presumably matrix RNP, in the nucleoplasm.

As the reaction proceeds, the enzyme continues to digest the RNA in the granules, thus breaking up the matrix, probably into the 200 A units identified by electron microscopy. Evidence will be presented later (section G), that undegraded RNA is also liberated with, and protected by, this 200 A protein complex. I suggest that this particle then spontaneously polymerizes into the 800 A unit, which in turn aggregates to form the fibres visible in cytological preparations. Further evidence, presented later, suggests that there exists another major protein component, distinct from the 200 A complex, in both the fibres and the loop matrix.

Isolation of chromosomes into 2 µg/ml cytochrome C, a basic protein of low molecular weight and similar in many respects to RNase, has no effect either on the integrity of the loop matrix, or the polymerisation of material from the nuclear sap, which argues that fibre formation is a result of the enzymatic digestion of RNA, and not simply a nucleating effect of a small basic protein.

The suggestion from these observations therefore, is that the material which aggregates into fibres after RNase digestion, has its origin in the chromosomal matrix. However, some qualification of this conclusion is required, since it is also possible to produce a dense network of fibres from older oocytes in which the loops have almost completely retracted. Clearly therefore, digestion of RNP present in the nuclear sap, as well as that coming from the loop matrix, contributes to the network of fibres. It would seem reasonable enough to expect such a dual source of origin, because the RNP free in the nuclear sap is assembled on the lateral loops earlier in oogenesis.

The fact that polymerisation is so extensive, together with the evidence from electron microscopy as to the nature of the resulting fibres, suggests that the 800 A repeating units have a uniform, and probably fairly simple composition, and therefore that the components which make up this complex are of widespread origin throughout the nucleus. Moreover, the dependance of the polymerisation on the enzymatic action of RNase suggests that the components of the fibres were originally associated with RNA.

The main theme of this thesis is the analysis of protein associated with nuclear RNA. In the light of the above observations, one possible approach to this problem therefore is to digest nuclear RNP with ribonuclease, but to prevent subsequent formation of fibres, thus keeping the liberated proteins in solution. Analysis of the supernatant proteins by gel electrophoresis after such treatment should show the nature of the proteins associated with nuclear RNA.

RNase can be made up in 2 M urea without affecting the activity of the enzyme. Isolation of chromosomes into RNase/Urea again produces complete stripping of the loop matrix, but in this case, no polymerisation of the released proteins occur. Evidence from light microscopy suggests that after this treatment, the loop matrix is completely solubilised (Fig. 9). In the next section, I present the results of electrophoretic analyses which show the effect of solubilising nuclear RNP with RNase/Urea. Examination of the electrophoregrams after this experimental procedure has yielded information concerning the nature of the protein associated with nuclear RNA.

A feature which has emerged from these digestion experiments is that sensitivity of the chromosomal matrix to RNase is short-lived. Isolation of chromosomes into saline for periods of time in excess of 15 minutes before addition of RNase results in progressive resistance of the matrix to enzymatic degradation. For this reason, when collecting nuclei for electrophoresis, it has been necessary to isolate them directly into the solubilising medium, which, as an analytical technique, suffers from the inherent disadvantage that any changes in the banding pattern occurring as a result of the solubilisation procedures are superimposed on an already complex mixture of proteins. An alternative procedure for solubilizing the chromosomal matrix, which is not subject to changes occurring after isolation has been developed by Dr. R.J. Hill. It is based on the method described by Hill et al., (1971). This procedure has the advantage that the proteins in the chromosomal matrix can be solubilised after thorough washing to remove the proteins of the nuclear sap. Details of this method are given below.

(ii) Guanidine HCl/2-mercaptoethanol

Available evidence indicates that denaturation of the loop matrix in preparations of isolated chromosomes is through the formation of protein disulphide linkages (see accompanying paper, Hill et al., 1973).

Dissolution of the denatured protein can be achieved by exposing chromosomes, which have been allowed to lie in saline for 1 - 2 hours, to a solution of 4 M guanidine hydrochloride/0.1 M 2-mercaptoethanol buffered to pH 8. The effect of incubation for a few minutes in this solution, is shown in Figure 10. Chromomeres and loops are no longer distinguishable, and traces of cores are all that remain of nucleoli.

Moreover, after alkylation of reduced sulphydryl groups by incubation with sodium iodoacetate for 15 minutes (Crestfield *et al.*, 1963) the reagents can be dialysed away against 7 M urea, and the proteins remain in solution. This is an important consideration for subsequent analyses, since neither high salt concentrations nor the presence of a reducing agent are compatible with successful electrophoresis.

(D) Electrophoretic analysis of solubilised proteins

(i) Proteins solubilised by RNase/Urea

In the experiment detailed below, the proteins in the low-speed supernatant from nuclei isolated into unbuffered 3 : 1 have been compared with the proteins in the low-speed supernatant obtained by isolating nuclei into the RNase/urea solubilising medium described in the previous section. RNase itself of course is present in the sample in very low quantity (about 0.2 µg) and does not contribute significantly to the banding pattern.

Collecting nuclei necessarily entails some dilution of the final medium by the small volume of washing medium (unbuffered 3 : 1) transferred with each nucleus. For this reason nuclei were collected in double strength medium i.e. 4 µg/ml RNase, 4 M urea - thus ensuring that an effective concentration of reagents is maintained throughout the isolation. This problem does not arise with the unsolubilised supernatant since in this case, nuclei are both washed and collected in unbuffered 3 : 1. In this experiment the smaller of the two gel sizes has been used, and 10 nuclei were collected for each sample. After centrifugation for 10 minutes at 3000 rpm the supernatants were withdrawn from each preparation and electrophoresed according to the procedure

described earlier. The results are recorded in Fig. 11, gels (a) and (b). At the outset, I should point out that the banding pattern obtained from the untreated nuclear sap in this experiment is somewhat unusual in that the sharp band normally found at position 0.06 is absent. As I will discuss later, I suspect that this band is a degradation product resulting from RNase digestion of nuclear RNP during isolation and therefore the presence of this component is somewhat variable. The absence of band 0.06 after treatment with RNase/urea, on the other hand, is a reproducible effect of the solubilisation procedure, which I will also attempt to explain in a later discussion. This aside, the main feature I would like to draw attention to in Fig. 11 is the response of band 0.56 to the experimental treatment. Comparison of the two electropherograms (a) and (b) shows that the single major difference in the protein composition of the two samples is the marked enhancement of the protein content of band 0.56 as a result of isolating nuclei into RNase/urea. It will be recalled from the observations made earlier that the effect of RNase/urea on lampbrush chromosomes is to cause complete dissolution of the lateral loops (Fig. 9). Correlating this cytological observation with the biochemical analysis in the present experiment suggests that solubilization of the loop matrix releases a single major species of protein which migrates, in the electrophoretic system used here, with a mobility of 0.56. Augmentation of this band after solubilization with RNase/urea is a reproducible effect. The possibility that minor components of the loop matrix are solubilized by this procedure, but are not present in sufficient quantities to be detectable, is not eliminated by the present data.

Gel (c) in Fig. 11 shows an electrophoretic analysis of the supernatant obtained after precipitation of the fibrous web by ribonuclease. In this case 10 nuclei were isolated into 4 µg/ml RNase, and then left for 45 minutes to allow fibre formation to reach completion. The preparation was centrifuged, and the supernatant was withdrawn and electrophoresed alongside samples (a) and (b). Comparison of the supernatant proteins after precipitation of the fibres, with the pattern in gel (b) in which aggregation of fibres was prevented by 2 M urea, gives some information about the protein composition of the fibres. As in the comparison between gels (a) and (b) there is a marked difference in the intensity of band 0.56, which suggests that some of the protein in this band becomes incorporated into the fibres; in addition, the protein in band 0.19, which is present to the same extent, both before and after solubilisation of the chromosomal matrix (gels a and b), completely disappears when fibre formation is allowed to occur, (gel c). Summarising the information in Fig. 11 therefore, I think it suggests that there is one major species of protein (0.56) present in the chromosomal matrix, and at least two major species of protein present in the RNase-induced fibres, of which one (0.56) is derived from the chromosome and possibly the nucleoplasm, while the other (0.19) is derived predominantly from the nucleoplasm.

The appearance of band 0.06 in gel (c) is consistent with the idea mentioned earlier, that this represents a degradation product following RNase digestion. As I will argue later, I suspect that this protein is also a component both of the chromosomal matrix, and of the RNase-induced fibres.

An additional feature, which is apparent in this experiment is the substantial reduction in stainable material trapped at the origin of gel (c). This observation is unexplained at present. Presumably, this material is protein which also becomes incorporated into the fibrous web.

Using a completely different procedure for solubilising the chromosomes, further evidence for the simplicity of the proteins in the chromosomal matrix has been obtained.

(ii) Proteins solubilised by Guanidine hydrochloride/2-mercaptoethanol

Low-speed centrifugation of nuclei isolated in unbuffered 3 : 1 solution yields two fractions, the nucleoplasmic proteins described in section A, and a preparation of centrifugable material containing chromosomes, nucleoli and membranes (Fig. 12) loosely termed the "chromatin fraction".

In the following experiment proteins have been prepared for electrophoretic analysis from total nuclei, nucleoplasm and washed chromatin, by a 30 to 60 minute treatment with 2 µg/ml pancreatic ribonuclease in 2 M urea (introduced as a precaution to degrade RNA and minimise any possible RNA-protein interactions during electrophoresis), followed by dissolution of all structures in 4 M guanidine hydrochloride/ 0.1 M mercaptoethanol. Finally the proteins were S-carboxymethylated (Crestfield et al., 1963) to prevent random reformation of disulphide bonds, and dialysed against 7 M urea. Gel electropherograms in 7 M urea at pH 9 are depicted in Fig. 13. Total nuclear material is resolved into some twelve bands. Of these, one (1.30) is very much depleted in the

nucleoplasmic fraction, and a second, (2.65) appears to be present in slightly decreased proportions in the nucleoplasm. Both of these components are very much enriched in the chromatin fraction.

Approximate calculations, based on the assumptions that the nuclear membrane may be considered as two unit membranes and that membrane proteins have generally been found to be heterogeneous (Lenard, 1970; Fairbanks et al., 1971) indicate that the major protein components of the chromatin fraction probably originate from the chromosomes and nucleoli.

A fuller account of this work is provided in the accompanying paper, (Hill et al., 1973). In developing the present argument the interesting feature to emerge from this analysis is the simplicity of the major protein components of the chromatin fraction. The relationship between these proteins and the single protein liberated by RNase/urea treatment is the subject of experiments to be described later.

Before this, however I will discuss briefly a preliminary attempt to determine more precisely the intranuclear localisation of the two major proteins in the chromatin pellet. For reasons detailed above, it seems likely that these two proteins are derived from the chromosomes, or nucleoli, or both. In the experiment described here, use has been made of the fact that in young oocytes the nucleoli are firmly attached to the nuclear membrane, and can therefore be isolated and washed free from the chromosomes before solubilisation. A comparison between nucleolar plus membrane proteins obtained in this way and the proteins from the total nuclear pellet is shown in Fig. 14. The supernatant proteins are included for reference. Elimination of the chromosomal contribution to the pellet produces a marked reduction in the protein

content of the slower of the two major bands (1.30) but has only marginal, possibly insignificant, effect on the faster moving band (2.65). Taken at face value this suggests that the protein in band 1.30 is present in both nucleoli and chromosomes, whereas protein in band ~~1.30~~ 2.65 is preferentially localised in the nucleoli. I emphasize, however, that these conclusions are based on the results of a single experiment and further study will be necessary to show whether or not this effect is repeatable. For the present, therefore, the conclusions prompted by these data remain tentative. Further information bearing on the intranuclear distribution of the 2 major components of the pellet is expected in the future when the complementary experiment to this is performed, in which the protein from isolated chromosomes separated from nucleoli and membranes, is solubilised and examined.

(E) Molecular weight determination of some selected proteins

Electrophoretic analyses of solubilised protein have provided information along two lines. First, two species of non-histone protein (1.30 and 2.65 in Fig. 13) predominate in the chromatin pellet; and second, a single major species of non-histone protein (0.56 in Fig. 11) is found associated with nuclear RNA. In this section I have attempted to relate these two lines of evidence by considering the molecular weights of the proteins in question. Molecular weights were determined by SDS gel electrophoresis using the procedure of Shapiro et al. (1967). Once again I would like to express my thanks to Dr. R.J. Hill for help in collecting these data.

(i) Proteins in the chromatin pellet

The experimental procedure was as follows: 50 nuclei were isolated into unbuffered 3 : 1 and the chromatin pellet was prepared by low-speed centrifugation for 10 minutes. After removal of the supernatant, the material in the pellet was washed in saline, and reduced and alkylated as described previously. The solution of proteins so obtained was then dialysed against 0.1% SDS in 0.01 M phosphate buffer pH 7.2, and electrophoresed in the continuous SDS system described under Materials and Methods. To determine the molecular weights of proteins from their electrophoretic mobilities, calibration gels were included using known molecular weight marker proteins treated in the same way as the sample. The sample and the standard proteins were run simultaneously to ensure as far as possible identical electrophoretic conditions. About twelve bands are distinguishable in the solubilised pellet (Fig. 15a), of which two are particularly prominent. From their mobilities in the SDS system, the molecular weights of these two major components of the chromatin pellet are estimated to be 110,000 and 43,000 (Fig. 15b).

(ii) Protein from band 0.56 liberated by RNase/urea treatment

Characterisation of this protein was more difficult. No experimental assay has yet been devised to allow its identification and extraction from bulk preparations, so I decided to isolate the protein on the basis of its known electrophoretic mobility, by cutting the appropriate section from the gel after electrophoresis, and recovering the protein by diffusion. Fortunately this protein becomes well separated from the rest during electrophoresis and it has therefore been possible to take the required band without significant contamination.

In practice, slight variation in the relative mobility of this protein is found, and therefore its position in an unstained gel cannot be predicted with absolute confidence. For this reason, nuclei sufficient for two gels were isolated into RNase/urea, and two identical samples were run simultaneously. After electrophoresis, one gel was frozen immediately, and the other was stained for protein in the usual way. Aligning the two gels enabled the appropriate section to be cut accurately from the unfixed gel, and this was confirmed subsequently by staining the remainder of the experimental gel. The excised slice was then placed on a siliconised slide, and chopped into small pieces with a razor blade. The fragments were collected and transferred to a siliconised vial containing 0.25 mls of 0.1% SDS, and protein was left to diffuse into this medium for 6 hours at 37°C. At the end of this time, the vial was centrifuged and the supernatant withdrawn. The extraction was continued overnight with a further 0.25 mls of 0.1% SDS. The two supernatants were then pooled, and evaporated to about 40 µl in a stream of nitrogen. (It is essential that the proteins are concentrated as much as possible at this stage since in a continuous buffer system of electrophoresis no stacking gel can be included). As before, the molecular weight of the protein under investigation was deduced from its mobility in the SDS gel by comparison with marker proteins of known molecular weight. The results are presented graphically in Fig. 16. The protein solubilised by RNase/urea and extracted from band 0.56 migrates in the SDS gels as a single electrophoretic species with an estimated molecular weight of 116,000.

The similarity between the mobility of this protein in SDS and the larger of the two major components of the chromatin pellet, is striking, and at least is suggestive evidence that the same protein has been characterised in both cases. At first sight, however, the contrasting mobilities of the two proteins in the Ornstein Davis discontinuous system (compare bands 0.56 in Fig. 11 and 1.30 in Fig. 13) seems inconsistent with such an assertion. The difference between the electrophoretic conditions in these experiments is that after complete solubilisation of nuclear proteins shown in Fig. 13, electrophoresis was conducted in gels containing 7 M urea whereas analysis of the effect of RNase/urea in Fig. 11, was performed in standard gels, without urea. Whether the effect of urea on the protein in band 0.56 could account for the observed difference in mobility, has been investigated in the following experiment.

(F) Effect of urea on the electrophoretic mobility of the protein liberated by RNase/urea

A frequent modification of the Ornstein Davis procedure of electrophoresis is the inclusion of urea in the monomer solutions before polymerization of the gels. This is in no way detrimental to the process of electrophoresis, though it does produce slight reduction in the mean pore size of the polymerized gel, which must be borne in mind when analysing the results. In the present experiment, the electrophoretic mobility of the protein liberated by RNase/urea has been compared in two situations, identical in every respect except for the presence or absence of 2 molar urea in the gels. The larger of the two gel sizes has been used, for which 25 nuclei from 0.7 mm oocytes are sufficient to produce a strong banding pattern. As described earlier, collecting this number of nuclei means that the volume of saline transferred to

the solubilizing solution causes significant dilution of the reagents, and for this reason, nuclei were isolated into a measured volume of 'double strength' medium (4 µg/ml RNase, 4 M urea) and the final concentration of reagents adjusted to a 2 µg/ml RNase, 2 M urea by suitable addition of saline at the end of the isolation.

In this way 50 nuclei, sufficient for the two electrophoretic analyses, were collected in 0.2 ml 4 µg/ml RNase, 4 M urea, and the final volume made up to 0.4 ml, by addition of saline. This pool of protein was then divided into two identical samples, one of which was run in the standard gel, and the other in standard gel containing 2 M urea. Preparations of untreated nuclear sap were also co-electrophoresed with the two samples to indicate the position of the augmented band in each case. The four electropherograms are shown in Figure 17.

Several points of interest emerge from this experiment. What is immediately apparent is the extent to which the mobility of the protein liberated by treatment with RNase/urea is influenced by the presence of urea in the gel. In non-urea gels, the protein exhibits the mobility characterised previously of 0.56* with respect to the buffer front (cf. Figs. 5 and 11). On electrophoresis in gels containing 2 M urea, however, the mobility of this protein is reduced to the much lower value of 0.40. There can be little doubt that the two bands 0.56 (in Fig. 17 a and b) and 0.40 (in Fig. 17 c and d) contain the same protein, since both exhibit the characteristic augmentation as a result of solubilization of loop matrix by RNase/urea.

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* The mobility of the augmented band in Fig. 17 (a) and (b) is actually 0.66. The discrepancy with earlier experiments, I think merely reflects a slight difference in acrylamide concentrations in the monomer solution before polymerisation, producing in this case a gel with a slightly larger pore size. The relative mobilities of the proteins in the nucleus remain unchanged. For simplicity the proteins will be referred to by their nominal mobilities.

A component of this retardation is certainly the somewhat smaller pore size characteristic of urea gels, referred to earlier. But there is clearly also a specific effect of urea on this particular protein, which changes its mobility relative to the other proteins of the nucleus. Such an effect, I suggest, is most easily explained in terms of a conformational change in which the molecule undergoes a transition in the presence of urea from a tightly packed configuration, to a more extended one, with a corresponding decrease in electrophoretic mobility. Moreover, this is presumably a reversible effect, since protein isolated into RNase/urea, and electrophoresed without urea (gel (b) in Fig. 17) returns to the fast-migrating conformation during stacking in the non-urea environment.

This property of the protein is of interest to the present discussion in that it could explain the paradoxical finding, referred to earlier, that the two proteins isolated by entirely different procedures, but postulated to be the same on the basis of molecular weight determination, have such widely differing mobilities when electrophoresed in the two contrasting pH 9 discontinuous systems of Ornstein and Davis. With the knowledge that the electrophoretic analysis of the chromatin pellet was conducted in 7 M urea, whereas the analysis of the nuclear proteins after RNase/urea treatment was performed in the standard gel, such a difference in mobility, in the light of the present experimental data, is to be expected. It is consistent with the view that the protein from band 0.56 after RNase/urea treatment, and the protein from band 1.30 extracted from the pellet, are the same. In the future, I hope it will be possible to recover sufficient protein from the gels after electrophoresis to allow an amino-acid analysis of the two proteins. For the present, identity between these two proteins is indicated, but remains unproven.

The other major protein component of the chromatin pellet, which was shown by SDS gel electrophoresis to have a molecular weight 43,000, has not as yet been positively identified in electropherograms following RNase/urea treatment of nuclei, although several fragments of circumstantial evidence lead to its tentative identification as the diffuse band 0.69 - 0.76. The arguments on which this assertion is based will be fully dealt with later. In anticipation of the discussion on this subject, however, mention can profitably be made here of the effect of urea on the electrophoretic behaviour of band 0.06, as revealed by the results of the present investigation. This particular protein migrates very slowly during electrophoresis in standard gels, and forms an unusually sharp band. Furthermore, it is clear from the data in Fig. 17 that the protein in this band disappears on exposure to 2 M urea, which suggests that what is seen as a slowly migrating protein is actually a complex of smaller subunits. No definite information has yet been obtained about the electrophoretic distribution of the dissociated subunits, though it should be a fairly simple operation to dissect out band 0.06, as was done for band 0.56, and re-run the protein after dissociation in urea. This experiment is also planned for the future.

(G) Association of newly synthesised RNA with proteins of the nucleus

The original intention in conducting this experiment was to obtain supporting evidence for the conclusion reached earlier that the protein liberated by RNase/urea is associated *in vivo* with nuclear RNA. It was thought that even though RNase was present in the solubilising medium, and that digestion of the RNA was in all probability a necessary part of the solubilization reaction, that nevertheless sufficient RNA might be protected by the protein to prevent its complete degradation, and thus

to allow its detection in the gel. Staining gels in 0.2% orcein, or 0.2% acridine orange, after electrophoresis of nuclear proteins solubilised in RNase/urea, failed to demonstrate the presence of RNA, so a more sensitive approach was undertaken in which RNA was labelled with ^{3}H-uridine before isolation of protein. The experimental procedure is given in detail below.

In this experiment the sample was not prepared by solubilisation in RNase/urea as it was for the stained gels referred to above, but in RNase alone. The reason for omitting urea was to prevent the change in tertiary structure demonstrated in the previous section, which if it were allowed to occur, would presumably expose the putative RNA normally protected by the protein in its native conformation. On the other hand, it has been shown previously that isolating nuclei into 2 µg/ml RNase alone results in formation of the fibres, and therefore precipitation of the protein around which most interest centred. From this reasoning the optimum situation would be one in which stripping of loop matrix was achieved without the subsequent formation of fibres. By isolating chromosomes into various dilutions of RNase, and monitoring the effects by light microscopy, an enzyme concentration was established in which these conditions were fulfilled. This concentration was 10^{-9} gm/ml.

Freshly excised oocytes were incubated for 6 hours in 0.5 ml of Steinberg medium containing 0.5 mCi ^{3}H-uridine (sp. act. 30 mCi/mM). 30 nuclei isolated from 0.7 mm oocytes were then collected in 10^{-9} gm/ml RNase in TBS/Mg^{2+}/Ca^{2+} pH 7.2, centrifuged, and the supernatant electrophoresed on the standard gel. Detection of radioactivity in the gel was carried out as described in the Materials and Methods section. The results are presented in Fig. 18. No radioactivity was found associated

with band 0.56. Examination of the distribution of radioactivity in the rest of the gel, however, revealed a peak of labelled RNA co-migrating with band 0.06. Clearly this cannot be pure RNA migrating fortuitously with these characteristics, since all such molecules would be degraded by the RNase. It therefore follows that such RNA must be associated in some way with the protein of band 0.06. Bearing in mind the information given in the previous section on the effect of urea, the anatomy of this band begins to emerge as a fragment of RNA associated with, and protected by, a number of protein subunits, stabilised by hydrogen bonding. Such molecular species have been identified before in the nuclei of other biological material (Samarina et al., 1966). These authors found later that after mild RNase treatment the large heterogeneous RNP particles of the nucleus are converted quantitatively to a 30S particle consisting of an RNA fraction of about 200,000 molecular weight, associated with about 20 identical protein subunits of molecular weight 40-45,000. Negative staining of the 30S fraction followed by examination in the electron microscope revealed a uniform population of particles, with a diameter of approximately 200 A.

The mean pore size of a 7% acrylamide gel is estimated to be about 60 A. (Ornstein, 1964). A 200 A particle would therefore enter the gel with extreme difficulty. However, an RNP particle with a high negative charge density by virtue of its RNA component, and therefore a strong driving force towards the anode, might conceivably enter the gel and migrate very slowly. Moreover, the combination of large size and high charge density would presumably produce a very sharp band, as indeed is observed in the case of band 0.06. It is an intriguing possibility that the RNP migrating as band 0.06 and the 30S particle of Samarina

et al. may represent comparable structures. The relationship between these two entities will be considered in more detail in the next chapter.

C H A P T E R IV.

GENERAL DISCUSSION

The first observations on ribonucleoprotein complexes in animal cell nuclei came from electron microscope studies on the giant nuclei of dipteran salivary glands (Beermann & Bahr, 1954). In these nuclei the chromosomal loci active in transcription can be identified cytologically by the characteristic 'puffed' appearance of the chromatin, the most notable examples of this phenomenon being the so-called Balbiani rings. In sections through such regions of the chromosome, dense accumulations of granules were seen, the granules being of uniform size, about 400 A in diameter. The integrity of these granules was markedly affected by RNase treatment prior to fixation, but totally unaffected by DNase. Similar particles were seen associated with the lampbrush chromosomes of amphibian oocytes, and also with the nuclear envelope (Gall, 1956). The idea arose that the newly transcribed RNA becomes associated with protein, and that it is transported to the cytoplasm as a ribonucleoprotein complex. Subsequent work has confirmed these conclusions, (see for example, Stevens & Swift, 1966).

Another approach to the study of this problem was reported in 1966 with the first successful isolation of a ribonucleoprotein complex from animal cell nuclei (Samarina et al., 1966). The complexes they described had a sedimentation coefficient in sucrose of 30S and consisted of RNA and protein only. It has since been shown that this 30S particle is actually a degradation product resulting from endogenous RNase activity, and that if the isolation is performed in the presence of an

RNase inhibitor, a heterogeneous distribution of particle sizes is found in the gradient with sedimentation coefficients ranging from 30S to 300S. Usually a number of discrete components are found, with sedimentation coefficients of 30S, 45S, 57S, and 70S, the other peaks being less reproducible. In general, the distribution is found to depend on the quality of the RNase inhibitor. If RNase activity is suppressed altogether, and the inhibitor isolated from rat liver supernatant (Roth, 1958) is most effective in this respect, the 30S peak virtually disappears and most of the RNP sediments in the 80S - 100S zone. In the absence of an RNase inhibitor, or after mild deliberate RNase treatment, these large complexes are quantitatively converted to a homogeneous population of 30S monomer particles as found in the original isolation. These results have led on to the speculation that the high molecular weight ribonucleoproteins in the nucleus have a polysome-like structure, consisting of linear chains of 30S subunits (Samarina et al., 1967a; 1968).

Confirmation of this idea comes from electron microscope observation of material isolated from different regions of the gradient. In shadowed preparations, the 30S peak appears as a uniform population of single particles 200 A in diameter. The 45S peak consists of dimers; the 60S peak predominantly of trimers; the 70 - 75S peak of tetra-pentamers and so on. In the 100 - 120S zone, long chains of particles were seen and in some well extended examples, 8 - 10 monomers were resolved. Mild RNase treatment reduces the polysome-like structures into monomers (Samarina et al., 1968). These data prove the polysome-like conformation of the particles containing nuclear RNA.

The ease with which nuclear RNP can be isolated has made possible an analysis of its properties and composition. Investigation of this RNA shows it to have many of the characteristics expected of messenger, namely high AU content, high hybridisability with homologous DNA, ability to stimulate amino-acid incorporation into proteins in a cell-free system, and heterogeneous distribution in sucrose gradients (Samarina et al. 1966). At least part of this RNA therefore corresponds to messenger, but since the current tests for messenger activity are rather imprecise, these authors prefer to call this high molecular weight, non-ribosomal RNA 'DNA-like RNA' (or 'D-RNA'). I will adhere to this nomenclature in the ensuing discussion.

Almost all of the high M.W. D-RNA synthesised in the nucleus becomes complexed with protein, thus contributing to the heterogeneous population of particles described above. All such species of D-RNP when centrifuged to equilibrium in CsCl have a buoyant density of 1.4 g/cc. Since the buoyant density of RNA is 1.9 g/cc, and that of protein is about 1.2 g/cc, the proportions of RNA : protein can be estimated to be about 1 : 4. The properties of the protein component of the nuclear RNP will be discussed later. Briefly it consists of a single species, with a molecular weight of 40-45,000.

In the present work two protein components of the nucleus are implicated in their association with nuclear RNA.

In the experiments with RNase, I found that an enzyme concentration of 2 µg/ml produced complete stripping of the chromosomal matrix, followed a few minutes later by the formation of a pan-protease-sensitive fibrous web. Electron micrographs show these fibres to be aggregates of a repeating unit of approximately 800 A diameter, the latter in turn

consisting of a number of 200 A subunits. The internal structure of the 200 A is not resolvable, but it seems likely that this is of chromosomal origin, and that it is analogous to the 30S particle described by Samarina et al (1966). This idea will be discussed later.

There appears to be at least one other protein component present both in the web and the chromosomal matrix, which in standard 7% gels shows a mobility of 0.56 with respect to the buffer front. Extracting the protein from the gel has made it possible to estimate its molecular weight by a second electrophoresis in SDS. The molecular weight of the protein as determined by this method was found to 116,000. A protein with a similar molecular weight (110,000) was shown to be a major constituent of the chromatin pellet (Fig. 15). On the basis of this similarity in size an identity between the two proteins is indicated. However no definitive conclusions can be drawn from the present data on this point, and support for this idea must await the results of amino-acid analyses of the two proteins - an experiment referred to earlier.

Preliminary study of the intranuclear localisation of the proteins in the chromatin pellet has been initiated. The result of a single experiment comparing the proteins of the total pellet and the proteins solubilised from nucleoli and membranes alone, is shown in Fig. 14. The suggestion from this as yet unrepeated result, is that the larger of the two major protein components of the chromatin pellet is associated with both the chromosomes and the nucleoli.

In summary therefore, a protein with a molecular weight of 116,000 has been isolated from oocyte nuclei, which for two reasons is

thought to be associated with RNA. First, because its solubilisation seems to be dependent on the enzymatic digestion of RNA; and secondly, because a protein of similar molecular weight has been discovered in the chromatin pellet, in which the bulk of the material is made up of chromosomal and nucleolar RNP.

No evidence was obtained from the incorporation studies with ^{3}H-uridine to corroborate the postulated association of the protein in band 0.56 with RNA, although since RNase was present in the isolation medium, absence of RNA under these conditions cannot be taken to mean very much. On the other hand the experiment did demonstrate the presence of newly synthesised RNA bound to, and apparently protected by, another component of the nucleus, namely that protein which migrates in standard gels as a very sharp band with a mobility of 0.06. It appears that this component of the nucleus is actually a ribonucleoprotein complex, and in the following discussion evidence is presented which suggests that such a particle may be analogous to the 30S ribonucleoprotein characterised by Samarina et al.

(1) Size and composition

The RNA of the complex described above becomes rapidly labelled in vitro by ^{3}H-uridine (section 3 (G)), and is probably of chromosomal origin. It therefore corresponds to the D-RNA defined by Samarina et al. The size of the 30S particle as revealed by negative staining is about 200 A (Samarina et al., 1968), and it will be recalled that this is the estimated size, first of the smallest resolvable unit in the RNase-produced fibres (Fig. 7) and secondly, that of the particulate unit

seen in the chromosomal matrix (Fig. 8). I therefore suggest that the 200 A unit identified within the chromosomal matrix is released by treatment with RNase and that, on electrophoresis, migrates as the ribonucleoprotein found in band 0.06. While there is no unequivocal evidence to support this notion, the circumstantial evidence that such a particle might be expected to exhibit just these characteristics (i.e. a slow moving, very sharp band in a gel with an average pore size of 60 A), together with the evidence to be discussed on p. 66 concerning the protein composition of the 30S particle, makes such an assumption reasonably justified.

(2) Dissociation in urea

Destabilisation of both the 30S RNP complex of Samarina et al. and band 0.06 occurs in the presence of urea. In the case of the 30S particle, the breakdown is reversible and the RNP reaggregates if the urea is dialysed away (Samarina et al., 1967b). This experiment has not yet been deliberately attempted in the case of the protein isolated from newt oocyte nuclei, but in occasional experiments in which the proteins were solubilised in RNase/urea, and then run on gels without urea, traces of band 0.06 can be observed, suggesting that during stacking without urea, some reaggregation of the protein subunits occurs to produce a complex with an electrophoretic behaviour identical to the original. This property of the protein merits further investigation under better controlled conditions of reassociation.

(3) RNase digestion

The characteristic response of the two RNPs to varying concentrations of RNase is perhaps the most compelling evidence that they represent comparable structures.

According to Lukanikin (1969) both the 30S particle, and the heterogeneous D-RNP of the nucleus show a buoyant density in CsCl of 1.4 g/cc. Since the buoyant density of RNA in CsCl is about 1.9, and that of protein is about 1.25 g/cc, the particles with a density of 1.4 g/cc are estimated to contain about 20% RNA and about 80% protein. Mild digestion with RNase leads to a slight decrease in buoyant density to about 1.35 g/cc, reflecting a loss of about a quarter to a third of the RNA from the particles. Further digestion by RNase produces almost complete degradation of the RNA and simultaneously induces aggregation of the protein. Thus after intensive digestion, most of the radioactivity incorporated into RNA is found in the form of oligonucleotides at the top of the sucrose gradient, while the aggregated protein is recoverable at the bottom of the centrifuge tube (Lukanidin, 1969).

These observations are in close accord with the results of RNase digestion documented in Fig. 18. It was shown that in very low concentrations of RNase (10^{-9} gm/ml), conditions under which aggregation of protein does not occur, a peak of radioactivity is found associated with the protein in band 0.06. Under conditions of more intensive digestion (2 µg/ml RNase), precipitation of fibres was shown to occur in isolated chromosome preparations, but no activity was found associated with band 0.06. Thus in both cases the RNA is partially protected by the associated proteins, but under conditions of intensive digestion, in which the RNA component of the particle is degraded, aggregation of the proteins occurs.

(4) Protein composition of the 30S particle

The ease with which the 30S fraction can be purified from nuclear preparations, and the quantity of material so obtained, has made possible a detailed examination of the protein associated with the 30S particle. Dissociation of the protein subunits and release of the RNA, can be accomplished either by addition of urea, or by increasing the salt concentration to greater than 0.7 M. The properties of the proteins released in this way have been examined by Krishevskaya & Georgiev (1969).

In polyacrylamide gel electrophoresis, Krishevskaya & Georgiev found the characteristic distribution of dissociated subunits shown in Fig. 20a (their Fig. 2). Usually three main bands (A, B, C) and about five minor bands can be distinguished, all moving to the cathode at pH 4.5. After reduction with mercaptoethanol, essentially all of these bands are quantitively converted into the single component B. Comparing the mobilities of the components leads to the conclusion that component B is an extended, reduced polypeptide chain; component A consists of two B chains joined by disulphide bridges; and component C is a single chain with an intramolecular S-S bond (see Fig. 20b; their Fig. 3). All of these configurations are formed from B, probably during the preparation of the proteins for electrophoresis, after denaturation with urea, and all can be converted back to B by treatment with mercaptoethanol, as seen in the electropherograms in Fig. 20a.

Thus, the protein in the 30S particles consists of a number of identical polypeptide chains. Further analysis of this protein has shown it to be almost neutral or slightly basic with a molecular weight determined by electrophoresis in SDS of 40,000 to 45,000 daltons.

From the present experimental data on newt oocyte nuclei, two facts have emerged which are relevant to the discussion at this point. First, an examination of the proteins in the chromatin pellet has revealed two major fractions, of which the faster-migrating component has a molecular weight of 43,000 as determined by SDS gel electrophoresis (Fig. 15). Second, in attempting to solubilise protein of isolated chromosomal preparations which had been allowed to stand for about 30 minutes or more, the inclusion of a reducing agent such as mercaptoethanol, dithiothreitol, or thioglycollate was essential if complete dissolution of chromosomal structures was to be obtained (Hill et al., 1973). This strongly suggests that the matrix protein contains reduced sulphydryl groups, and that after isolation, progressive formation of S-S bonds occurs within the chromosomal matrix leading to the observed increase in resistance of the loops to solubilisation procedures (see page 43). Considering these two observations in the light of the properties of the 30S particles discussed above, it is tempting to conclude that the protein component of the 30S particle is related to one of the proteins of the chromosomal matrix, and further, that the 30S particle is analogous to the 200 A unit observed in electron micrographs of lampbrush chromosomes. As I have suggested above, on electrophoresis in 7% polyacrylamide gels the 200 A particle of the chromosomal matrix migrates as a ribonucleoprotein particle with a mobility of 0.06. From these three considerations it would follow that the proteins associated with the 200 A particle in the matrix, and the RNP in band 0.06 should be the same, and at least very similar to the protein component of the 30S particle characterised by Samarina et al.

The prediction which follows from this conclusion is that the dissociation of the RNP of band 0.06 by electrophoresis in urea releases a slightly basic protein of molecular weight 43,000 daltons, which under

the electrophoretic conditions employed, should have a rather high mobility and be readily detectable in the gel. Failure to detect such a protein after dissociation of band 0.06 (see for example Fig. 17) has not yet been accounted for. However, the discovery that proteins in both the chromosomal matrix and the 30S ribonucleoprotein possess free -SH groups and the circumstantial evidence equating these components with the RNP in band 0.06 suggests the following possible, if somewhat speculative explanation. Dissociation of the nuclear RNP by urea, I suggest, releases into the electrophoretic sample a population of identical protein molecules each containing reduced sulphydryl groups. It is well known that during electrophoresis in urea gels, interactions between such side-chains, within a population of identical proteins, can produce a continuous distribution of protein in the final electropherogram (Mackinlay & Wake, 1964). Thus in the present case, random formation of disulphide linkages between proteins during separation could be responsible for the failure of the released protein to migrate as a single electrophoretic species, and form a discrete band after disaggregation in urea.

Extending this line of argument a little further, I suggest that the diffuse band of protein often found migrating between 0.69 and 0.76 (see Fig. 5) is actually protein released either naturally, or as an artifact of isolation, from association with RNA. If the protein is indeed analogous to the protein of the 30S particle, then the diffuse nature of the band could be explained in terms of a randomly occurring transition, during electrophoresis, from the reduced form of the polypeptide, to the faster migrating configuration in which an internal S-S band has been formed. This then would produce a continuous range of mobilities between two extremes; on the one hand, where sulphydryl groups

remained reduced throughout the separation, and on the other, where disulphide bond formation had occurred at some time before the start of electrophoresis. This interpretation is of course entirely analogous to that depicted in Figure 20b and invoked by Krimovskaya & Georgiev to explain the difference in mobility between bands B and C in Figure 20a.

Speculation along such tenuous lines may be undesirable; hppefully, however, it will soon be rendered superfluous by the results of the experiment mentioned earlier, in which protein is extracted from band 0.06, and analysed by a second electrophoresis in urea, after dissociation of the protein, and alkylation of reduced sulphydryl groups. From the above argument, I anticipate that this will yield a single protein with a mobility of 0.69 in the standard gel system, and a molecular weight of 43,000 daltons. The possibility that other minor proteins may be associated with RNA, but in amounts too low to be detected, is not ruled out by these data.

In conclusion, the evidence, though rather circumstantial, seems to indicate a chromosomal original of the RNP in band 0.06, and a similarity between this fraction and the 30S particle isolated from a variety of nuclei by Samarina et al.

In passing, I should add that a 30S particulate fraction has been isolated from newt oocyte nuclei by sucrose gradient fractionation (Rogers, 1968). The claim from this work is that the 30S particle represents a ribonucleoprotein precursor of the 40S ribosomal subunit, and while no convincing evidence is presented in support of this claim, it nevertheless remains a possibility which cannot be overlooked.

The protein component of the nuclear RNP is implicated in two aspects of the behaviour of newly synthesised D-RNA, namely its processing from a newly formed polynucleotide into functional messenger, and transport of this processed molecule into the cytoplasm. I will now consider the role of the RNA-associated proteins in these two aspects of nuclear physiology.

There is a growing body of evidence that newly synthesised RNA in animal cell nuclei has a high molecular weight, and heterogeneous distribution of between 20S and 80S in sucrose gradient analyses (Warner et al., 1966). This "heterogeneous nuclear" RNA is metabolically active and rapidly reaches a high specific activity in pulse-labelling experiments with ^{3}H-uridine. The majority of this RNA is unstable and is degraded, with a half life of a few minutes, into acid-soluble fragments without leaving the nucleus. A DNA-like base composition has been demonstrated for this fraction of the RNA (Soeiro et al., 1966), and several lines of evidence indicate that it is the precursor of stable messenger RNA which eventually reaches the cytoplasm (Scherrer et al., 1966; Stevens & Williamson, 1972; Williamson et al., 1973). Estimates vary regarding the proportion of newly synthesised D-RNA which leaves the nucleus as stable messenger; in salivary glands of Rhynchosciara (Armelin & Marques, 1972) approximately 10% of RNA labelled in a 10 minute pulse becomes associated with cytoplasmic polyribosomes, which seems to be about average. This general scheme of RNA metabolism appears to operate in all eukaryotic organisms thus far studied, and although no convincing explanation has yet been put forward to account for such an apparently inefficient process, what does seem likely, is that the protein, discussed above, which becomes associated with the rapidly

labelled D-RNA of the nucleus is in some way instrumental in the selective degradation of the primary transcription product which gives rise to stable messenger. The recent finding that protein isolated from nuclear D-RNP has RNase activity (Neissing & Sekeris, 1970) lends credibility to this notion although at the same time it raises the problem of how certain sequences within the giant D-RNA are protected from degradation. Whether this is achieved through packing of the RNA, modification of bases, or by attachment of different proteins along the molecule, and how it relates to the polysome-like structure of newly formed D-RNP are all questions which must await further experimentation.

The second aspect of RNA behaviour in which its associated proteins are implicated is the transfer of the processed molecule from the nucleus to the cytoplasm. In the first place, there is every indication that the RNA migrating across the nuclear membrane does so as a ribonucleoprotein complex. Evidence for this comes from several sources. In preparations of isolated nuclei, movement of RNP particles across the nuclear membrane has been demonstrated in the presence of ATP (Ishikawa, 1969; Schumm & Webb, 1972) and this is in accord with the observation that the nuclear pores are sites of ATPase activity (Yasusumi & Tsubo, 1966). In addition to this, electron microscope studies on metabolically active nuclei have repeatedly demonstrated RNP particles "caught" in different stages of penetration of the nuclear pores (Stevens & Swift, 1966; Monneron & Bernard, 1969).

The question arises whether there are specific transport proteins exclusively involved in transferring processed RNA across the nuclear membrane. As yet, this question has not been resolved. A likely candidate for such a role, however, has been described by Goldstein from evidence

gained from a series of nuclear transplant experiments in Amoeba proteus (Goldstein, 1958; Byers et al., 1963). In this work, strong evidence is obtained for a protein fraction which shuttles back and forth, across the nuclear membrane, and it is tempting to speculate that this protein is engaged in transporting RNA from the nucleus to the cytoplasm. Indeed, in a series of experiments designed to elucidate the intracellular role of this 'cytonucleoprotein' fraction, donor nuclei, labelled with ^{3}H-leucine, were injected into host amoebae in which RNA synthesis had been interrupted with D-actinomycin. It was found that the ratio of donor : host nuclear radioactivity was greater in the experimental situation than in the control in which labelled nuclei were transplanted into amoebae actively synthesising RNA (Goldstein, 1965). Although this was not repeated sufficiently often to be statistically significant, and the problems of using D-actinomycin apart, the observed increased ratio would be expected if the migrating protein had the function of transporting newly synthesised RNA across the nuclear membrane.

Up to now the discussion has been concerned solely with the ribonucleoprotein complexes obtained from animal cell nuclei. The first indication of cytoplasmic RNP with template activity came from the work of Hoagland & Askonas (1963). The particles described by these authors had sedimentation coefficients ranging from 20S to 70S, and contained protein, and an RNA component capable of stimulatory activity in a cell free system. On the basis of these data, it was concluded that the cytoplasmic particles recovered in the post-microsomal fraction contained mRNA. Since this discovery, two distinct classes of cytoplasmic mRNP have been reported.

True mRNP can be isolated from polyribosomes dissociated by treatment with 5 mM EDTA (Perry & Kelley, 1968; Henshaw, 1968). This treatment reduces the polyribosomes to 60S and 45S ribosomal subunits plus another component, usually of heterogeneous distribution in sucrose gradients (20S-65S), containing rapidly labelled D-RNA and protein in a ratio of 1 : 2 or 1 : 3. Fixation of this rapidly labelled material with formaldehyde, and centrifugation to equilibrium in CsCl, produces a sharp band with an average buoyant density of 1.45 g/cc, suggesting an RNA content of about 40%. Evidence that the rest of the complex consists of protein has been presented by Tennermann & Lebleu (1969).

The other class of rapidly labelled D-RNP in the cytoplasm was discovered in particulate form in embryonic tissue of the loach by Spirin et al. (1964); Spirin termed the particles, 'informosomes'. These ribonucleoproteins are not associated with ribosomes and are found mainly in the post-ribosomal region of the sucrose gradient, sedimenting in 6 or 7 discrete peaks from 20S - 70S. More recently, some very large informosomes with sedimentation coefficients of up to 110S have also been reported (Spirin, 1969). In general, when analysed on CsCl gradients, they are found in a sharp peak with a density of about 1.4 g/cc although the smaller informosomes tend to have a broader distribution between 1.35 and 1.52 g/cc. Although embryonic tissue is the richest source of informosomes, their detection in adult tissues has been claimed on several occasions (Perry & Kelley, 1966; Henshaw & Loebenstein, 1970). Very much lower quantities of material are found, however, and the possibility that this material has leaked from the nucleus during extraction has not yet been eliminated (Perry & Kelley, 1968; Olsnes, 1970).

The composition of these two cytoplasmic ribonucleoprotein fractions, and how they relate, both structurally and functionally, to each other, and to the D-RNP in the nucleus, is a subject of current debate. Certainly each fraction contains D-RNA with a high hybridisability to chromosomal DNA (Penman et al., 1963; Spirin & Nemer, 1965); an interesting question concerns the nature of the associated proteins in each structure, and the role played by these proteins in regulating the activity of the RNA. Currently, it seems most likely that the informosomal RNP represents an inactive messenger fraction (Spirin, 1966) which is intermediate between the processed D-RNP leaving the nucleus, and the mRNP found associated with polyribosomes. Obviously such an intermediate could have either a very transient existence, and become rapidly incorporated into polyribosomes as in the case of those messenger RNA's designated for immediate translation; or alternatively it could be stored for long periods of time before its ultimate expression, as is known to be the case for haemoglobin mRNA (De Bellis et al., 1964), for silk fibroin mRNA (Smirnov et al., 1964), and for much of the RNA made during oogenesis.

It seems most probable that the activity of mRNA within the cell is controlled by its associated proteins. This line of reasoning has prompted several recent attempts to characterise the protein components of the various cellular RNP fractions.

The proteins associated with nuclear RNP have been extensively studied in a variety of organisms and this data has already been presented. The question arises whether this protein is restricted to some function within the nucleus, or whether it accompanies the D-RNA across the nuclear membrane, and becomes incorporated into the cytoplasmic forms of RNP.

In an attempt to answer this question, preliminary investigations have been carried out as to the nature of proteins associated with cytoplasmic RNPs. So far, these have yielded conflicting results.

In the case of the protein associated with mRNA released from polyribosomes, rabbit reticulocytes have provided the most convenient source of material (Lebleu et al., 1971; Blobel, 1972). EDTA treatment of reticulocyte polyribosomes yields a single 15S peak containing D-RNP, which can be dissociated into a homogeneous 9S RNA fraction later shown to be globin messenger (Lane et al., 1971), and two proteins. Some discrepancy is found in the reported sizes of these two proteins. According to Lebleu et al., the proteins have molecular weights of 130,000 and 68,000 whereas Blobel finds that the two major components have molecular weights of 78,000 and 52,000, with a third, possible contaminating protein estimated to be 115,000. Clarification of these differences must await further study. What does seem clear however, is that none of the proteins are related to the major component of nuclear RNP which has a molecular weight of 43,000, and this conclusion is substantiated by some recent immunological studies of Lukanidin (1972) in which he failed to find a reaction, even using a very sensitive assay procedure, when antibodies made against 30S nuclear particles, were challenged with mRNP isolated from rat liver polyribosomes. In direct conflict with these results however, are the experimental observations of Schweiger & Hannig (1970). They succeeded in isolating polyribosomal mRNP by a novel technique of 'free flow' electrophoresis, and then examined the complexed protein by a second electrophoresis in acrylamide gels. They found a single protein which was electrophoretically identical to the protein isolated from nuclear RNP. Unfortunately, as with all the experiments in which a bulk isolation of cytoplasmic RNP is the first step, the

inevitable criticism which arises is that leakage of D-RNP from the nucleus could contaminate the preparation. For the moment therefore, although this remains a polemic issue, the balance of evidence suggests that the major protein component of the nuclear RNP is restricted to the nucleus, and that as the RNA passes across the nuclear membrane some change occurs in its accompanying protein.

As far as I know, no successful attempt has yet been made to study the proteins associated with informosomes.

In the oocyte, I think the situation will prove to be somewhat more complicated. In this cell there is now a great deal of evidence that two classes of messenger RNA are synthesised: one which is utilised during oogenesis, and one which is stored in a repressed form in preparation for early development. I find it an attractive idea that this latter class of RNA molecules is retained by the oocyte nucleus during oogenesis, and is only released into the cytoplasm when the germinal vesicle breaks down at maturation. I envisage that this material then becomes incorporated either into the informosomes described by Spirin et al., or perhaps into an inactive polyribosome fraction, analogous to that described in sea urchin eggs by Monroy et al. (1965). Although somewhat speculative, this notion would make sense of the observation that the nucleus continues to increase in size and RNA content throughout oogenesis (Davidson et al., 1966) even though its synthetic activity declines. It is also consistent with the observations of Gall & Edstrom (1963) on the base composition of nucleoplasmic RNA; although base composition seems to vary considerably from one nucleus to another there is a tendency towards high uracil values, which excludes the possibility that a significant proportion of the nucleoplasmic RNA is ribosomal

precursor. Thirdly, it is supported by the EM observations of Brachet & Malpoix (1971) on maturation of amphibian oocytes, in which ribosomes were found invading the nucleus as the membrane breaks down, perhaps incorporating mRNP into inactive polysomes. I therefore suggest that the maternal mRNA used ultimately to programme the early development of the embryo is not released into the oocyte cytoplasm, but is retained by the nucleus until maturation, when it is incorporated either into inactive polysomes or informosomes. Whether any of the protein components of nuclear RNP survive maturation and become incorporated into the inactive forms of cytoplasmic RNP is unknown. This is one of the questions I hope to investigate in future experiments.

Concluding remarks

The following series of events seem to be common to all eukaryotic messenger RNAs:-

(1) release of the newly synthesised polynucleotide from contact with its homologous DNA template;

(2) processing of the giant D-RNA molecule to produce a functional messenger;

(3) transport of the processed molecule into the cytoplasm;

(4) translation according to a specified schedule;

(5) degradation.

Very little can be said at present concerning the manner in which these events occur, and are regulated within the cell, though it seems reasonable to suppose that an understanding of these processes will depe-

on knowing something of the nature of the proteins associated with RNA at each stage of its history. To date, the surprising feature to emerge from several studies, including this one, is the essential simplicity of the protein component of cellular RNP; in part this must surely reflect the inadequacies of available techniques for the detection of protein, for it is difficult to believe that the specific behaviour of each RNA molecule within the cell, for example, the specific derepression of maternal messenger RNA during early development, can occur without some element of individuality being embodied in each RNP complex. The way in which such specificity is both conferred on the molecule, and recognised by the cellular apparatus, are problems which are fundamental to an understanding of eukaryote physiology.

SUMMARY

The aim of this thesis is to extend existing knowledge of the physiology and biochemistry of the amphibian oocyte nucleus.

Experimental work has been conducted along two main lines. First, in chapter II, I have attempted to identify the site of origin of the protein in the oocyte nucleus; and second, in chapter III, I have studied the proteins of the nucleus by electrophoresis in polyacrylamide gels. In chapter III I have been particularly concerned with the proteins associated with nuclear RNA. Some of the conclusions I have reached are undeniably extravagant; they are advanced as working hypotheses only for by and large they lead to predictions which can be tested by further experiment.

The main findings are summarised below:-

Chapter II.

1. An autoradiographic study of the movement of rapidly-labelled protein within the oocyte has provided evidence that nuclear protein is synthesised in the cytoplasm. Freshly isolated oocytes were incubated *in vitro* with ^{3}H-amino-acids for either 1 or 2 hours; further protein synthesis was then inhibited by addition of cycloheximide, and oocytes were left to incubate for increasing periods thereafter before being fixed and embedded. Quantitative autoradiography on sectioned oocytes showed a progressive increase in the ratio of nuclear/cytoplasmic labelling during the incubation with cycloheximide. From these data I have suggested that newly synthesised protein is transferred from the cytoplasm into the nucleus.

2. Approximate calculations based on these data, suggest that in oocytes of 0.7 mm diameter 10% of the total protein synthesized during any given period is destined to enter the nucleus, but that before doing so, it spends about 30 minutes in a cytoplasmic pool.

Chapter III

1. Isolation of nuclei from amphibian oocytes is, with practice, a rapid and straightforward operation. A single nucleus from a 0.7 mm oocyte contains 1.2 µg of protein, and consequently, sufficient protein can be obtained by manual collection of small numbers of nuclei to allow analysis of nuclear proteins by gel electrophoresis.

2. Development of the amphibian oocyte is accompanied by substantial increase in the volume and protein content of the nucleus. Electrophoretic analysis of the protein composition of the nucleus at 4 different stages in development has shown that there are some 8 major acidic proteins in the nuclear sap, and that all these proteins are present throughout oogenesis.

3. Two procedures for solubilising the RNP matrix on the lateral loops of lampbrush chromosomes have been developed. Exposure of freshly isolated chromosomes to a saline solution containing 2 µg/ml RNase and 2 M urea results in complete dissolution of the RNP matrix, but leaves chromomeres and interchromomeric strands apparently intact. Further evidence suggests that ribonucleoprotein particles in the nuclear sap are also solubilised by this treatment. Sensitivity to RNase/urea is short-lived however, and it seems probable that this is a result of the progressive crosslinkage of loop matrix by the formation of protein disulphide bonds following isolation of chromosomes into saline.

Solubilization of the crosslinked matrix has been achieved by an alternative procedure. Chromosomes isolated into an observation chamber containing Tris-buffered saline (TBS)/Mg^{2+}/Ca^{2+} pH 7.1, and left for 1-2 hours to allow disulphide bonds to form, can be solubilised by treatment with 4 M guanidine hydrochloride/0.1 M mercaptoethanol. Dissolution of virtually all cytological structures is complete within a few seconds.

The proteins solubilised by these two procedures have been examined by electrophoresis.

4. An electrophoretic comparison between total nuclear protein solubilised by RNase/urea, and the proteins of the untreated nuclear sap, has provided information concerning the protein composition of nuclear RNP. The characteristic result observed in such a comparison is the specific enrichment of a single protein (referred to by its relative mobility as 0.56) after solubilisation with RNase/urea. The result suggests that the protein in band 0.56 is a major component of nuclear RNP. Subsequent experiments have shown that this protein has a molecular weight of 116,000.

5. In an attempt to gain experimental support for the idea that protein in band 0.56 is associated with nuclear RNA, an experiment was undertaken in which oocytes were incubated <u>in vitro</u> with ^{3}H-uridine before isolation of nuclear protein. After electrophoresis the gel was examined for labelled RNA. No radioactivity was found associated with band 0.56. Unexpectedly, however, the experiment did show labelled RNA bound to another component of the nucleus, namely, a particularly sharp band with an electrophoretic mobility of 0.06. The evidence suggests that this

component of the nucleus is a RNP particle containing rapidly labelled RNA and protein. No information has yet been obtained about the properties of the protein component of band 0.06.

In summary therefore, two proteins are implicated in their association with nuclear RNA; one of them (0.56) because it appears to originate in the chromosomal matrix, and its solubilisation seems to be dependent on the enzymatic effect of RNase; the other, (0.06) because it is found associated with nuclear RNA after electrophoresis.

6. Centrifugation of nuclei at 1000 g for 5 minutes yields a preparation containing chromosomes, nucleoli, and nuclear membranes, loosely termed the "chromatin pellet". Electrophoresis of the proteins in the chromatin pellet after solubilisation with guanidine hydrochloride/2-mercaptoethanol, shows that it contains two major protein components. Molecular weight estimation by electrophoresis in SDS-acrylamide gels shows that the two proteins have molecular weights of 110,000 and 43,000.

7. The similarity in molecular weights between the larger component of the chromatin pellet and the protein extracted from band 0.56 after treatment with RNase/urea, suggests that the same protein has been isolated by both methods. Furthermore, there are some reasons for believing that the protein component of band 0.06 has a molecular weight of 40-45,000, and I suggest that this protein and the smaller of the two proteins in the chromatin pellet may also be one and the same species. Testing these assertions will be the subject of experiments to be undertaken in the future.

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Fig. 1.

A complete ovary of *T.o. carnifex* freshly isolated and lying in unbuffered 3 : 1 K/NaCl. Oocytes in all stages of development can be seen.

Scale, 1 mm.

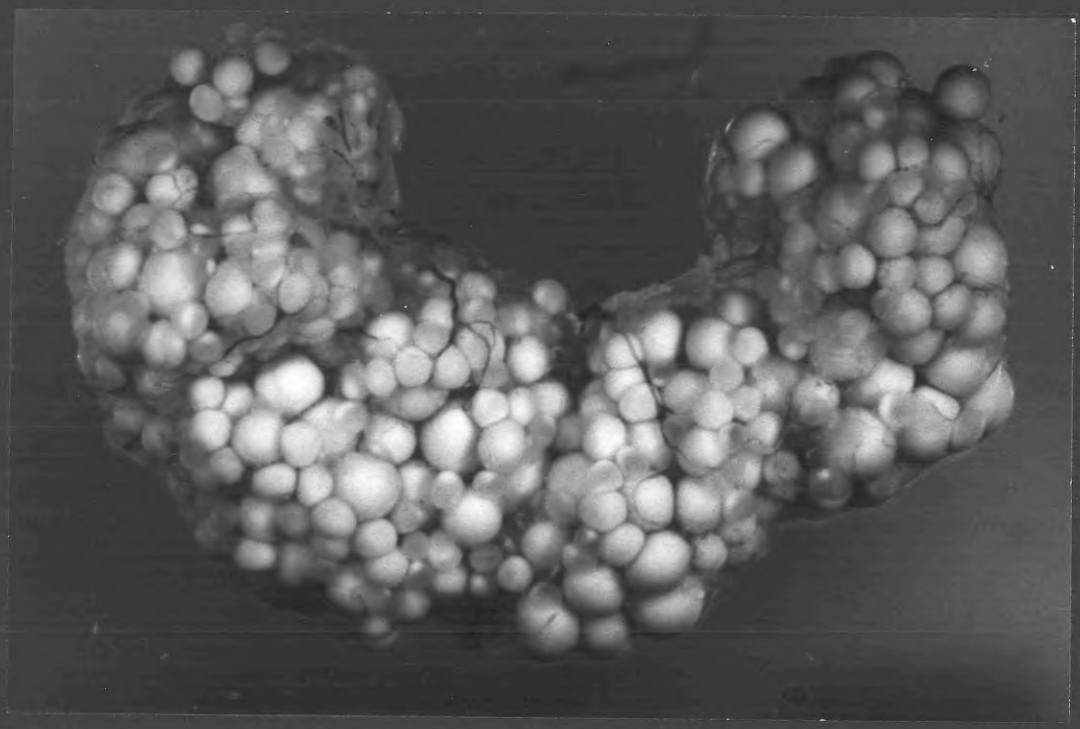


Fig. 3.

A graphical presentation of the data in table 2 showing the redistribution of newly synthesized protein within the oocyte during incubation with cycloheximide. Oocytes were pre-labelled with ^{14}C-amino-acids for 1 hour (x-x), or 2 hours (o-o) before addition of cycloheximide.

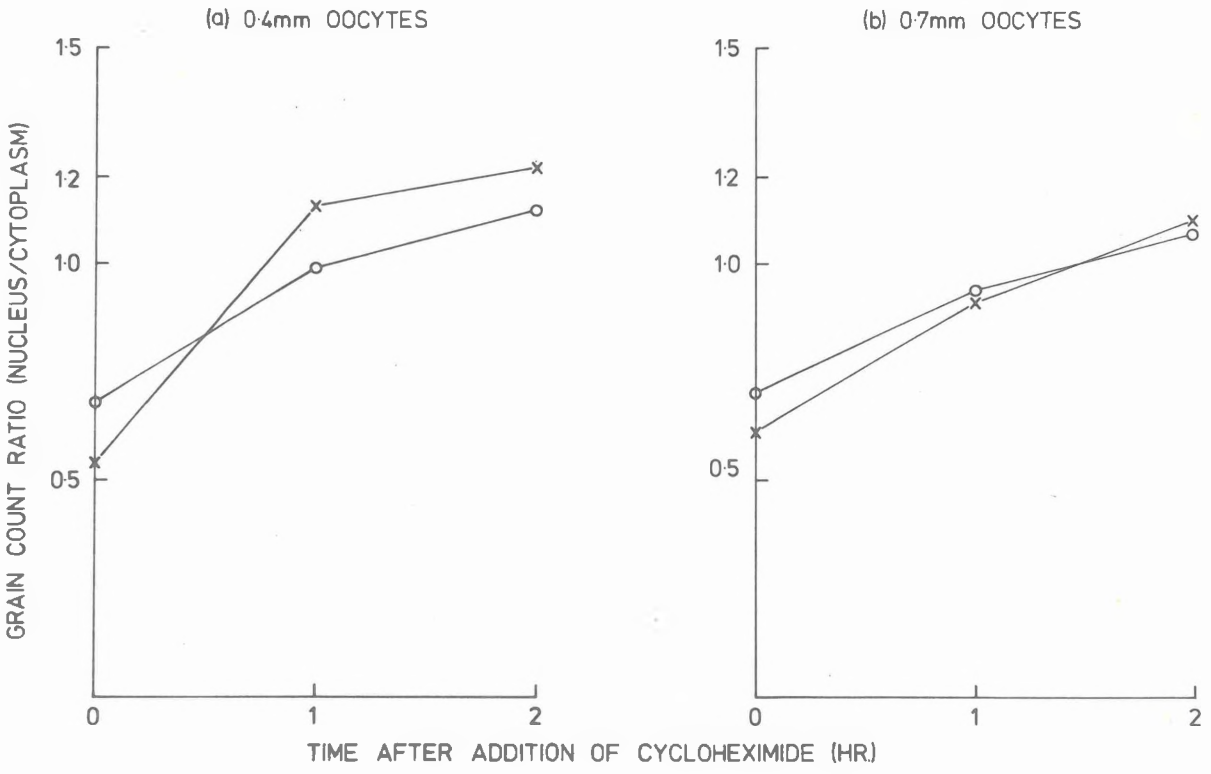

(a) 0·4mm OOCYTES
(b) 0·7mm OOCYTES
GRAIN COUNT RATIO (NUCLEUS/CYTOPLASM)
1·5
1·2
1·0
0·5
0
1
2
TIME AFTER ADDITION OF CYCLOHEXIMIDE (HR.)

Fig. 4.

The effect of *in vitro* incubation of oocytes on protein synthesising capacity. Oocytes were placed in 0.5 ml Steinberg's medium, at room temperature. At intervals after the start of incubation, a proportion of the oocytes were transferred to a second chamber containing 0.5 ml Steinberg's medium plus 25 µCi ^{14}C-amino-acids (sp. act. 52 mCi/mA) and left for a further 1 hour. The duration of incorporation is shown by the solid bars. At the end of each labelling period, individual 0.7 mm oocytes were ruptured on filter paper discs, and protein was precipitated with 5% TCA. Filters were washed twice in TCA and dried. Radioactivy was assayed by scintillation in a 0.04% PPO/0.005% POPOP/toluene cocktail. Results for individual oocytes (O) and mean values ( ) are shown.

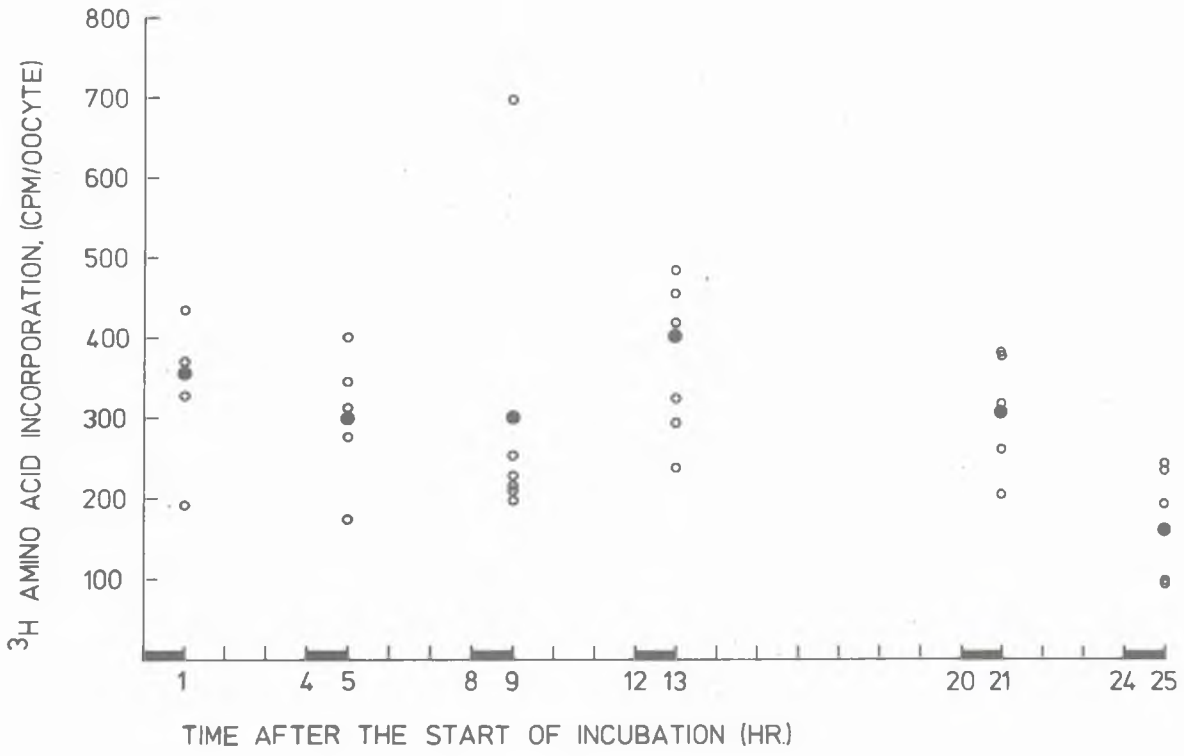

800
700
600
500
400
300
200
100
^{3}H AMINO ACID INCORPORATION, (CPM/OOCYTE)
1
4 5
8 9
12 13
20 21
24 25
TIME AFTER THE START OF INCUBATION (HR.)

Fig. 5.

Soluble proteins of the nuclear sap electrophoresed in the pH 9 discontinuous system of Ornstein & Davis. Nuclear proteins were isolated from oocytes at 4 different stages of development: (a) 0.5 - 0.6 mm diameter, (b) 0.6 - 0.7 mm diameter (c) 0.7 - 0.9 mm diameter, (d) 1.0 - 1.5 mm diameter. Numbers refer to relative mobilities.

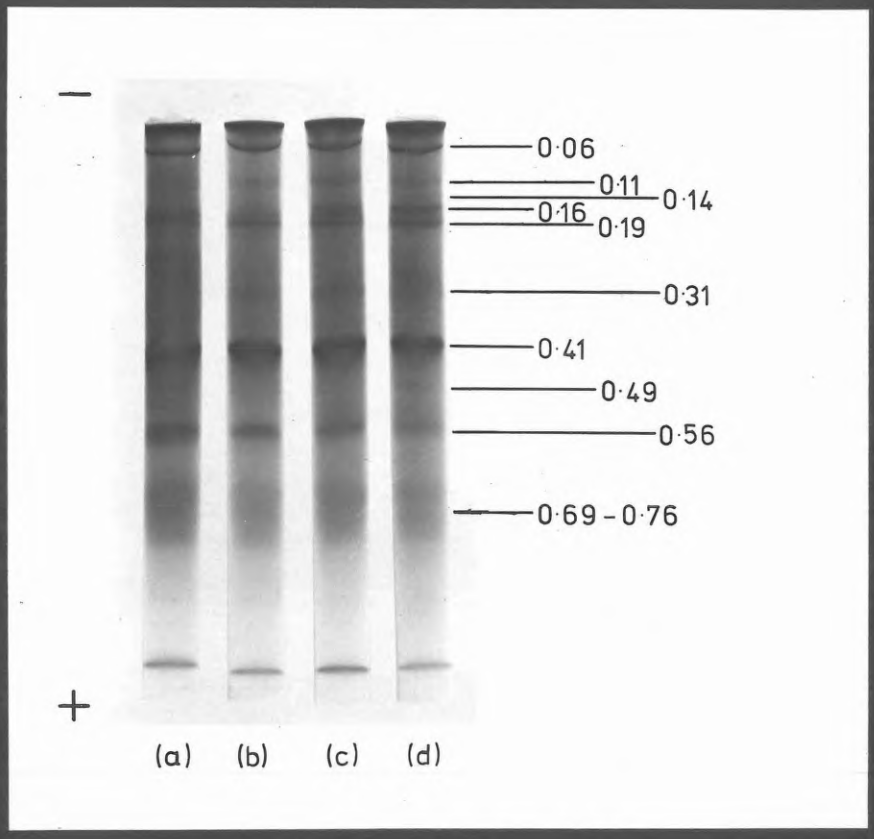
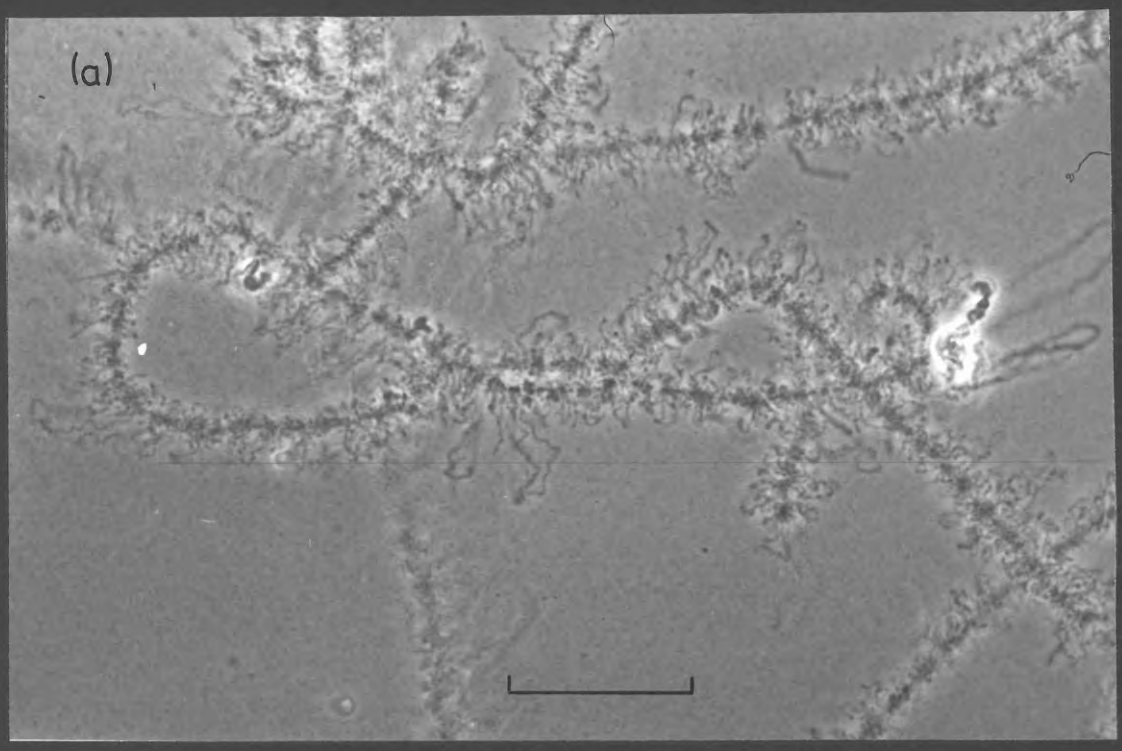
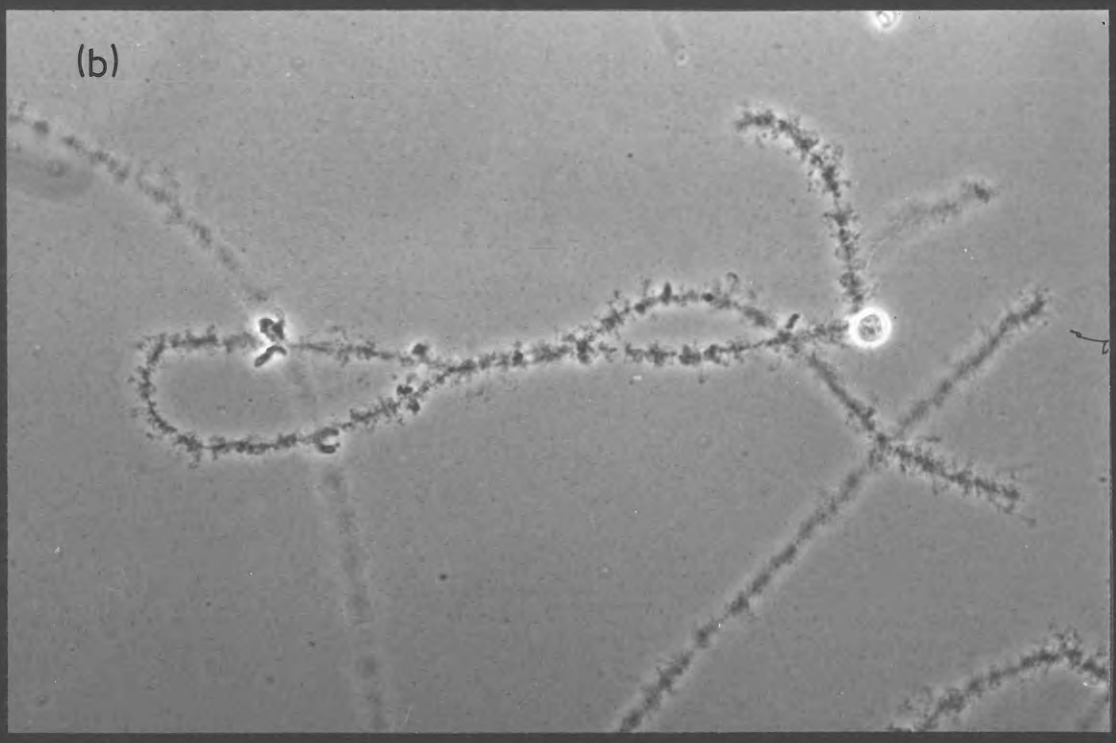

−
+
0·06
0·11
0·14
0·16
0·19
0·31
0·41
0·49
0·56
0·69 - 0·76
(a)
(b)
(c)
(d)

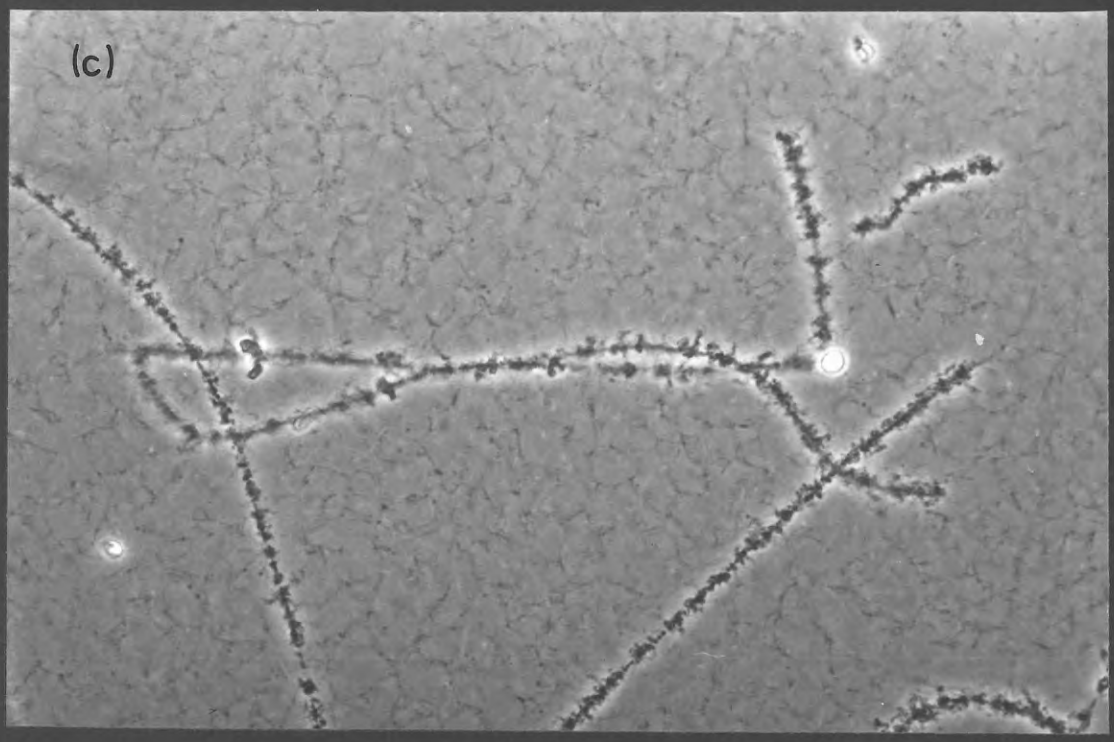
Fig. 6.

The effect of pancreatic ribonuclease on lampbrush chromosomes; (a) bivalent X of T.c. carnifex 7 minutes after isolation into an observation chamber containing TBS/Mg^{2+}/Ca^{2+}; (b) 1 minute after addition of pan-ribonuclease to a final concentration of 2 µg/ml; (c) 17 minutes after addition of ribonuclease, showing deposition of fibres; (d) 60 minutes after addition of pan-protease to a final concentration of 250 µg/ml, showing complete digestion of fibres.

Scale 50µ.


(a)


(b)


(c)

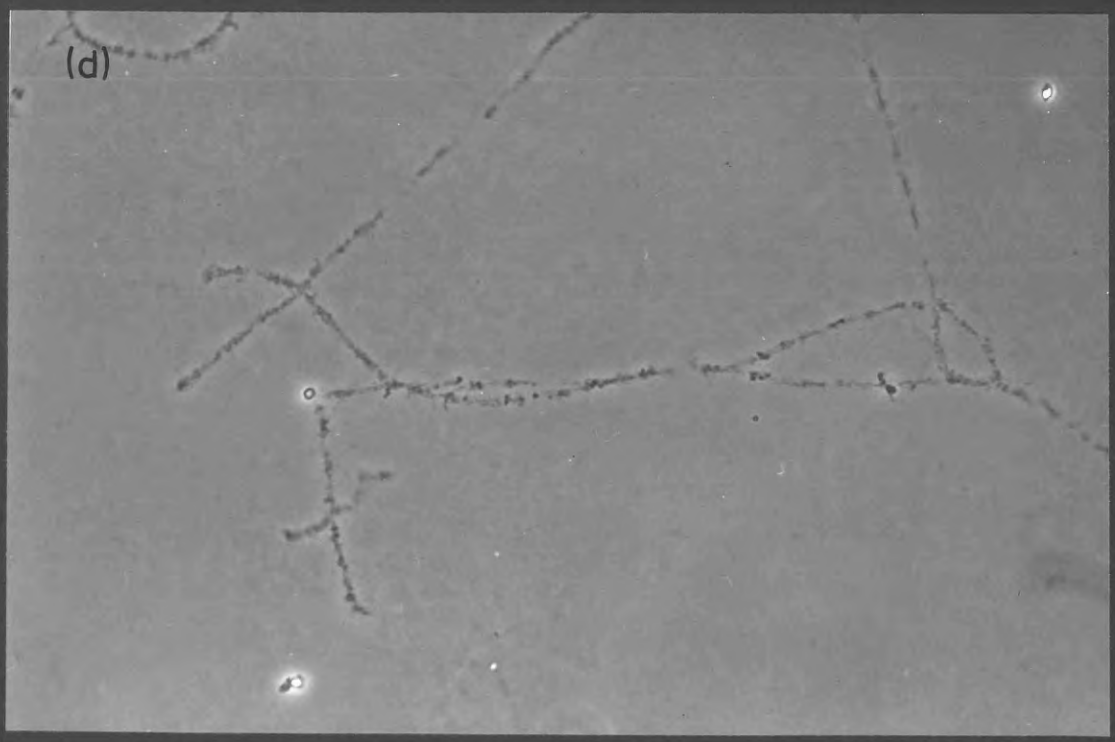

(d)

Fig. 7.

Negatively stained preparation showing the ultrastructure of the fibres formed after treatment with RNase. A repeating unit of approximately 600 A can be distinguished, which in occasional examples (arrows) can be seen to consist of subunits each of about 200 A in diameter.

Scale, 0.5μ.

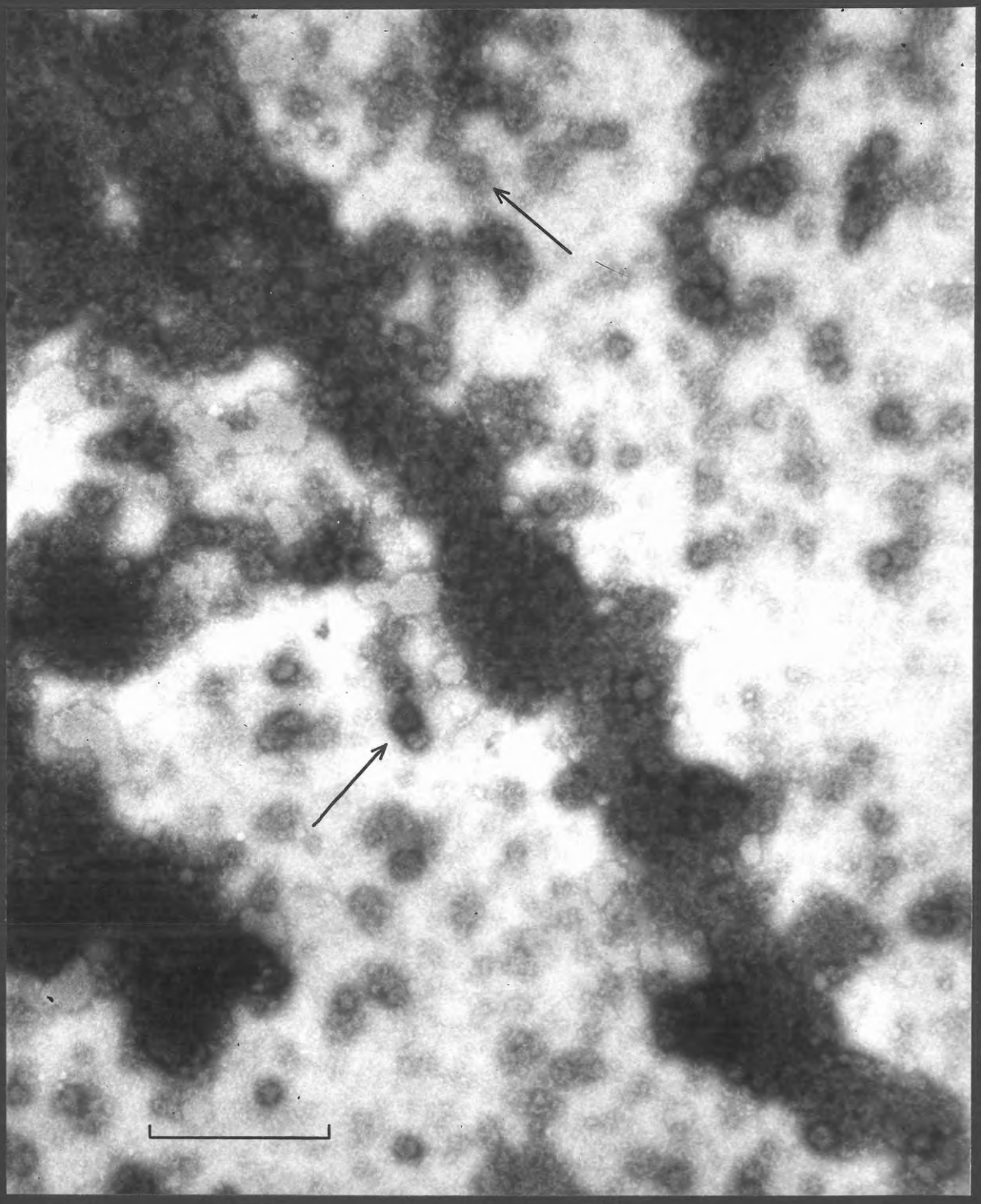


Fig 8.

Electron micrograph of a section through part of a lampbrush chromosome. Chromomeres (C) surrounded by the particulate loop matrix (M) can be seen. The particles in the matrix range from 200 A - 400 A in diameter.
Scale, 0.5μ.

Fig. 9.

The effect of RNase/urea on lampbrush chromosomes. A nucleus was isolated into an observation chamber containing 2 μg/ml RNase, 2M urea. The nuclear membrane was removed and the chromosomes were allowed to settle on to the glass coverslip. The chromosomes were then anchored to the glass by centrifugation at 1500 g for 5 minutes, and photographed as described in materials and methods. An entire bivalent (unidentified) is shown. The loop matrix is completely solubilised; chromomeres and interchromomeric strands remain. No polymerisation of fibres has occurred.
Scale, 100μ.

Fig. 8

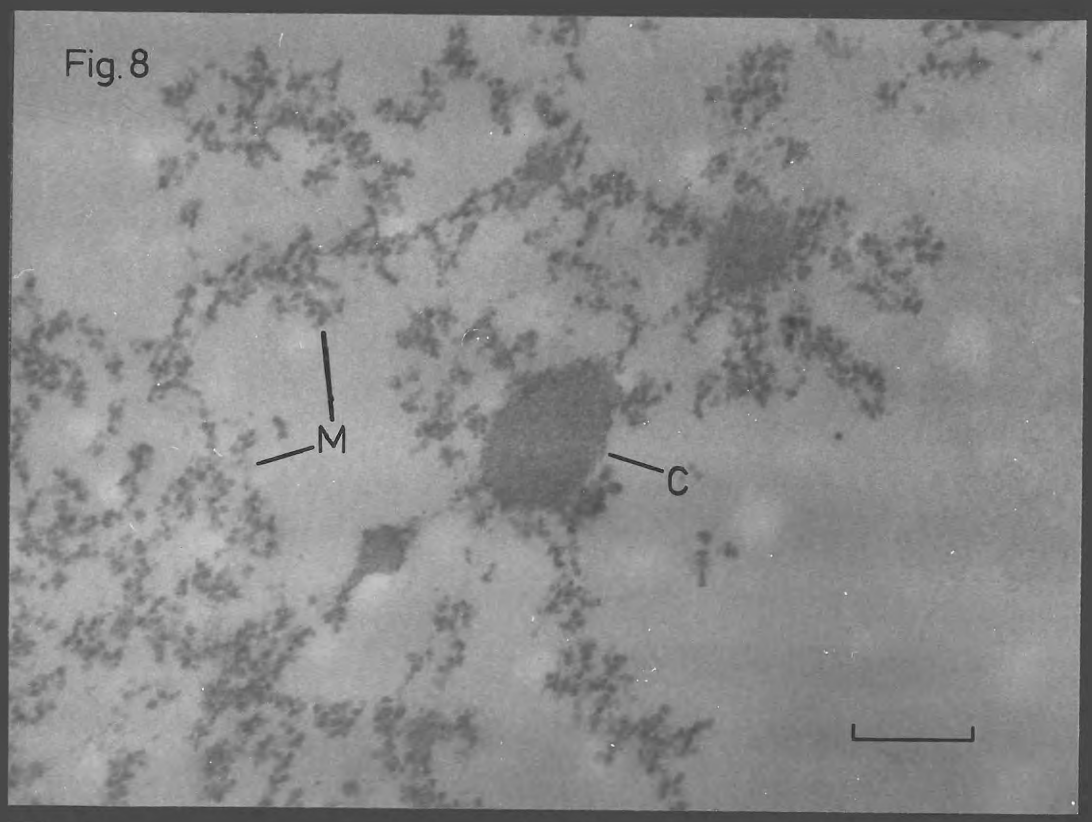


Fig. 9

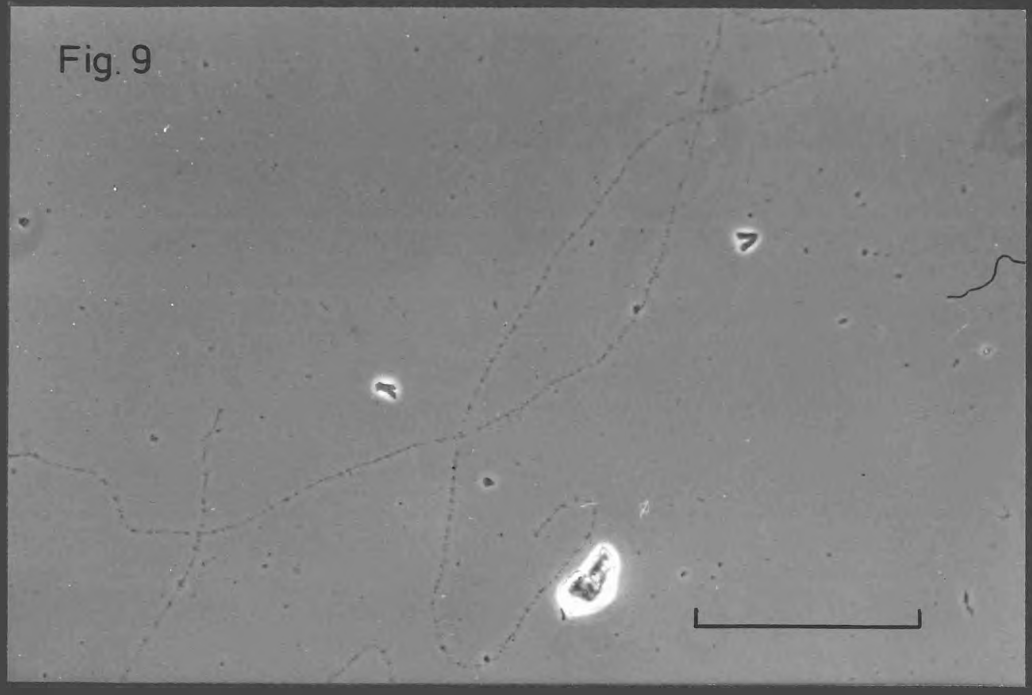
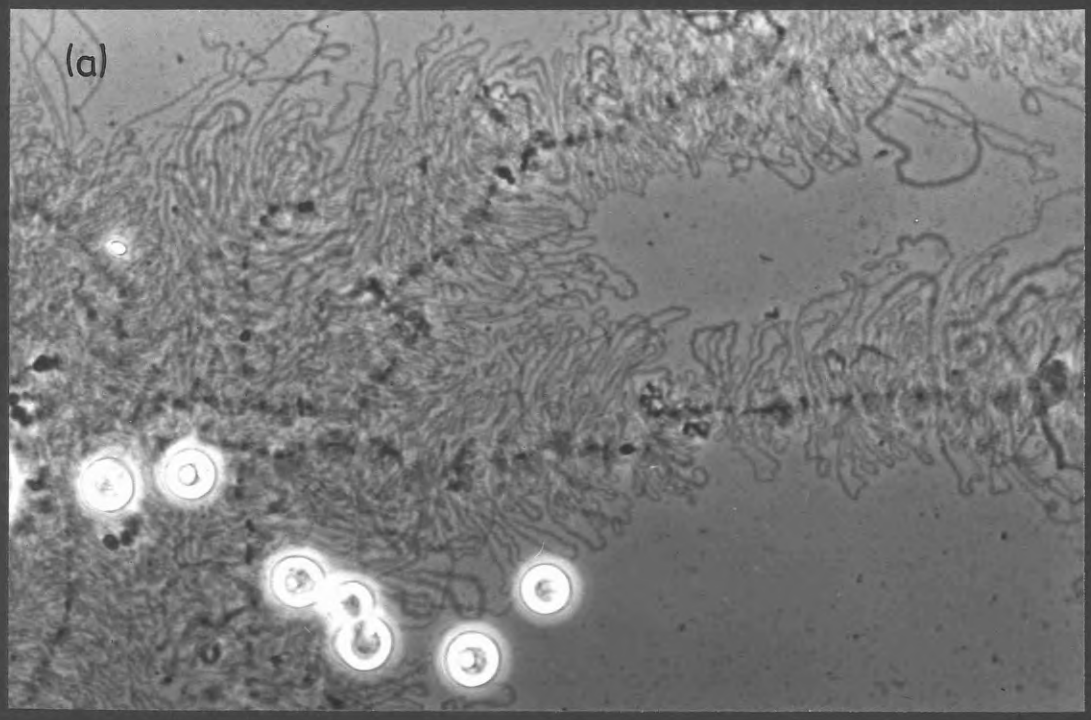


Fig. 10.

The effect of guanidine hydrochloride/2-mercaptoethanol on lampbrush chromosomes. Chromosomes were isolated into PBS/Mg^{2+}/Ca^{2+}, centrifuged, and left for 1 - 2 hours. Part of bivalent I is shown before (a) and after (b) solubilisation in 4 M guanidine hydrochloride/0.1 M mercaptoethanol. Dissolution of cytological structures is virtually complete within a few seconds.

Scale, 50μ.


(a)

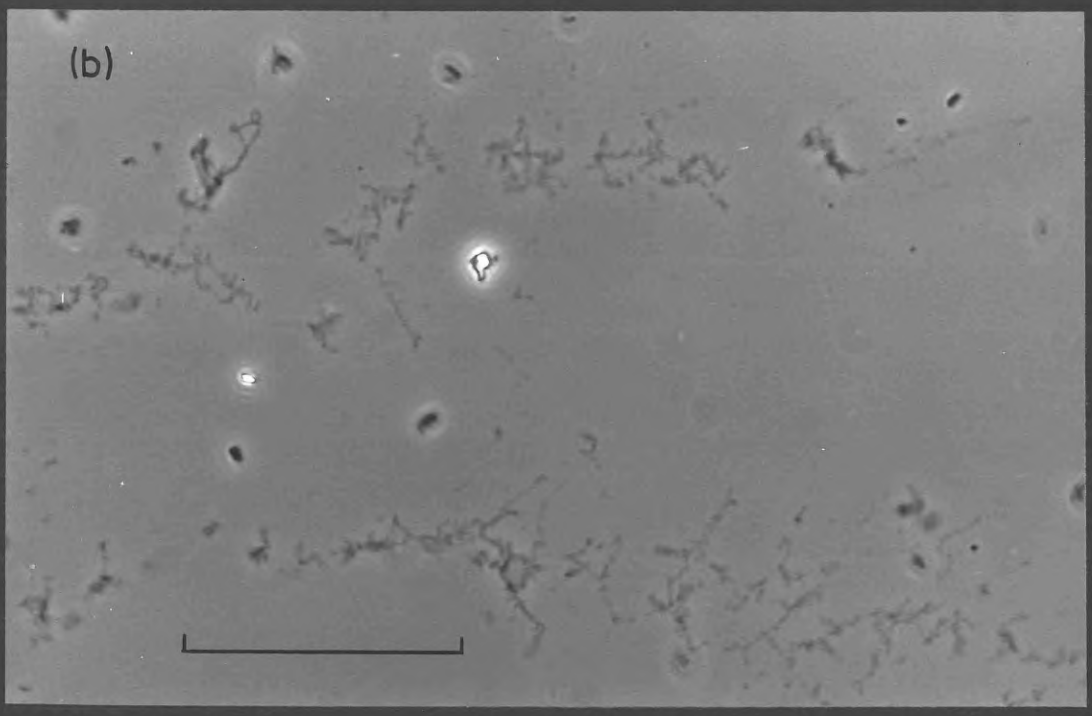

(b)

Fig. 11.

Gel electropherograms at pH 9: (a) soluble proteins of the nuclear sap; (b) total nuclear protein solubilized in 2 µg/ml RNase, 2 M urea; (c) supernatant proteins after precipitation of the fibrous web by 2 µg/ml RNase. 10 nuclei from 0.7 mm oocytes were collected for each sample.

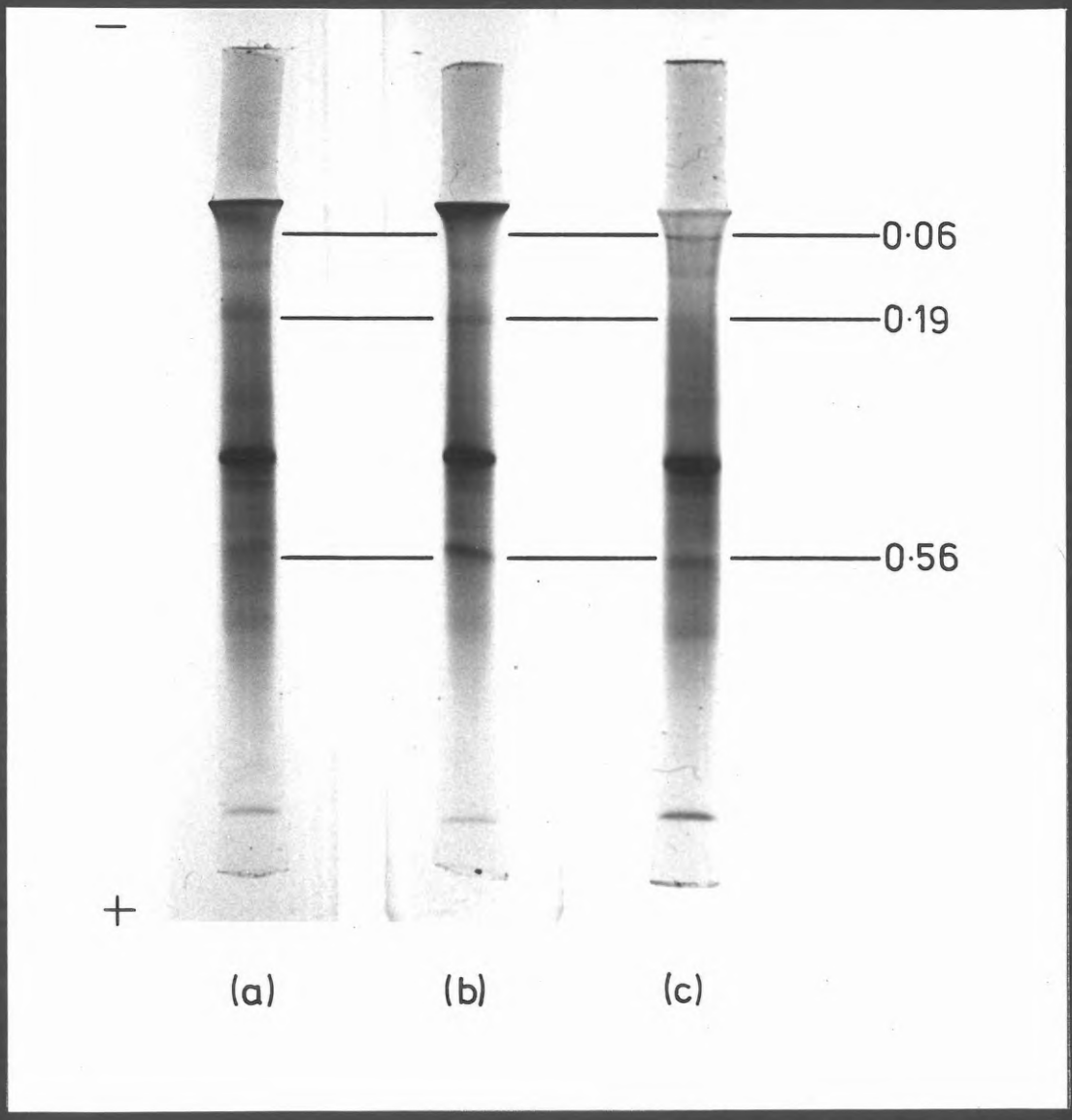

−
0·06
0·19
0·56
+
(a)
(b)
(c)

Fig. 12.

Oocyte "chromatin" prepared from a manually isolated germinal vesicle which was allowed to rupture in a solution of unbuffered 3 : 1 K/NaCl and fractionated by centrifugation. A mass of lampbrush chromosomes can be seen to have emerged from the ruptured nucleus lying to the left of the photograph. The bright objects associated with the nuclear membrane are nucleoli.

Scale, 100μ.

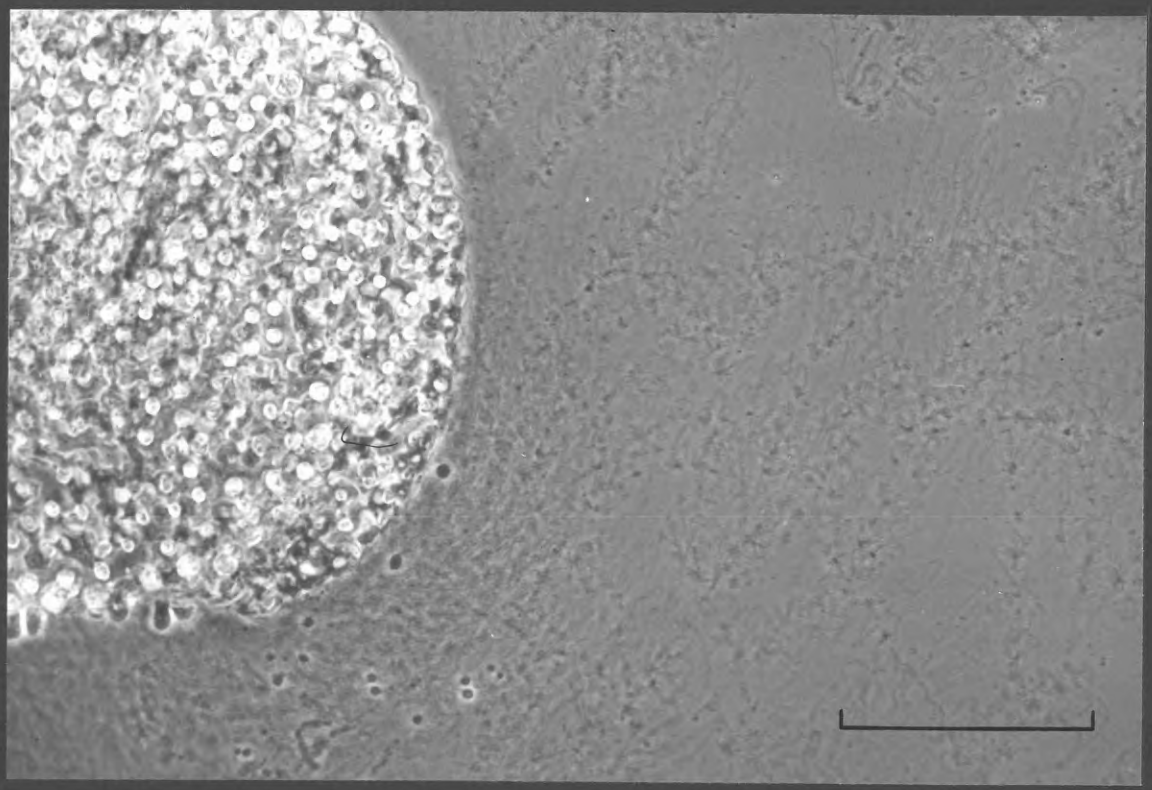


Fig. 13.

Gel electropherograms at pH 9 in 7 M urea: (a) proteins of the nuclear sap from 10 nuclei solubilised with 4 M guanidine hydrochloride/0.1 M mercaptoethanol; (b) total protein from 10 nuclei solubilised as in (a); (c) proteins of the chromatin pellet from 50 nuclei solubilised as in (a). In all cases, nuclei were obtained from oocytes of 0.7 mm diameter.

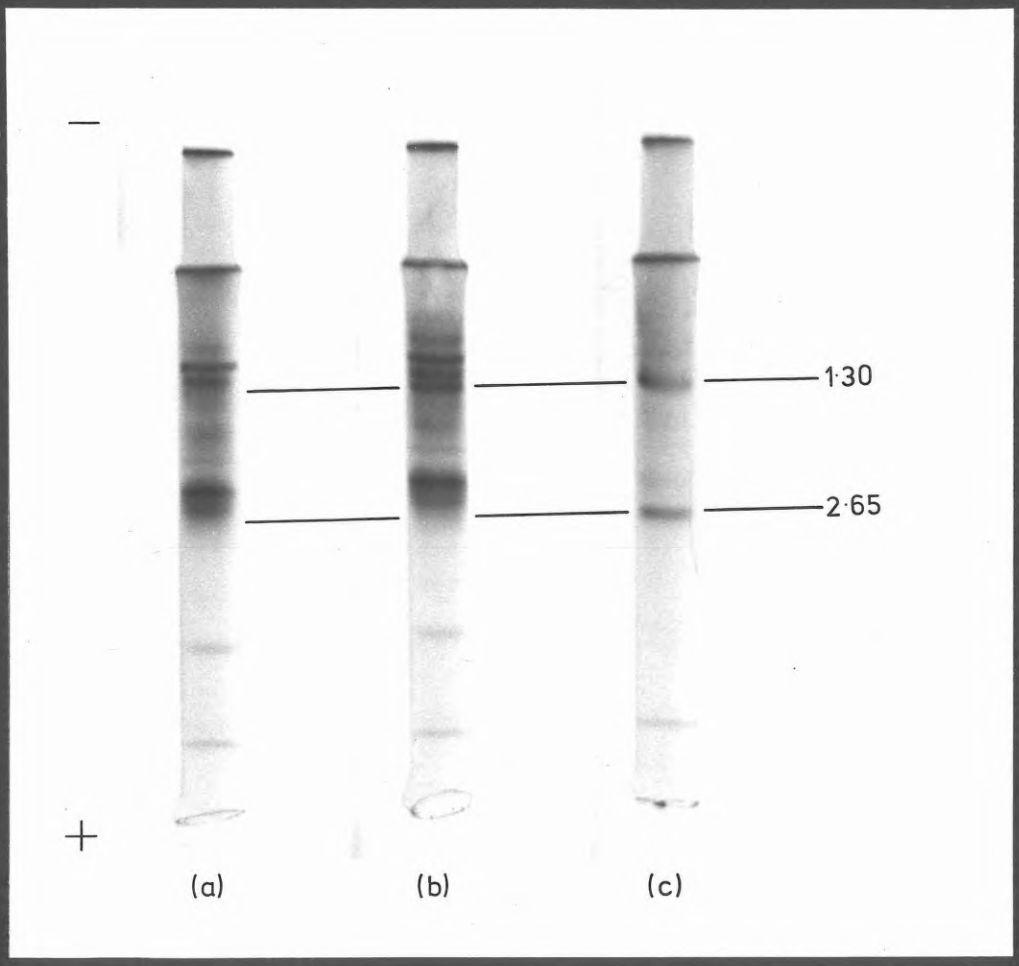

−
1·30
2·65
+
(a)
(b)
(c)

Fig. 14.

Gel electropherograms at pH 9 in 7 M urea: (a) proteins of the nuclear sap from 10 nuclei solubilised in 4 M guanidine hydrochloride/0.1 M mercaptoethanol; (b) proteins of the total chromatin pellet from 50 nuclei solubilised as in (a); (c) nucleolar plus membrane proteins from 50 nuclei solubilised as in (a). In all cases, nuclei were obtained from oocytes of 0.7 mm diameter.

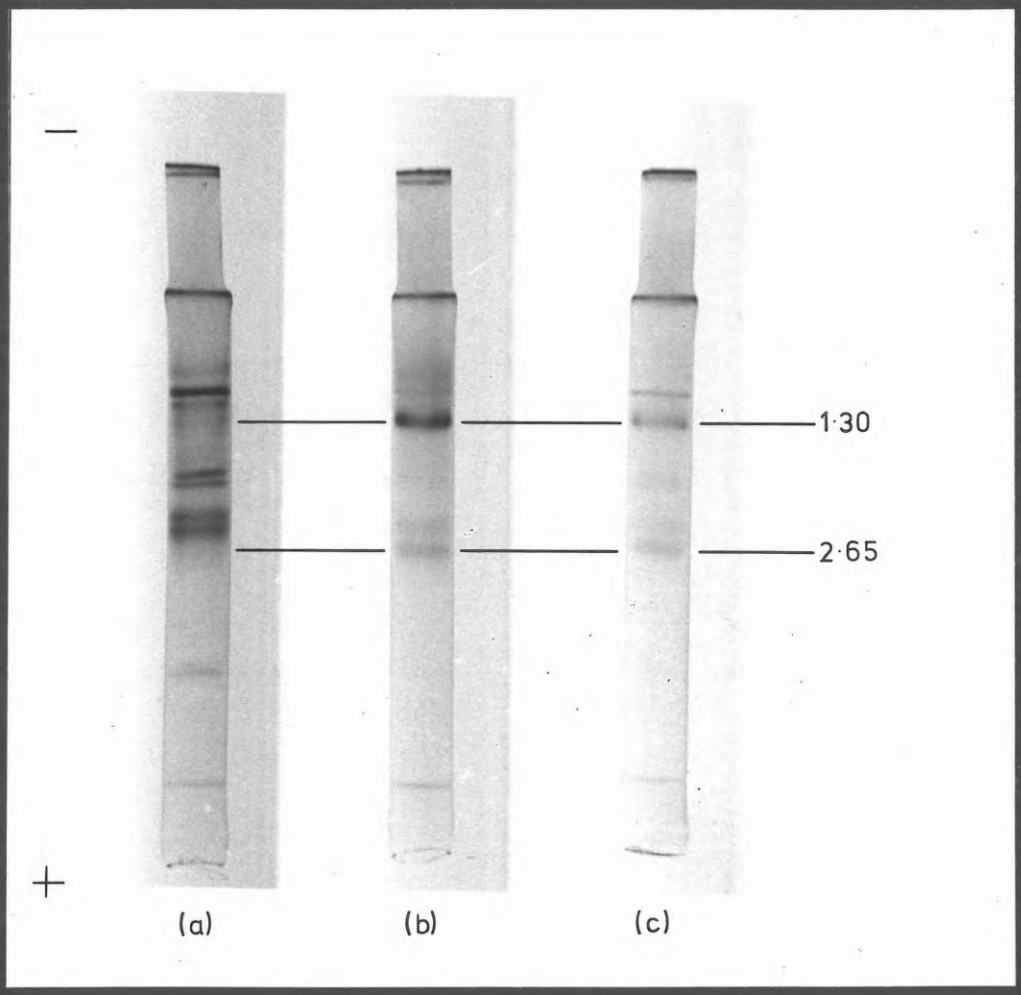

−
+
1·30
2·65
(a)
(b)
(c)

Fig. 15.

Molecular weight determination of the proteins in the chromatin pellet. The chromatin pellet from 50 nuclei was dissolved in 4 M guanidine hydrochloride/0.1 M mercaptoethanol and electrophoresed at pH 7.2 in the SDS-acrylamide system of Shapiro et al. (1967), (Fig. 15 (a)). Molecular weights of the two major components were estimated by comparison with the mobilities of standard proteins of known molecular weight, (Fig. 15 (b)).

(a)

(b)

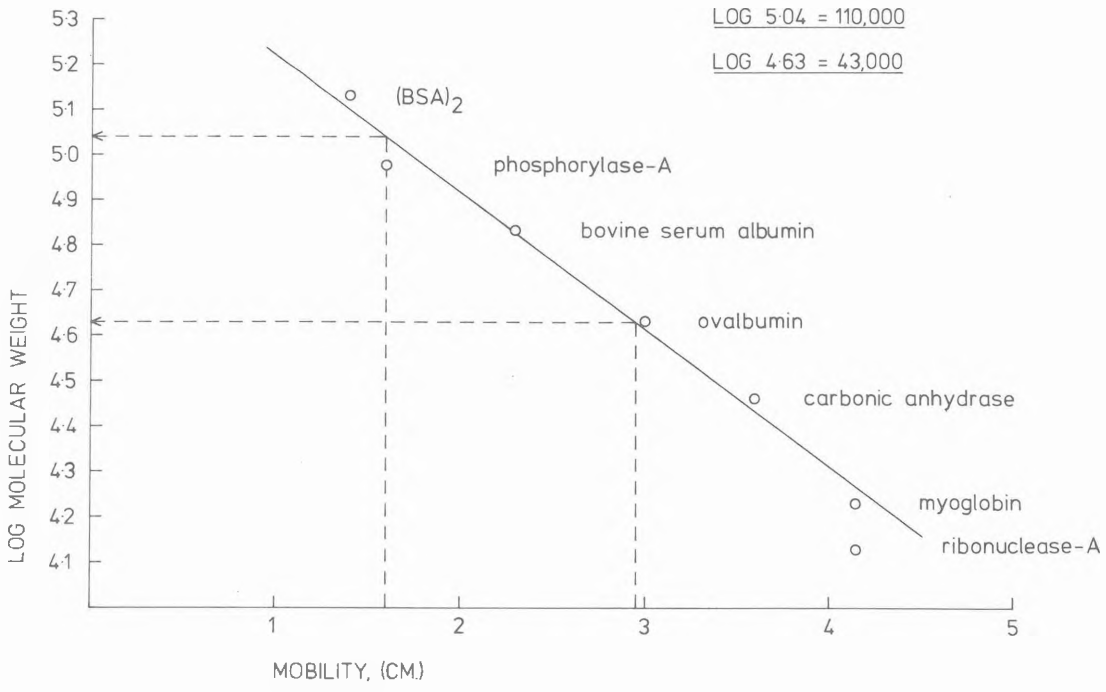

LOG 5·04 = 110,000
LOG 4·63 = 43,000
(BSA)2
phosphorylase-A
bovine serum albumin
ovalbumin
carbonic anhydrase
myoglobin
ribonuclease-A
LOG MOLECULAR WEIGHT
5·3
5·2
5·1
5·0
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
1
2
3
4
5
MOBILITY, (CM.)

Fig. 16.

Molecular weight of the protein extracted from band C.56, determined by a second electrophoresis in SDS-acrylamide system of Shapiro *et al.* (1967). As in the previous figure, the molecular weight was estimated by comparison with the mobilities of known molecular weight standards.

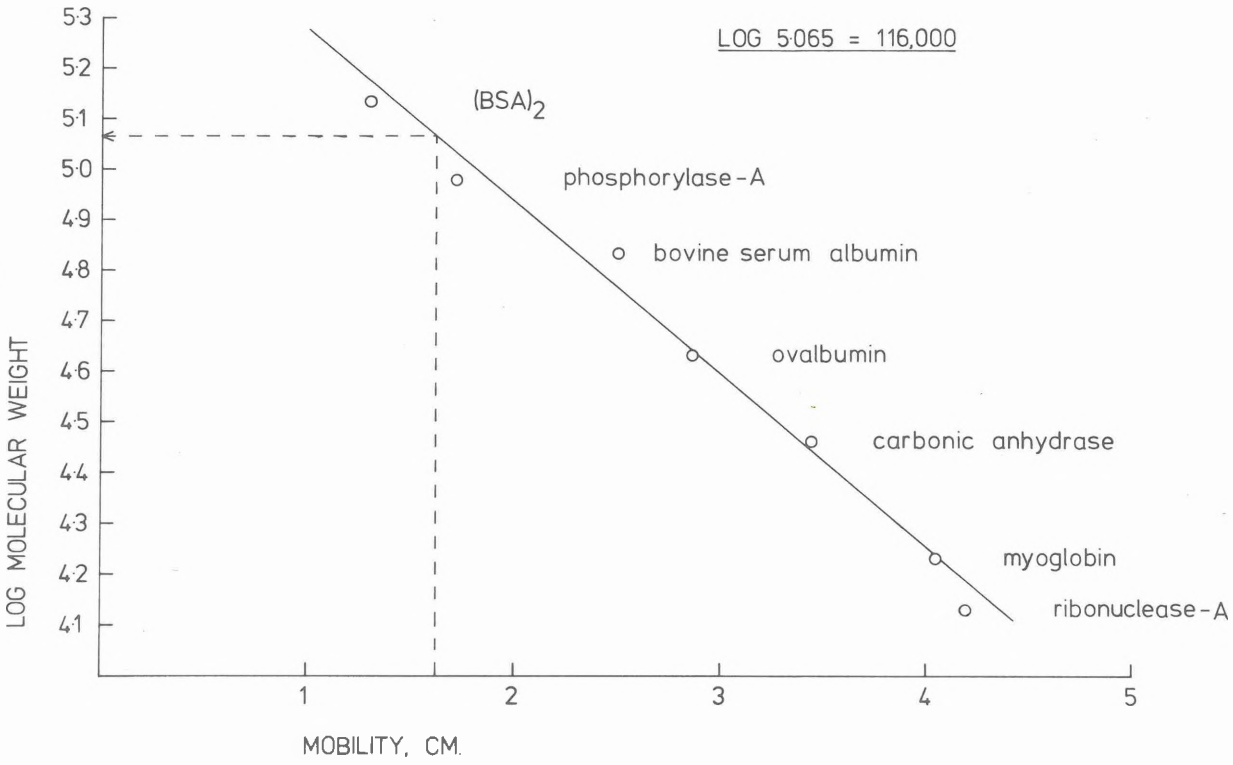
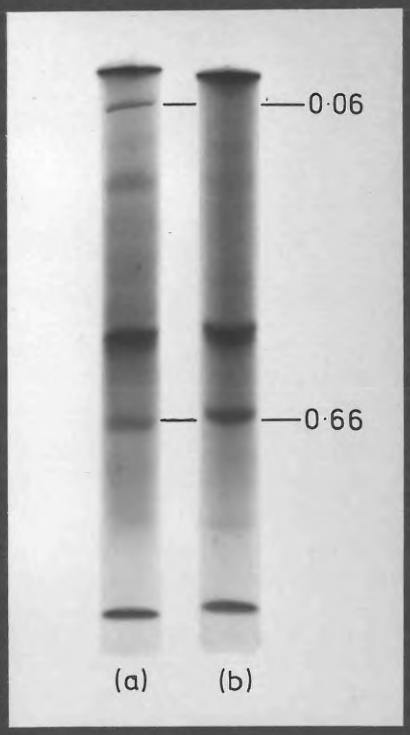

LOG 5·065 = 116,000
(BSA)2
phosphorylase-A
bovine serum albumin
ovalbumin
carbonic anhydrase
myoglobin
ribonuclease-A
LOG MOLECULAR WEIGHT
5·3
5·2
5·1
5·0
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
1
2
3
4
5
MOBILITY, CM.

Fig. 17.

Effect of urea on the mobility of the protein in band 0.56*. Gels (a) and (b) run at pH 9 without urea; gels (c) and (e) run at pH 9 in 2 M urea. The protein sample contained either soluble proteins of the nuclear sap (gels (a) and (c)) or total nuclear protein solubilised by 2 µg/ml RNase, 2 M urea (gels (b) and (d)).

* Relative mobilities in this experiment are not those established previously (see footnote on p. 53).


0·06
0·66
(a)
(b)

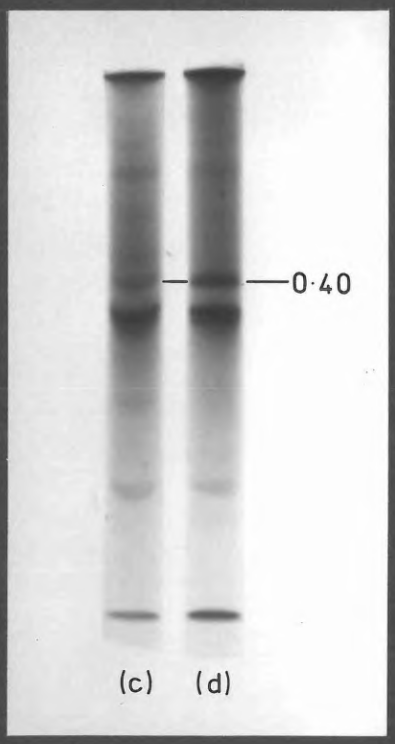

0·40
(c)
(d)

Fig. 18.

Association of rapidly-labelled RNA with the proteins of the nucleus. Oocytes were labelled for 6 hours *in vitro* with ^{3}H-uridine. Nuclei were collected in 10^{-9} µgm/ml RNase. The sample was electrophoresed at pH 9.

(——) Absorbance at 650 nm. (o–o) radioactivity.

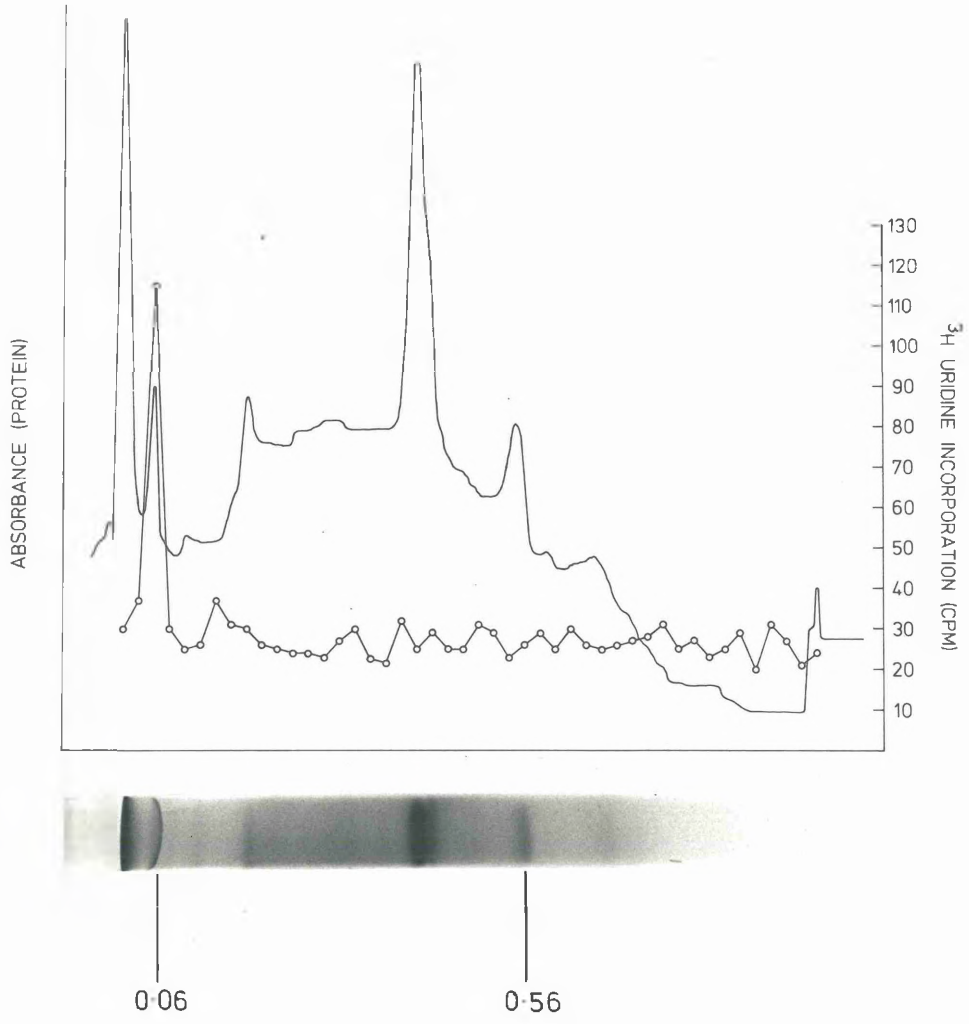

ABSORBANCE (PROTEIN)
130
120
110
100
90
80
70
60
50
40
30
20
10
^{3}H URIDINE INCORPORATION (CPM)
0·06
0·56

Fig. 19.

Estimation of the protein content of 20 nuclei by the Lowry procedure.

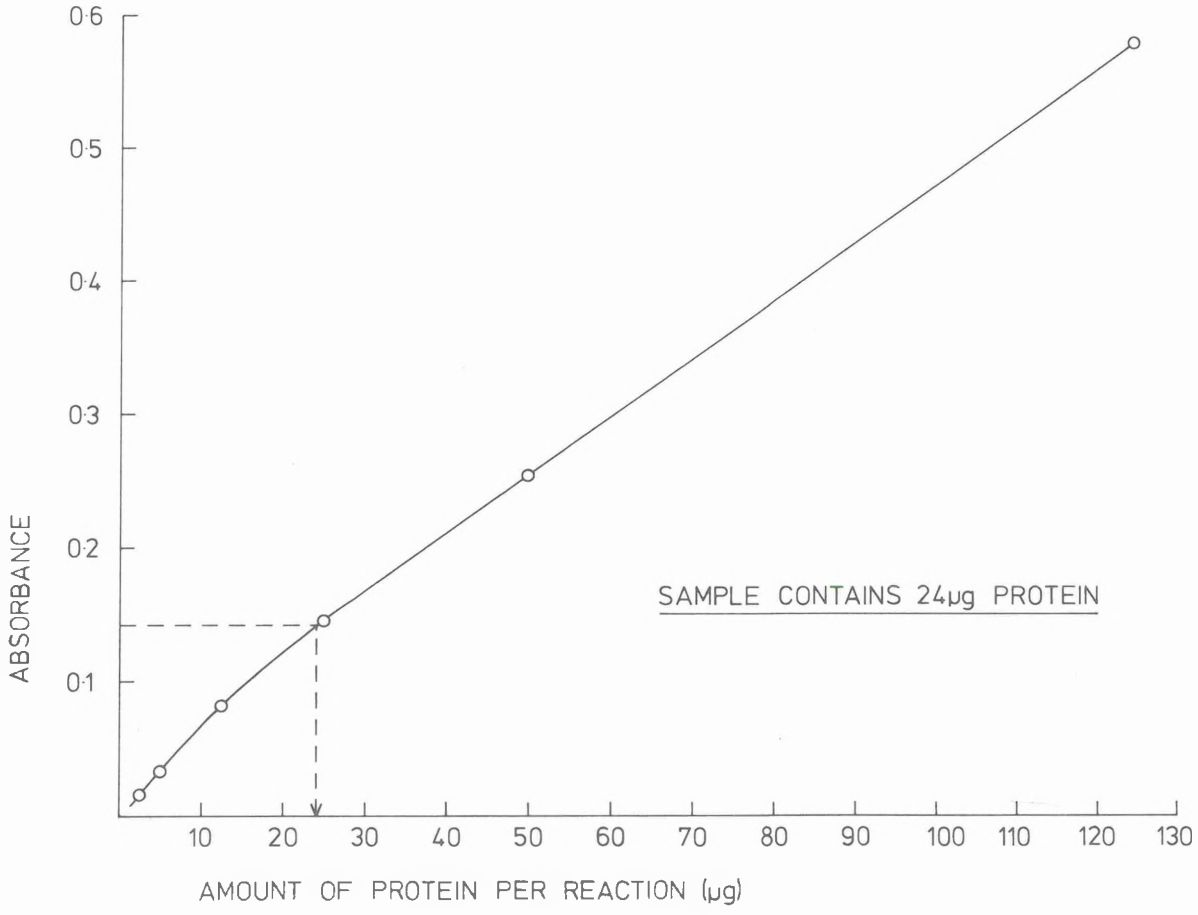

0·6
0·5
0·4
0·3
0·2
0·1
ABSORBANCE
SAMPLE CONTAINS 24µg PROTEIN
10
20
30
40
50
60
70
80
90
100
110
120
130
AMOUNT OF PROTEIN PER REACTION (µg)

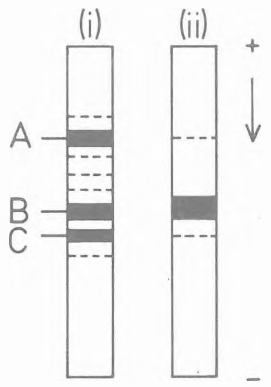
Fig. 20.

(a) Gel electropherograms at pH 4.5. Total protein of the 30S particle dissociated in 6 M urea, and electrophoresed either without treatment with mercaptoethanol (i), or after treatment with mercaptoethanol, (ii).

(b) Possible interconversions between the protein components of the 30S particle.

FIG. 20 Adapted from Krichevskaya and Georgiev (1969)

(a)



(b)