

The Distribution of Ribosomes in fission yeast and
Tetrahymena

by

Norman Maclean

Department of Zoology, University of Edinburgh

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GENERAL INTRODUCTION

The central problem of modern biology is how cells synthesise specific proteins. The machinery which carries out the synthetic process seems normally to commence with DNA, where the specific information is stored as an encoded memory trace in terms of base sequence along the length of the DNA molecule. A specific class of molecules, the messenger RNAs, copy the message in their own molecular base sequence and convey it to the actual site of synthesis which is the ribosome particle. The cooperation of ribosome and messenger RNA makes possible the production of specific proteins whose linear amino acid sequence is dependent on the sequence of bases on the messenger molecule (Watson, 1963). It is now some years since the notion that the ribosomes possess the encoded information for specific protein synthesis was abandoned. With its abandonment, the limelight passed from the ribosome to the messenger molecule.

Now despite the fact that ribosomes probably do not possess specific encoded information, their structural or supporting role in the synthetic process renders them extremely interesting particles. Many of the questions that can be asked about ribosomes turn out to be very illuminating questions regarding the whole synthetic process. How many ribosomes are present in a cell? Does the number of ribosomes increase linearly or stepwise during the cell cycle? In nucleated cells, are ribosomes confined to the cytoplasm, or, if present in the nucleus, are the nuclear ribosomes similar to cytoplasmic ones? This thesis is a

report of work designed to throw some light on these questions.

The arrangement and sequence of the work reported here demands some explanation. I first began by working with the fission yeast, Schizosaccharomyces pombe, with the intention of counting the number of ribosomes present in sections examined in the electron microscope. However, since no electron microscopy had at that time been carried out on this organism, I spent some time studying the general morphology as revealed by the electron microscope. This work is reported in Part I. Since this fission yeast grows in the main only in length, it is possible to use length-wise measurements to determine a particular cell's stage in the cell cycle between divisions. By aligning and cutting cells in longitudinal sections, and examining these sections in the electron microscope, the number of ribosomes present in the cell may be calculated and the average number for each stage in the cell cycle determined. Part II is concerned with this work.

Part III is very short and describes attempts to isolate nuclei from this fission yeast in order to study the nuclear ribosomes. The difficulties of isolating nuclei from yeast are very considerable and the attempt was eventually abandoned. I cast around for another organism which could be grown easily in axenic culture and from which it would be feasible to isolate intact nuclei. Micro-organisms provide material with easy and rapid culture and largely avoid problems of differentiation and inhomogeneity of cell type. Tetrahymena pyriformis of strain W have no micronuclei but possess a very large macronucleus, and were chosen as a source of bulk nuclear preparations. The

methods of isolating the nuclei from Tetrahymena are described in Part IV.

Part V is very preliminary work on the whole cell and isolated nuclear fractions, in which the behaviour of the ribosomes from these fractions was studied in the ultra-centrifuge. It is clear that the usefulness of information yielded by these experiments depends largely on the purity and integrity of the nuclear fraction and this problem is discussed in both Part IV and Part V. The question of how many, and what sort of ribosomes are present in nuclei seems to me to be enormously important, since it would help to resolve questions about how the messenger molecule comes off the DNA chain, how the messenger molecule is transported to the cytoplasm, where ribosomes are synthesised, and whether the messenger ribosome complex is formed in the nucleus, in the cytoplasm, or partly in both. The usefulness of a discrete nucleus in almost all non-bacterial cells is far from obvious.

During the course of my work on Tetrahymena I became very interested in reports which began to appear in the literature on the melting profile of DNA. This way of investigating DNA base ratio, double strandedness and molecular complexity is extremely simple, and, despite the fact that it does not come under the scope of the general title of this thesis, I have described in Part VI some experiments which I carried out on the isolation and purification of undenatured bacterial and tetrahymena DNA. The melting profile and attempts to restore double-strandedness are also dealt with. Bacterial DNA was prepared and used as a control, since experiments carried out by other workers had involved bacterial DNA

in heating and annealing experiments. Part VI is, therefore, a separate section not primarily concerned with the central theme of this thesis, and is included as an Appendix.

There is a great need at the present time for a more confined and precise use of the word ribosome. It would clarify molecular biology if the word were reserved for particles in the cell, measuring 100 - 300 A diameter, composed of RNA and protein, and which have been shown to be active as sites for the synthesis of proteins. However it is to be regretted that at this time such criteria are not generally attainable, and the use of the word ribosome in this and other sections of the thesis is defensible only on grounds of a common less precise usage.

PART I

An electron microscope study of the fission yeast

Schizosaccharomyces pombe

Introduction

Studies of budding yeast by light microscopy are numerous (Lindegren, 1949, Leupold, 1956, and Ganesan, 1959) and in recent years Hashimoto, Conti and Naylor, 1959, Yotsuyanagi, 1960 and 1962, Hirano and Lindegren, 1963, Vitols, North and Linnane, 1961, Hirano, 1962, Kohler, 1962, Tanaka, 1963, and Mundkur, 1961, have published electron microscope pictures of Saccharomyces cerevisiae. A most interesting paper on an electron microscope study of frozen etched cells of this yeast has been published by Moore and Muhlethaler, 1963, electron micrographs of Schizosaccharomyces octosporus have been published by Conti and Naylor, 1960, of Torulopsis utilis by Linnane, Vitols, and Newland, 1962, and of Pichia by van Iterson, 1962.

Work on the structure of Schizosaccharomyces pombe is sparse. The position of its nucleus was demonstrated by Ganesan and Swaminathan, 1958, using staining and light microscopy, and by Rustad, 1958, using fluorescent microscopy. Nuclear division in S. pombe has been studied by Sando and Tanaka, 1963, and by Schopfer, Wustenfeld and Turian, 1963. At the time of carrying out the work described in this thesis no electron microscopy on this organism had been published, but subsequently a paper by Tanaka, 1963, has appeared.

Material and Methods

Organism. Schizosaccaromyces pombe (N.C. Y.C. 132) is a fission yeast, cylindrical in shape, with the ends of the cell hemispherical. Normal cells vary between 8 μ and 20 μ in length, maintaining a fairly constant diameter of 3.5 μ . Although sexual reproduction by conjugation and resulting spore formation is known in this species, all the cells examined were growing vegetatively.

Culture. Cells were grown in 2% (w/v) Oxoid wort broth (Malt extract broth) in distilled water. All the cells described were in the exponential phase of growth from cultures containing between 3 and 5 x 10⁶ per ml. Cultures were grown at 33°C; the generation time at this temperature is slightly over two hours. The method of culture was to inoculate sterile litre flasks containing 400 mls. of medium, and the flasks were never aerated nor agitated during growth.

Electron microscopy - fixation. Cultures were harvested by centrifugation at 2700 g and fixed in the following ways:

1. Cells washed twice in 0.25M sucrose, buffered with veronal acetate at pH 7.2, followed by fixation for 1 hour at 0°C in 2% osmium tetroxide in the veronal acetate buffer.
2. Cells washed twice in 1.5M saline, followed by fixation for 1 hour at 0°C in 1.5% potassium permanganate in the 1.5M saline.

Embedding. Following fixation, cells were dehydrated in a graded series of alcohols from 20% to absolute alcohol, the dehydration being completed by three washes in absolute alcohol. Methacrylate was employed as an embedding medium, using a mixture of 92% butyl methacrylate, 7% methyl methacrylate and 1% benzoyl peroxide.

Material fixed in permanganate was much more prone to explosi^{on} during polymerisation than that fixed in osmium tetroxide, and Borysko's technique with pre-polymerised methacrylate and a high polymerisation temperature of 58°C proved essential to the embedding of such material. Osmium fixed cells were embedded in liquid met^hacrylate at 58°C. Some of the cells fixed with potassium permanganate were embedded in methacrylate with 1% benzoin replacing the benzoyl peroxide catalyst, and polymerisation was induced by irradiating the gelatin capsules with ultra-violet light for 12 hours. No difference in efficiency of polymerisation and preservation of cell structure could be detected between the two methods of ~~fixation~~^{polymerisation}, however.

Sectioning. Sections were cut with glass knives on a Huxley ultra microtome and floated on to a distilled water reservoir, from which they were picked up on carbon coated grids after expansion by contact with xylene vapour.

Staining. After sections had been transferred to grids and dried, the grids were immersed in a staining solution in order to improve contrast in the microscope. Both uranyl acetate and potassium permanganate were used in this way on different sections of both the osmium and potassium fixed material. Staining was carried out in petri dishes at 20°C for periods of $\frac{1}{2}$ to 1 hour.

Examination. A Siemens Elmiskop I was used in examining sections, with 40 or 60 Kv supply and a 30 μ objective aperture. Photographs were taken on Ilford N 40 plates at instrumental magnifications of 15,000 to 30,000.

Results and Discussion

Cell Wall.

Growth of a cell of S. pombe is lengthwise and usually at only one end of the cell. The growing end appears to be the end opposite to the most recent division scar, at the periphery of the cell plate. It is therefore not surprising to find that cell wall thickness varies from one part of the cell to another. The general dimensions of the cell wall along the length of the cell are between 1000 and 2000 Å (Figures 3 & 15). Old division scars may result in areas of greater thickness. Some electron micrographs reveal two layers to the wall (Figure 6), a thin outer coat of about 400 Å which appears coarse and granular when fixed, and a thick inner layer of around 1000 Å and with very low electron density in unstained sections. The work of Vitols, North and Linnane, 1961, reveals a cell wall of similar appearance in Saccharomyces cerevisiae.

Cell membrane.

As noted in S. cerevisiae (Vitols, North and Linnane) the cell membrane revealed by potassium permanganate fixation and subsequent uranyl acetate staining differs in appearance from the other membranes of the cell occurring in the cytoplasm and the nucleus. Unlike these membranes which all show a faint inner light layer, separating two clearly defined electron opaque layers, the cell membrane consists of a very pronounced central light layer bounded by two more indistinct dark lines. This structure measures about 80 Å, which is the general dimension for the single membrane

occurring in most cells fixed with permanganate. Figures 4 and 6 demonstrate this membrane fairly well. Material fixed with osmium tetroxide shows only poorly defined cell membranes, and both Agar and Douglas, 1957, and Vitols, North and Linnane, 1961, have commented on the difficulty of fixing yeast cells with osmium.

Between the cell wall and the cell membrane is to be found a layer of material staining darkly with uranyl acetate and sending protrusions of varying size into the cell (Figures 5 & 6). While remaining outside the protoplast, this layer does not seem to be an integral part of the cell wall. A similar layer of material has been noticed by Lindegren, 1963, in S. cerevisiae and he identified it as nucleoprotein on the basis of its dark staining with uranyl acetate. However, I feel that staining specificity is insufficient evidence for determining chemical composition in this case.

Cytoplasmic membranes and vesicles.

Throughout the cytoplasm of S. pombe there is a membranous system, part of which is organised into closed vesicles, but which also includes apparently loose membranes which are dispersed throughout the cytoplasm (Figures 1 & 6). The possibility that some of these open membranes represent vesicles disrupted during fixation or embedding cannot of course be ruled out. Recent studies of bacterial cytoplasm have revealed the frequent presence of whorled formations of unit membranes in the cytoplasm (Glauert and Hopwood, 1960), and the work of Linnane, Vitols and Newland on Torulopsis grown under anaerobic conditions has revealed a

strikingly similar picture. They suggest that yeast cells grown under anaerobic conditions do not develop mitochondria, but instead possess this straggling membranous and vesicular system. Now the whole question of fungal mitochondria is extremely confused, and although it has now been amply demonstrated that budding yeasts do possess mitochondria-like vesicles with numerous cristae (Hirano and Lindegren, 1961, Yotsuyanagi, 1962) some of the structures so designated are open to question. In any event, none of the membranous vesicles in S. pombe appear to possess cristae and their function remains to be discovered. It is also apparent from the cells fixed in osmium tetroxide (Figures 14 & 15) that ribosomes do not occur inside these vesicles. However, in the work of Yotsuyanagi, 1962, on budding yeast at different stages of growth, it appears that true mitochondria with obvious cristae only appeared in cells entering or in stationary phase, and that log phase cells had normally no mitochondria whatever, but only some scattered membranes. Since the cells of S. pombe under study were from log phase cultures it would appear that the lack of mitochondria is not an isolated example.

In the light of the work of Vitols, North and Linnane, it is unfortunate that the cells which I have used in this work were all grown in conditions in which the respiratory state of the cells is uncertain (see Methods). It would be interesting to establish the absence or presence of mitochondria in S. pombe grown under reliably aerobic conditions.

As found by Koehler, 1962, in S. cerevisiae, there is often present in S. pombe a double membrane extending around the

periphery of the cell in close proximity to the cell membrane itself. This is particularly obvious in Figure 7.

Storage granules.

Living cells of S. pombe, when viewed by phase contrast microscopy, display highly refractile particles which are especially frequent near to the ends of the cell (Figure 1). These particles are found to give staining reactions suggestive of lipid material (Mitchison, J.M., personal communication) and the dimensions and location of these particles in the light microscope are in keeping with the holes present in sections after fixation for electron microscopy and apparent in Figures 1 and 2. The correlation between similar holes and lipid storage material has been suggested in work on bacteria by Wyse, Newman and Socolofsky, 1961. They do not appear to be bounded by membranes and are present also in the electron micrographs of budding yeast made by other workers (Koehler, 1962). The large vacuole present in budding yeast, situated near to, and in the past often confused with its nucleus (Lindgren, 1949), is absent from S. pombe.

Nucleus.

The nucleus is fairly central, of dimensions 3 μ in length and 2 μ in breadth (Figures 2 & 3). In material fixed with osmium tetroxide or potassium permanganate its contents are less electron dense than is the cytoplasm.

Nuclear ^{envelope} ~~membrane~~.

It is less easy to achieve clarity of definition of the ^{membranes} nuclear ^{envelope} ~~membrane~~ than it is of the cell membrane, but in none of the sections is a double unit membrane suggested. By this is meant a structure measuring about 150 Å and consisting of three lines of electron dense material alternating with two lines of rather electron-transparent material, the central dark line normally being the thickest (Robertson, 1959). Nuclear pores are suggested in Figures 2 and 7. The results of the osmium tetroxide fixation on the nuclear ^{envelope} ~~membrane~~ and indeed on many of the cytoplasmic membranes ^{suggests the presence of a space between two membranes.} ~~is very curious in S. pombe.~~ It will be seen from Figures 8 and 9 that these membranes are ~~very~~ poorly defined ^{the enclosed space} and less dense to electrons than either the nuclear or cytoplasmic contents. ~~The reason for this apparent osmiophobic reaction is obscure.~~

Nucleolus.

When fixed with potassium permanganate, the nuclear contents of S. pombe are uniformly unorganised and rather less dense than the cytoplasm, but with osmium tetroxide fixation, a large and very dense nucleolus becomes visible (Figures 8 & 9), measuring 1.2 - 1.4 μ in diameter. No enclosing membrane can be detected at its surface. In all osmium fixed cells it is seen to be finely particulate in structure and in some sections an unknown and coarsely ^{fibillar} ~~granular~~ organelle is associated with it (Figures 8 & 9). Since all the recent electron micrographs on S. cerevisiae have involved fixation with potassium permanganate, the presence

of a nucleolus in this cell has not been demonstrated, but Edwards, Hazen and Edwards, 1959, have displayed it very clearly in the yeast-like cells of Histoplasma fixed with osmium tetroxide. The work of Mundkur, 1961, on S. cerevisiae using freeze-drying fixation displays a dark and highly granular object in the nucleus which is probably the nucleolus. In S. pombe the nucleolus is finely particulate, the particle size being around 100 \AA and distinctly smaller than that of the cytoplasmic ribosomes.



Figure 1. Cells of Schizosaccharomyces pombe in log phase growth, viewed by phase contrast. Photograph by Prof. J.M. Mitchison.

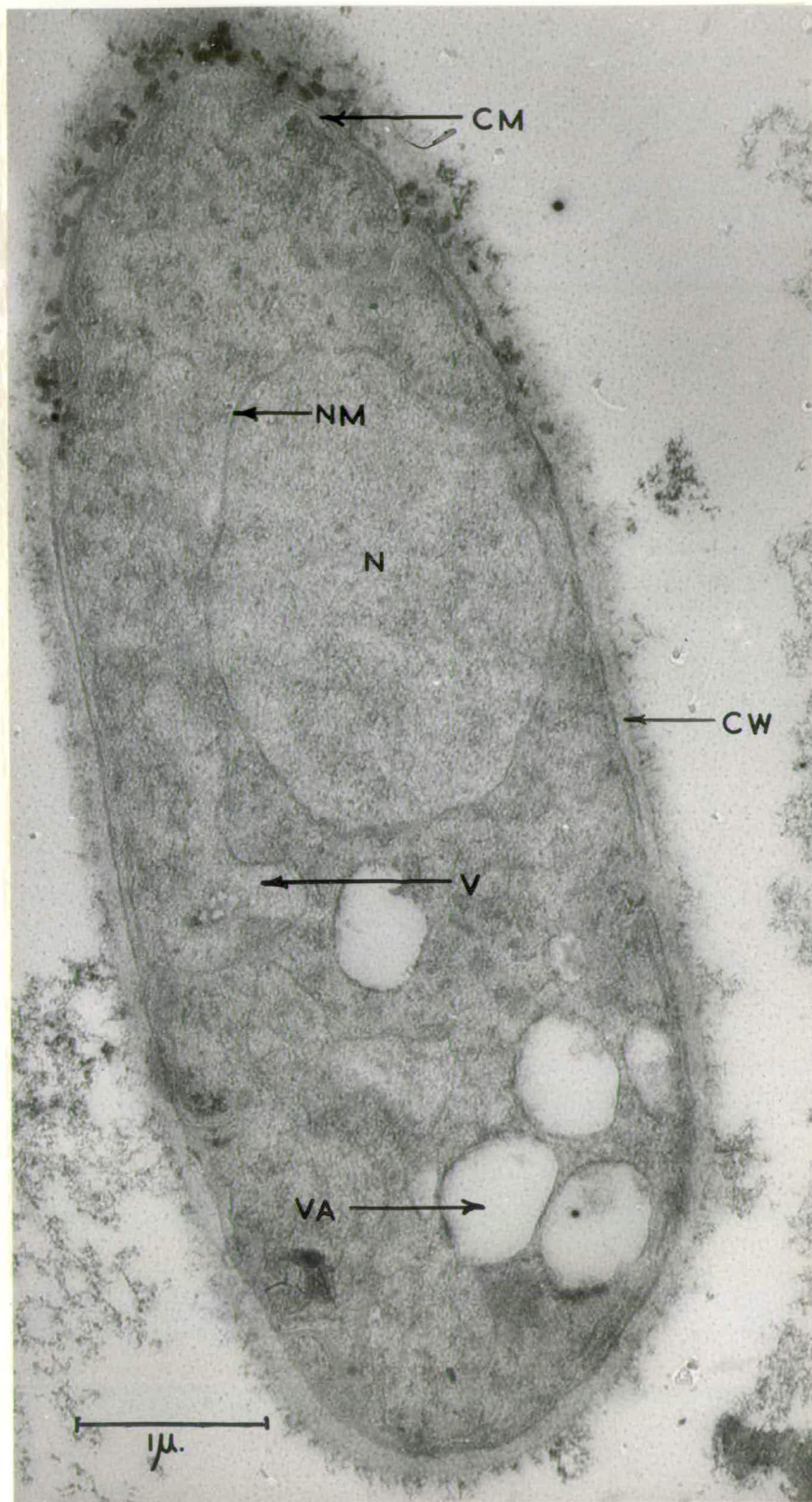


Figure 2. Cell fixed in Potassium permanganate, and stained after fixation with potassium permanganate. CM = cell membrane; CW = cell wall; N = nucleus; NM = nuclear membrane; V = vesicle; VA = vacuole.

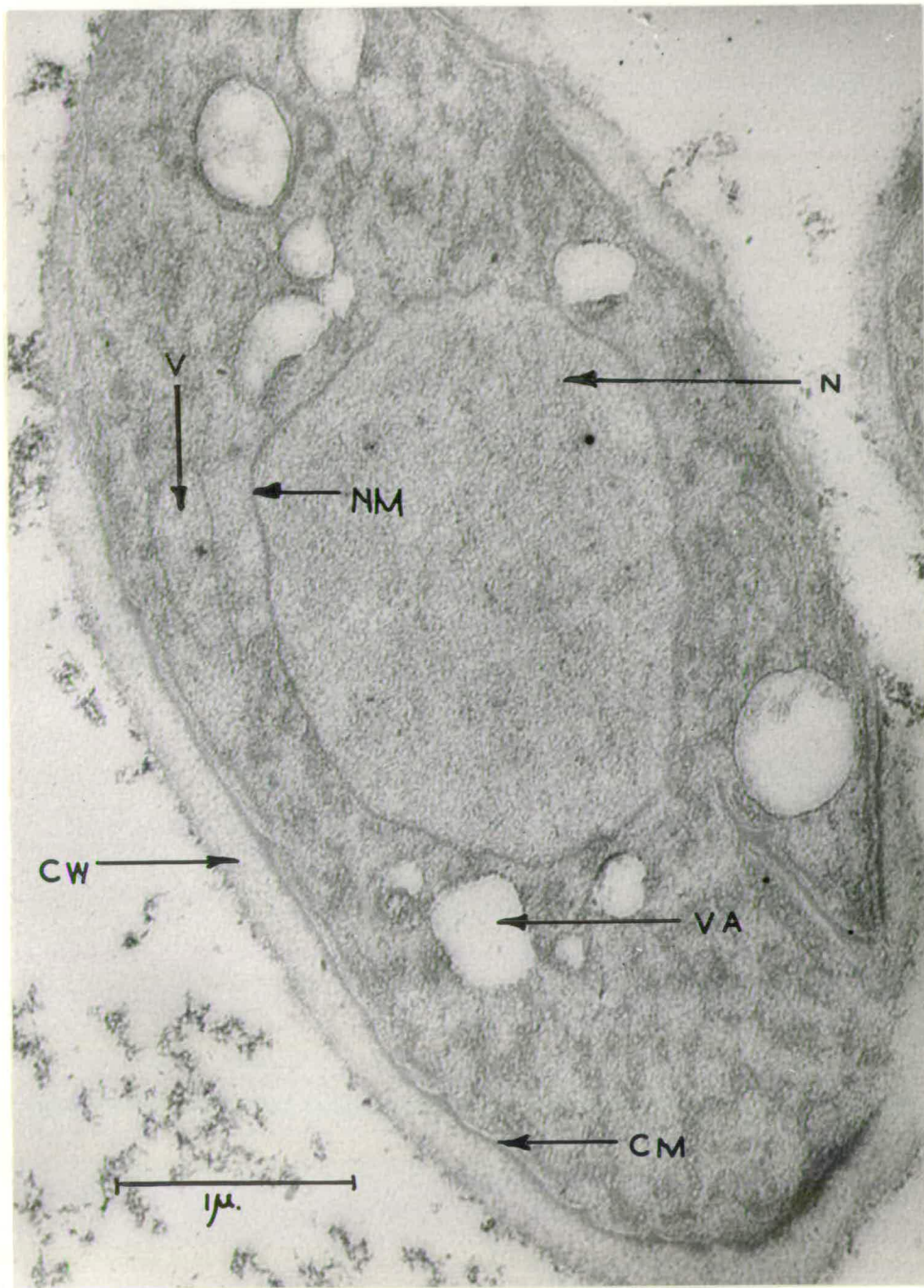


Figure 3. Cell fixed in Potassium permanganate, and stained after fixation with potassium permanganate. CM = cell membrane; CW = cell wall; N = nucleus; NM = nuclear membrane; V = vesicle; VA = vacuole.

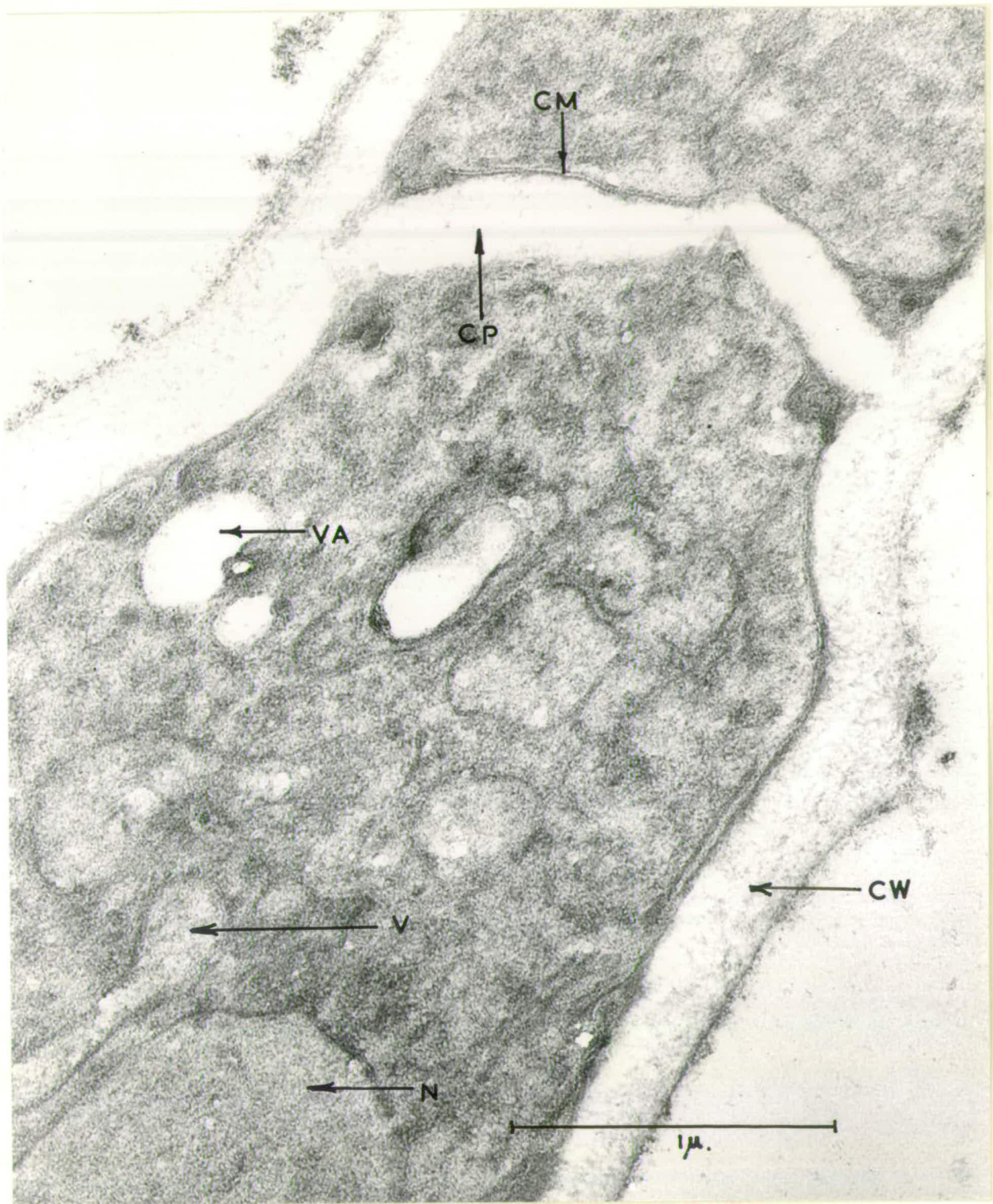


Figure 4. Section of cell showing cell plate. Fixation with potassium permanganate. CM = cell membrane; CP = cell plate; CW = cell wall; N = nucleus; V = vesicle; VA = vacuole.

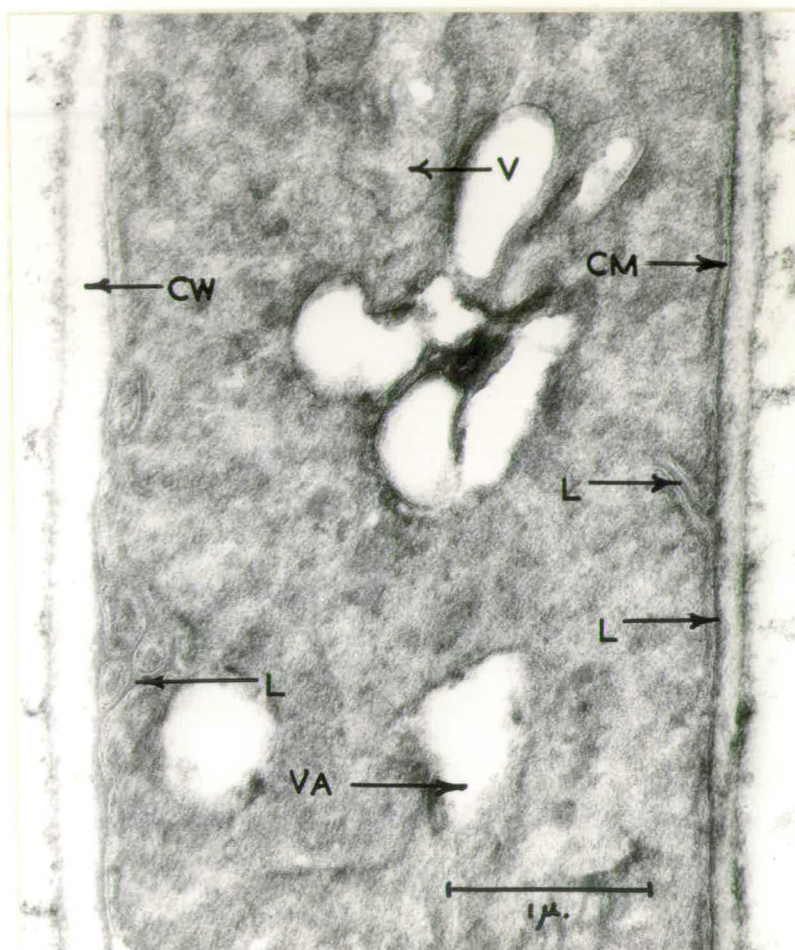


Figure 5. Part of cell section fixed with potassium permanganate showing the intrusion of fingers of the material, which normally lies between cell wall and membrane, into the cell. CM = cell membrane; CW = cell wall; L = layer of material between cell wall and membrane; V = vesicle; VA = vacuole.

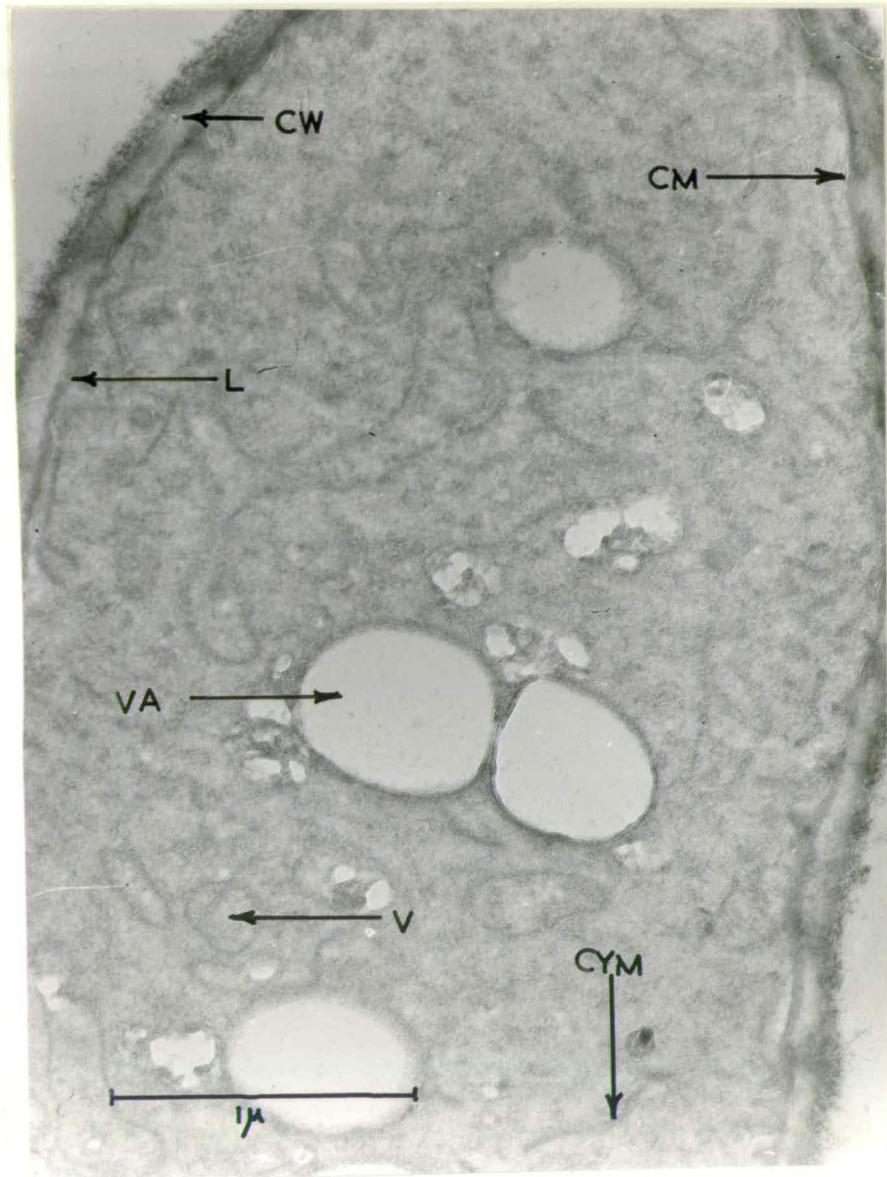


Figure 6. Cell fixed with potassium permanganate. CM = cell membrane; CW = cell wall; CYM = cytoplasmic membranes; L = layer between wall and membrane; V = vesicle; VA = vacuole

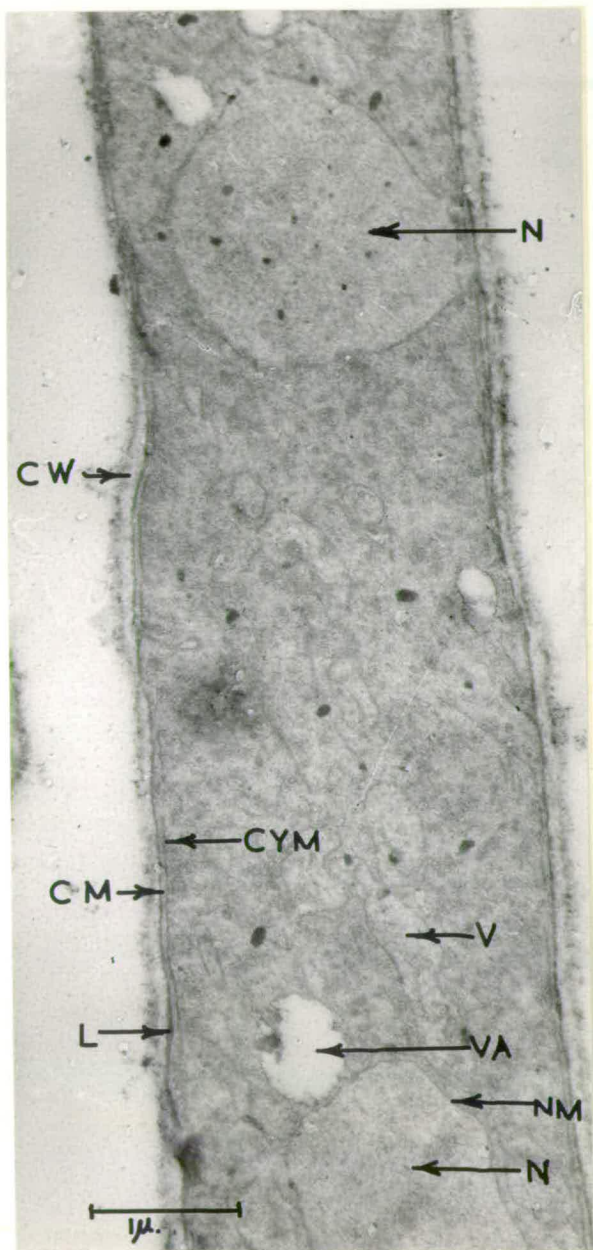


Figure 7. Cell fixed with potassium permanganate. CM = cell membrane; CW = cell wall; CYM = cytoplasmic membranes; L = layer between wall and membrane; V = vesicle; VA = vacuole; N = nucleus; NM = nuclear membrane.

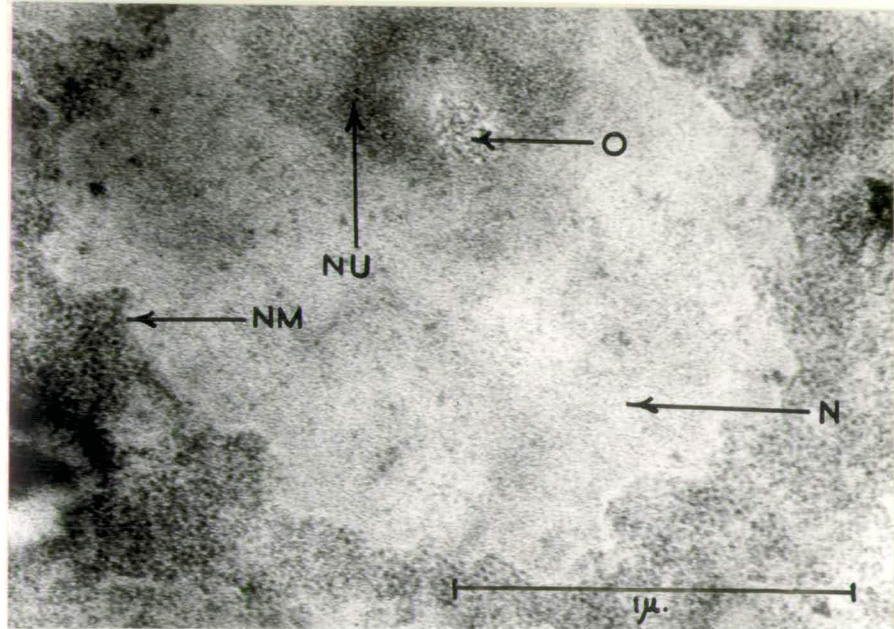


Figure 8. Part of cell fixed with 2% osmium tetroxide and stained with uranyl acetate. N = nucleus; NM = nuclear membrane; NU = nucleolus; O = granular organelle associated with nucleolus; note ribosomes in the cytoplasm.

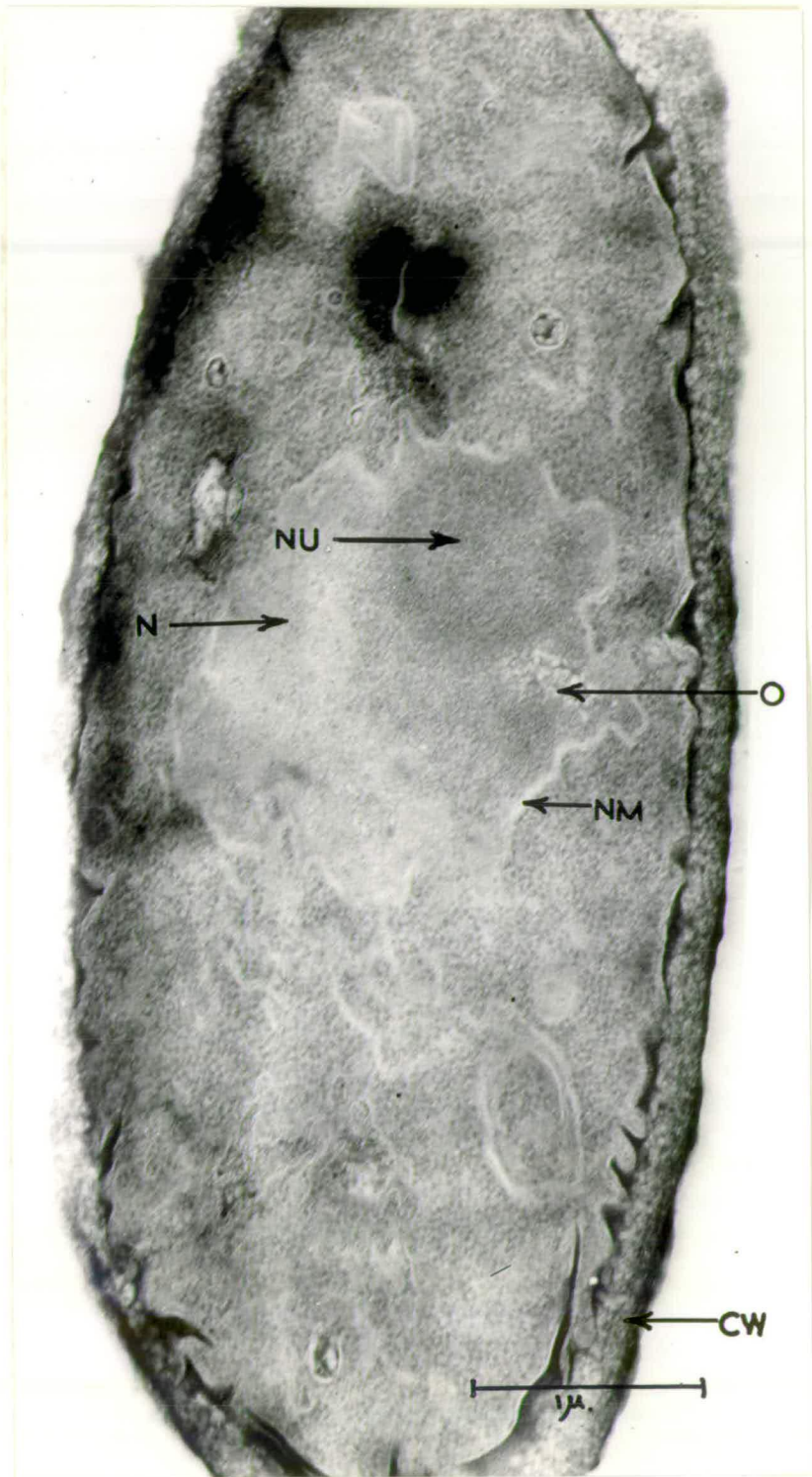


Figure 9. Cell fixed in 2% osmium tetroxide, stained with uranyl acetate. CW = cell wall; N = nucleus; NM = nuclear membrane; NU = nucleolus; O = granular organelle associated with nucleolus; note ribosomes in the cytoplasm.

PART II

An electron microscope study of ribosomes in *S. pombe*

Introduction

By centrifugation of homogenised cells, Claude, 1943, obtained a supernatant which, on high speed centrifugation at 20,000 r.p.m. yielded a pellet of granules rich in RNA. This, the microsomal fraction, was later derived from animal and plant cells of various kinds (Brachet and Jeener, 1944). With the development of electron microscopy, search was made for the counterpart of microsomes, as obtained by ultracentrifugation, within the morphology of the cell. Certain workers (Lehmann, 1955) did claim to have found them, but the overwhelming weight of opinion held that they did not exist as separate entities within the cell, but that microsomes constituted fragments of the cytoplasmic membrane system, the ergastoplasm of Garnier and Bouin, 1897.

With the increasing use of the electron microscope, very many workers became interested in the cytoplasmic fine structure and in the use of the electron microscope to elucidate its organisation. Sjostrand and Hanzon, 1954, de Robertis, 1954, and Porter, 1953 and 1956, were prominent in this field and the membrane system was given the name of endoplasmic reticulum by Porter, 1954. Further details were construed by Palade, 1953, 1955 and 1956, who, in his now famous work, drew attention to the large number of small granules lying on or adjacent to the surface of the endoplasmic reticulum. The regularity of granule size - about 150-200 Å diameter - suggested that they represented a basic cytoplasmic

component. At first termed Palade's small granules, and identified by their high electron density in osmium-fixed tissue, these particles were soon recognised as representing the microsomal particles brought down by ultracentrifugation of the microsome fraction after treatment with the detergent sodium deoxycholate. However, the term 'microsomal particle' proved inconvenient, since these particles may be recovered by ultracentrifugation or demonstrated by electron microscopy in cells such as bacteria which may not possess an endoplasmic reticulum nor yield a microsome fraction. Even in tissues well endowed with both endoplasmic reticulum and microsomal particles, the electron microscope suggests that many of the particles are free in the cytoplasm and not associated with the membrane system, while the endoplasmic reticulum of some cells lacks these particles altogether (Porter, 1961). The particles were therefore more usefully termed ribosomes, a word suggested for them at the first symposium of the Biophysical Society, 1958.

Claude, 1943, discovered that the microsomes were rich in RNA and Barnum and Huseby, 1948, calculated that RNA accounted for some 9% of the total microsomal dry weight. When separation of ribosome fractions became feasible, it was also found that they were characterised by an even higher RNA content (Schachman and Pardee, 1952) and it is now clear that RNA makes up about 50% of the ribosomal dry weight, and that ribosomal RNA constitutes up to 80% of the total cell RNA (Watson, Schlessinger and Hollingsworth).

The appearance of ribosomes in electron micrographs, both of osmium fixed tissue and of ultracentrifuge fractions of cell homogenates, convinced most workers that they were a genuine

component of living cells. But, in the course of work done by a freeze-drying fixation technique on pancreas tissue, Sjostrand and Baker, 1958, found no trace of ribosomes and tentatively challenged the authenticity of these particles. This work has been followed up by Hanson, Hermodsson and Toschi, 1959, and Grunbaum and Wellings, 1960, also working on pancreas cells, and embedding the frozen dried tissue in methacrylate. They report findings similar to those of Sjostrand and Baker, 1958, and assert that the ribosome is an artefact of osmium fixation and high-speed centrifugation, both techniques serving to give nucleoprotein a structure and organisation which it lacks in the living cell. However, the application of freeze-drying fixation techniques by other workers has resulted in the appearance of ribosomes in the electron micrographs of frozen dried tissues of pancreas, liver and bone marrow, but they are less well defined than in osmium fixed material (Seno and Yoshizawa, 1960, and Bullivant, 1960). It seems, then, that ribosomes are a genuine cellular component, but that some staining method is often necessary to demonstrate their presence in the electron microscope.

In recent years research on ribosomes has been very intensive, particularly since they came to be identified as the cellular site for most or all protein synthesis. For a time, the ribosomes were presumed to possess intrinsically the genetic information for specific protein synthesis, until the identification by Volkin and Astrachan, 1956, of a non-ribosomal RNA fraction with, in contrast to the bulk of RNA, a base ratio resembling that of DNA. The demonstration by Brenner, 1961, that this DNA-like RNA became

associated with ribosomes, explained the experimental data which related protein synthesis to ribosomes, and led to the concept of messenger RNA expounded by Jacob and Monod, 1961. This left the ribosome with the role of being a largely inert assembling site for proteins.

Now during this explosive phase of research on protein synthetic machinery, research on ribosomal structure and function has been pursued along three main lines. Firstly, a large number of experiments have involved the use of pulse-labelling with radioactive isotopes to demonstrate the time sequence of RNA and protein movement, and to identify ribosomes active in protein synthesis. Secondly, ribosomal fractions have been separated from cell homogenates by ultracentrifugation and their behaviour in the centrifuge in the presence of various ions used to determine their size and sedimentation constant. Thirdly, and of considerable interest to us here, is the work in which the electron microscope has been used to resolve ribosomal fine structure.

Numerous papers include electron micrographs of ribosomal pellets fixed in osmium tetroxide and photographed at rather low magnification, e.g. from pea seedlings (Setterfield et al., 1960), from rat liver (Moule et al., 1960) and from guinea-pig pancreas (Seikevitz and Palade, 1960). But more elegantly, the technique using phosphotungstate as an electron stain has been adopted by Hall and Slayter, 1959, and Huxley and Zubay, 1960, to elucidate something of the shape and size distribution amongst ribosomes at high magnification. And, very recently, with the idea that ribosomes function largely or exclusively in aggregate groups

strung along a molecule of messenger RNA (Barondes and Nirenberg, 1962; Warer, Rich and Hall, 1962; Gierer, 1963), electron microscopy has verified the existence of ribosome aggregates or poly-ribosomes in actively synthesising cells (Rich, 1963; Slayter, 1963).

The work described in this section of the thesis was aimed at answering two questions. Firstly, how many ribosomes on average does a cell of S. pombe have, and secondly, how are the numbers of ribosomes in the cell related to the cell's growth and development through the cell cycle. The visualisation of the ribosomes in cell sections with the electron microscope renders them countable, and since factors such as the thickness of section and length of cell can be calculated, some simple arithmetic gives a figure for the total ribosome count per cell. Since the stage in the cell cycle can be assessed in S. pombe cells by measuring length, correlation of ribosome counts with cell length on longitudinal section yields information about the relationship of ribosome number to stage in the cell cycle. The work of Mitchison, 1957, in estimating RNA and dry-mass increases during cell growth in S. pombe makes estimations of ribosome numbers in this cell more meaningful.

Some of the experiments on comparative fixation techniques for visualising ribosomes in the electron microscope were carried out during my final year as a B.Sc. student in this department; since they are relevant, they are included in this thesis.

Material and Methods

Cells were grown and harvested as described in Part I.

Electron microscopy - fixation methods designed to visualise ribosomes:

1. As described in Part I, cells were washed twice in 0.25 M sucrose buffered with veronal acetate at pH 7.2, followed by fixation for one hour at 0°C in 2% osmium tetroxide in the buffered sucrose solution.

2. This method was used as recommended by Afzelius, 1959. Cells were washed twice in distilled water and then fixed for 1 hour at 0°C in a solution of 40% (w/v) osmium tetroxide in pure carbon tetrachloride and successive washings in increasing concentrations of alcohol prior to embedding.

3. 4% formaldehyde in veronal acetate buffer pH 7.2 was used as a fixative for 30 mins. at room temperature after cells had been washed twice in the buffer. Fixation was followed by normal dehydration and embedding.

4. After two washes in veronal acetate buffer, cells were concentrated by low speed centrifugation and smeared on one surface of a square cover slip size 0. Within one minute, the cover slip coated with cells was dropped into a tube of liquid propane within a bath of liquid nitrogen. This initial quenching temperature is -185°C. The propane was quickly poured off and the tube transferred to a bath of solid CO₂ and alcohol, giving a drying temperature of about -50°C. Prior to immersion in this bath, a glass cold finger, filled with liquid nitrogen, was fitted into the tube containing the cover slips. With this arrangement water

driven off from the cells condenses on the cold finger. A vacuum pump was fitted to the tube to remove air and achieve rapid dehydration and the apparatus left for 24 hours, with occasional replenishment of the liquid nitrogen in the cold finger. Following dehydration, the cover slips with cells were rapidly transferred to another vacuum tube containing frozen methacrylate embedding medium, and after pumping down the temperature was gradually raised to permit slow penetration of the cells by the methacrylate. When at room temperature, the cells, now impregnated with methacrylate, were scraped from the cover slips into liquid methacrylate and the monomer polymerised in the usual way.

Embedding. Cells fixed by freeze-drying were embedded as described above under Fixation. The material fixed with 2% osmium tetroxide, 40% osmium tetroxide and 4% formaldehyde were all embedded as described in Part I of this thesis for tissue fixed in 2% osmium tetroxide.

Sectioning. This was carried out as described in Part I, except that the sections from material fixed by freeze-drying were floated on to a reservoir of 10% acetone instead of water.

Staining. No staining procedures were applied to the cells fixed with 40% osmium tetroxide, 4% formaldehyde or by freeze-drying, but some of the sections cut from material fixed with 2% osmium tetroxide were stained with uranyl acetate as reported in Part I.

Preparative procedure for producing longitudinal sections.

An estimate of the stage of a single cell of S. pombe in the cell cycle can be assessed from its length, but if lengthwise measurements are to be made on sections viewed in the electron microscope, it is

imperative that the sections be truly longitudinal. This implies that the cells must be aligned in the embedding procedure so that they are flattened in a monolayer, and present their total length to the microtome knife at a right angle. The following method of preparation was employed.

Cells were harvested by centrifugation, washed twice in 0.25 M sucrose buffered with veronal acetate at pH 7.2 and then spun down in the sucrose to a concentration of approximately 2×10^6 cells per ml. Drops of this suspension were now placed on square cover slips size 1, four drops per slide, and the solution evaporated almost, but not quite, to dryness. Placing the cover slips in a glass petri dish, cells uppermost, and some 8 cover slips per dish, drops of 2 % osmium tetroxide in the buffered sucrose were applied in sufficient quantity to flood each cover slip and the petri dish cover put in place. After half-an-hour at room temperature, the petri dish cover was removed and the dish flooded with distilled water. Numerous washings with water were carried out, each washing being drained off by means of a pipette. Washings with water were followed by washings in increasing concentrations of alcohol, ending with three washings in absolute alcohol. The cover slips were not moved during this procedure. After this thorough dehydration the petri dish was flooded once with liquid methacrylate which, on removal, was replaced by a final pool of liquid methacrylate which covered the cover slips to a depth of $\frac{1}{4}$ ". This methacrylate was made in the proportions 91% butyl methacrylate, 8% methyl methacrylate and 1% benzoyl peroxide, since the plastic tends to be soft on polymerisation of a small depth of liquid.

Polymerisation was carried out at 58°C with the petri dish cover in place and was normally complete in 24 hours. A somewhat similar technique is described by Koehler, 1961, for use with cells growing on agar.

When polymerisation was complete, the bottom section of the petri dish was carefully splintered with a hammer and removed, leaving a flat block of polymerised methacrylate with the cover slips applied to the flattened surface of the plastic. A sharp scalpel was used to cut up the plate of polymerised material into smaller pieces, and the cover slips were removed by placing small pieces of solid CO₂ on them, thus causing them to break away from the plastic layer. When the cover slips were removed, the monolayer of cells which had been applied to them was found to be embedded in the surface layer of plastic. Using a low power binocular microscope to locate the groups of cells in the block surface, small cubes of the plastic of about $\frac{1}{4}$ " square on the top surface, were cut out with a scalpel and trimmed under the microscope to leave a flat face of about $\frac{1}{32}$ " square to be presented to the microtome blade. Great care was necessary in cutting these blocks since, if the microtome setting was bad, it was easy to lose all the cells in a few primary sections which were too thick to be of use. The blocks of plastic were held in a small chuck for cutting in the Huxley microtome, and sections were floated on to a water reservoir and picked up on coated grids in the usual way. ~~No post-fixation staining was employed with this procedure.~~ Sections showing silver interference colours were removed for examination in the microscope.

Counting the ribosomes. Numbers of ribosomes per cell were calculated from the sections in the following way. Since a certain degree of detail and contrast was invariably lost in printing and enlarging the photographic plates, the bulk of the ribosome counts were made by direct examination of the plates themselves. Using a high power hand lens for examination, the plates were illuminated from below by means of a cold-light illuminator and the particle number and distribution determined by placing a transparent plate, marked off in 1 mm square, over the photographic plate under examination. By this means, a cell section at a magnification of x 20,000 was subdivided into some 1,400 squares, permitting assessment of the comparative area occupied by cytoplasm, nucleus and vesicles, and the distribution and absolute numbers of the ribosomes themselves.

Among other assumptions discussed in the Results section, it was assumed that all cell sections represented $800 \overset{\circ}{\text{A}}$ of the cell when cut (Peachey, 1958) and thus the number of particles per section was multiplied by 37 to give the number per cell, an average cell thickness, minus cell wall, being taken as 3μ . Using the grid plate examination technique described, it was found that the mean area of cytoplasm to area of nucleus plus cell inclusions was 9:1, and the total ribosome number per cell was assessed for 100% cytoplasm and then rectified in accordance with this ratio.

Actual counts of ribosome distribution and numbers were made by numbering the squares on the grid and counting the ribosomes in 40 squares per section, the squares for examination being selected

at random by the use of random number tables. The haemocytometer-slide counting technique of scoring particles which fall on the line on two sides only was adhered to. As will be discussed under Results, the standard deviation was low and the mean of 40 squares, multiplied by the total number of squares covering cell minus cell wall, did, I believe, after adjustment for nuclear ratio, give a figure for total number of particles that was reasonably accurate.

Results

The presence of the ribosomes.

All the fixation methods employed have been successful in so far as they result in a granular cytoplasm, with a grain size of 100 to 200 \AA diameter. That these particles are in fact ribosomes is suggested by the following evidence. Firstly, all of the fixative methods demonstrate them in about the same distribution and size, with particles more or less absent from nucleus, vacuoles, cell wall and vesicles, but distributed freely in the cytoplasm. Secondly, it seems unlikely that freeze-drying fixation would produce an artefact of similar appearance to the other fixation methods employed. For, contrary to the prediction of Hanson and Hermodsson, 1959, and despite the considerable cell damage involved, fixation by freeze drying results in cytoplasm which appears to consist of particles of about 150 \AA diameter.

Of course, none of the micrographs illustrating the use of different fixatives are by any standards good pictures, and it is

clear that the particle size, electron density and density of distribution varies, due to specimen drift, varying section thickness and contamination with artefact. I have not included them in this thesis because I consider them technically satisfactory, but because they do seem to me to establish that the particles counted in the material fixed with 2% osmium tetroxide are present in cells fixed by a variety of other fixation methods.

The distribution of the ribosomes.

In contrast to cells possessing a well organised endoplasmic reticulum, where the ribosomes tend to be on or near to the membrane surface, the ribosomes of S. pombe are freely dispersed in the cytoplasm. Unfortunately, with potassium permanganate fixation, in which the cell membranes are most clearly defined, the ribosomes are not visible, while with other fixation methods which visualize ribosomes, the cell membrane system is not easily discerned. It has already been noted in Part I that ribosomes are not present inside the cytoplasmic vesicles. From the electron micrographs it seems fairly certain that they are entirely absent also from the nucleus, or present in extremely small numbers. In some sections (Figure 12) ribosomes do appear in the nucleus, but it seems possible that they arrive in that position during the passage of the microtome knife over the section. Some workers have claimed (Sirlin, 1966) that the nucleolus consists of a packed mass of ribosomes; certainly the nucleolus of S. pombe is coarsely granular, but it is very clear that the particle size in the nucleolus is smaller than that of the ribosomes in the cytoplasm. It is of

course quite possible that the reaction of fixative with ribosome is different in a nuclear environment from a cytoplasmic one: the nucleolar particles may, on the other hand, be partly formed ribosomes which do not yet possess the full complement of RNA or protein.

The numbers of ribosomes.

The total number of ribosomes estimated to be present in cells of different sizes is given in Figure 16. Estimation of the standard deviation of the grid counts ranged from ± 0.1 to ± 0.5 for 40 counts, and the mean count per 1 mm grid square ranged from 6 to 8.7. Photographic enlargement prints of some of the plates were made and a ribosome count was made from one of these in order to check the reliability of the plate counting. Using a large size grid square, a total cell ribosome complement of 220,000 was estimated. This figure in comparison with that arrived at by illumination of the original plate and counting by hand-lens, i.e. 266,000, gives some indication of the magnitude of the errors inherent in the technique which has been employed. Unfortunately, in no case were two sections from the same cell identifiable and suitable for counting. From the figures quoted in Figure 16, it will be seen that an S. pombe cell of average length 11 μ will have about 500,000 ribosomes.

Now although this method of counting ribosomes probably yields a figure of some usefulness in terms of the number of ribosomes present in an average cell of S. pombe, it is more doubtful whether the correlation of cell size to ribosome number is of much value. The graph suggests that a cell increases its ribosomes in a non-

stepwise fashion during growth, but as will be considered at greater length in the Discussion section, most of the errors inherent in the counting system would tend to disguise rather than demonstrate any deviation from linearity in production. And the number of cells used in the count is very small. Many months of work produced only 15 cells which sectioned thinly and provided a longitudinal section with ribosomes sufficiently well shown to be scored. Ribosomes are so thickly distributed in the cytoplasm of S. pombe that even at 800 Å section thickness, the particles begin to be so closely dispersed on the photographic plate, that the illusory appearance of an homogeneous cytoplasm will result. Figure 10 from freeze-dried cells illustrates this point, since here the section thickness is probably 1,500 Å.

Figure 16 shows that three cells of length 11 μ were counted and that their ribosome counts gave totals of 37⁹₈,000, 410,000 and 500,000. Now this may be due to errors in the method employed, but if it represents a genuine difference in ribosomal complement between cells of the same length, then it would be impossible to detect the true pattern of ribosomal increase without devising a system for studying one cell throughout the cycle. If the scatter of points on the middle of the graph is not due to experimental errors, the most likely explanation is that it reflects an inhomogeneity of the growing cell population. The fact that cell size varies between 6 and 20 μ has already been mentioned and if, in fact, some cells in the population reach fission size at 12 μ and others are 10 μ at the beginning of the cell cycle, then it follows that cells of 11 μ may be at the beginning or end of the

cell cycle, and might thus have very different ribosomal complements. It is noticeable that the scatter of points is maximal with cells between 8 and 11 μ length.

Discussion

Visualising ribosomes in cell sections.

During the introduction to this part of the thesis, I discussed briefly the fact that, with the exception of the objections of Sjostrand and others, based on fixation by freeze drying, most workers had been convinced of the authenticity of the ribosome by the appearance of osmium fixed tissues in the electron microscope. Although most numerous in tissues like pancreas and liver, ribosomes have been detected in cells of many kinds, including the gastric chief cell of the bat (Ito and Winchester, 1963), rat thyroid gland (Wissig, 1963), rat pigment epithelium (Darling and Gibbons, 1963), L strain cells (Dales and Franklin, 1963), rat neurone (Rosenbluth, 1962), amoebae (Schuster, 1963), tetrahymena (Elliot, Kennedy and Bak, 1962), the fungus Allomyces (Blondel and Turian, 1960) and numerous plant cell types such as Vicia (Lafontaine and Chouinard, 1963), Allium (Buvat and Carasso, 1957), Elodea (Buvat, 1957) and Dactylis glomerata (Poux, 1962). But, strangely enough, the group of cells in which ribosomes are not clearly defined in the electron microscope are the bacteria. The electron micrographs in some papers do demonstrate ribosomes in bacteria, but only rather vaguely (Cohen-Bazire and Kunisawa, 1963; Young and Fitz-James, 1962; Fitz-James, 1962), while many others represent the cytoplasm

as being rather uniform or very finely granular (Bladen and Waters, 1963; Okye and Murrell, 1962; Kellenberger and Ryter, 1958), with osmium fixation. It is possible that this phenomenon is due to the particular pH or ionic environment of the fixation method, since ribosomes may be separated from bacteria in the ultracentrifuge.

As mentioned by Koehler, 1962, osmium fixation is not an easy fixative to use with yeast cells, and most recent studies on yeast with the electron microscope have involved potassium permanganate fixation. However, it is interesting to find that, when osmium is used as a fixative, ribosomes are clearly demonstrated in the cytoplasm.

Counting methods.

The un^certainty of relationship between an electron micrograph and a living cell has discouraged people from making quantitative estimations on the basis of electron microscopy. Loud, 1962, has employed a grid sampling technique to analyse the ratios of different cytoplasmic inclusions in cells; Marks and others, 1963, have used electron micrographs of reticulocytes for a visual estimation of the percentage of ribosomes which are aggregated as polysomes. I know of no attempts to count the total number of ribosomes in cells from electron micrographs, and to do so is to make a number of assumptions which will be discussed here. The first important assumption is that all cell sections are, say, 800 Å in thickness. Any variation in thickness will result in a difference in the number of particles, and a section of 600 Å thickness will show half the number of particles of a section

1200 Å⁰ thick, due to the long "depth of focus" of the electron microscope and its translation of thickness into a single plane view. Secondly, it is supposed that all ribosomes in the cell are countable. Now it is conceivable that ribosomes may not all react to fixation in precisely the same way and variation in intensity of contrast between particles may lead to a low count. The third assumption is that all particles counted are ribosomes. Fourthly, it is assumed that each electron dense dot in the section of around 200 Å⁰ is one ribosome, whereas it is clear from the literature that ribosomes may be present as dimers; the resolution of the plates used in this study is not sufficient to give any information on the range of size of the particles counted. The last important assumption is that the proportions of cytoplasm to cytoplasmic inclusions such as nuclei, vesicles and vacuoles is uniform, both between sections of the same cell and sections of different cells, and does not vary far from the calculated ratio of 9:1 used in the calculations. The error here could well be around 5%.

The number of ribosomes in a cell.

An average cell of S. pombe in logarithmic growth would seem to possess about 500,000 ribosomes. Figures for ribosome complements of other cells include estimates of 90,000 for E. coli (Tissieres, Watson, et al., 1959), 10,000 for E. coli (Roberts, Britten, et al., 1958) and 5-10,000 in E. coli (Cowie, Spiegelman, Roberts and Duerkeen, 1961). A figure surprisingly close to my own appears in a paper of Kihara et al., 1961; they calculated

that a single yeast cell (Saccharomyces dobyanskii x Saccharomyces fragilis) contains 550,000 ribosomes, using an estimated molecular weight of ribosomal RNA and the known ribosomal RNA content of the cell. Now all of the estimates made by other workers have depended on data of molecular weight and the ribosomal RNA yield of ultracentrifuged fractions of cells. The total molecular weights of the ribosomal RNA, composed probably of two or three separate molecules, is accepted now as being about 1.7×10^6 for a 70 or 80 S ribosome (Spirin, 1963), and it is not difficult to assess from this that the amount of RNA present in such a ribosome must be around 2.7×10^{-18} gms (using Avogadro's Hypothesis). With 500,000 ribosomes in a cell, and each ribosome containing 2.7×10^{-18} gms RNA, a cell of S. pombe should possess 1.35×10^{-12} gms of ribosomal RNA, and assuming 80% of the total cell RNA to be ribosomal, 1.7×10^{-12} gms of total RNA. This figure is rather low according to the estimation of RNA content of this cell made by Mitchison and Lark, 1962, although they report that the RNA content varies widely with growing conditions.

The good agreement between my calculations for S. pombe and those of Kihara et al., 1961, for another yeast species, suggests that the particles counted as ribosomes in the electron microscope with osmium fixation agree rather closely in number with the ribosomes assessed in biochemical terms from ultracentrifuged fractions.

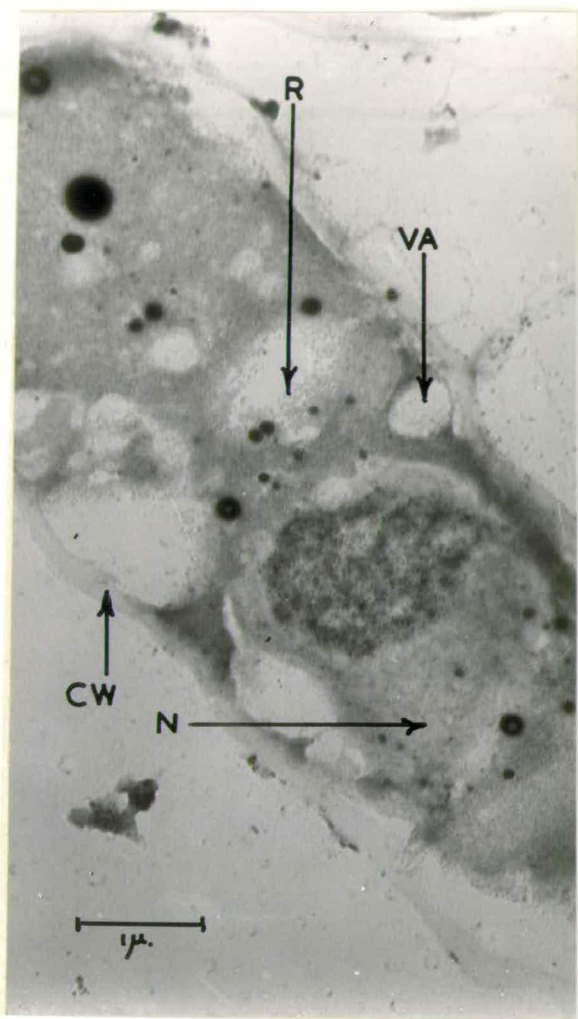


Figure 10. Section of cell fixed by freeze drying - no staining.
CW = cell wall; N = nucleus; R = ribosomes; VA = vacuoles.

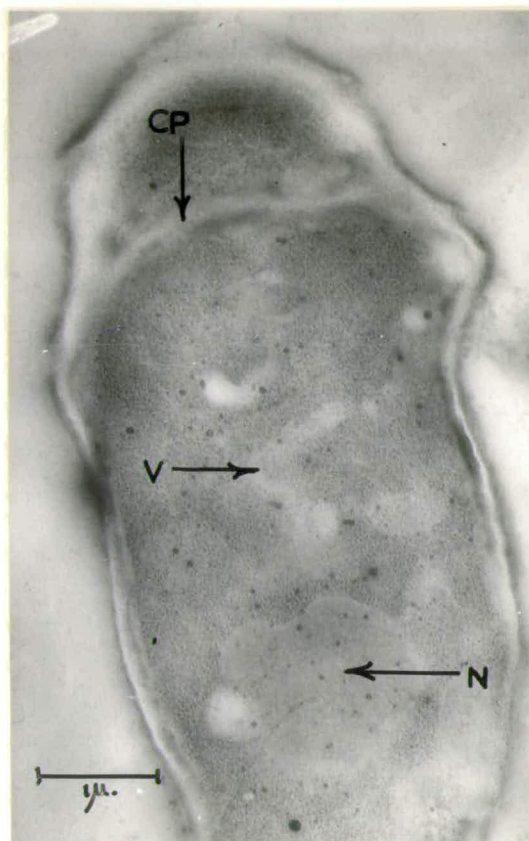


Figure 11. Fixation with 4% formaldehyde - no staining.
CP = cell plate; N = nucleus; V = vesicle; note ribosomes
in cytoplasm.

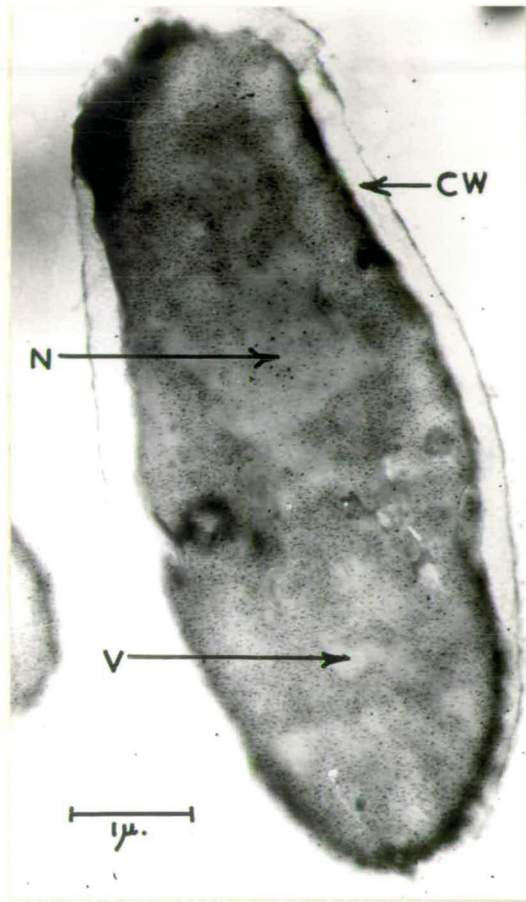


Figure 12. Cell fixed with 40% osmium tetroxide. CW = cell wall; N = nucleus; V = vesicle; note ribosomes in the cytoplasm.

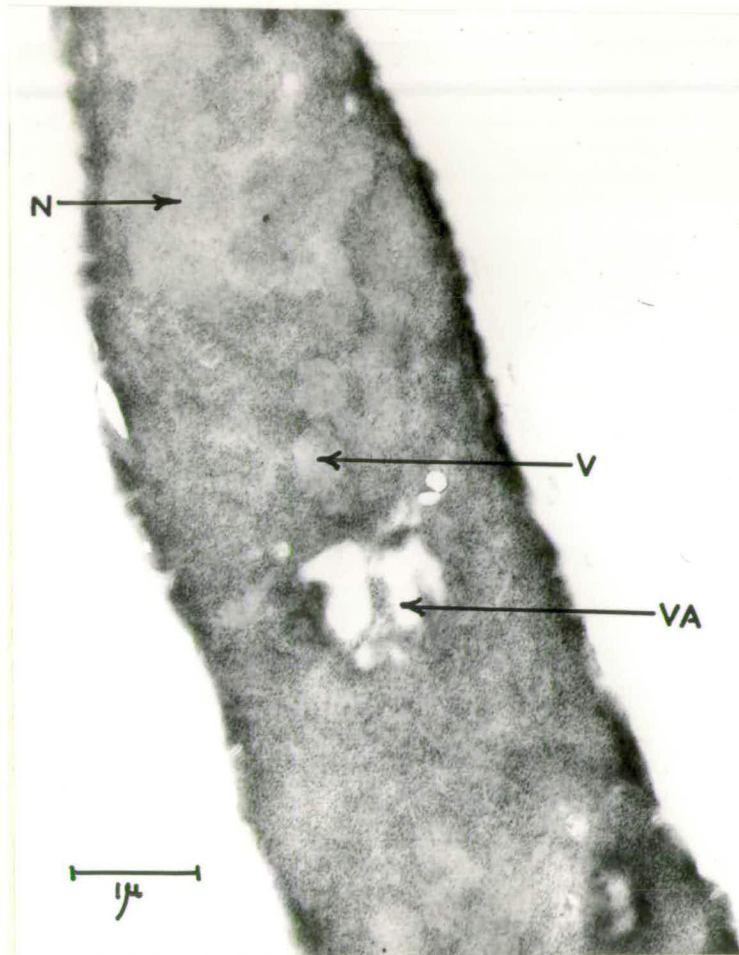


Figure 13. Longitudinal section of cell, fixed with 2% osmium tetroxide and stained with uranyl acetate. The cell wall is not sufficiently dense to appear in the prints. N = nucleus; V = vesicle; VA = vacuole; note ribosomes in the cytoplasm.

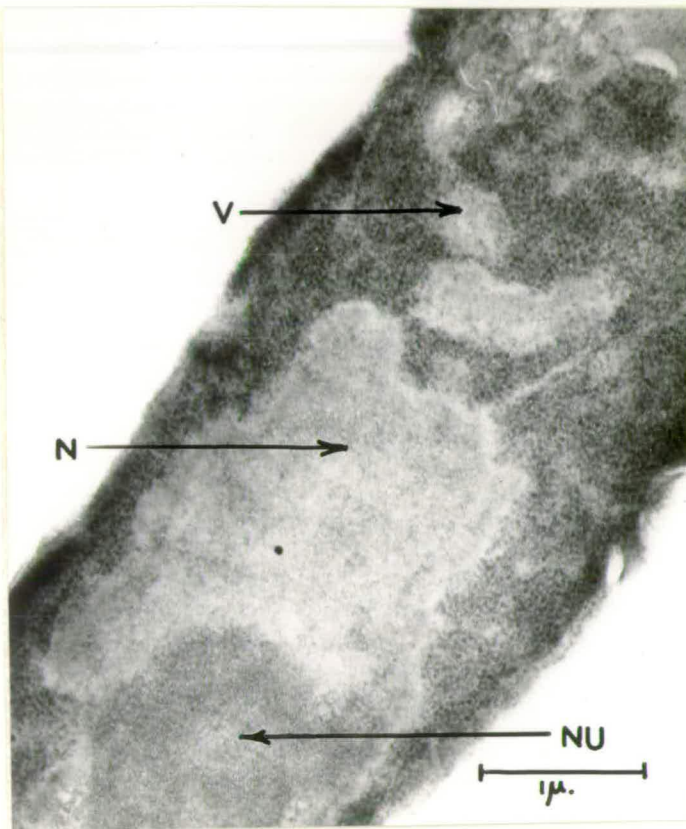


Figure 14. Part of a longitudinal section of a cell, fixed with 2% osmium tetroxide and stained with uranyl acetate. The cell wall is not sufficiently dense to appear in the prints. N = nucleus; NU = nucleolus; V = vesicle; note ribosomes in the cytoplasm.

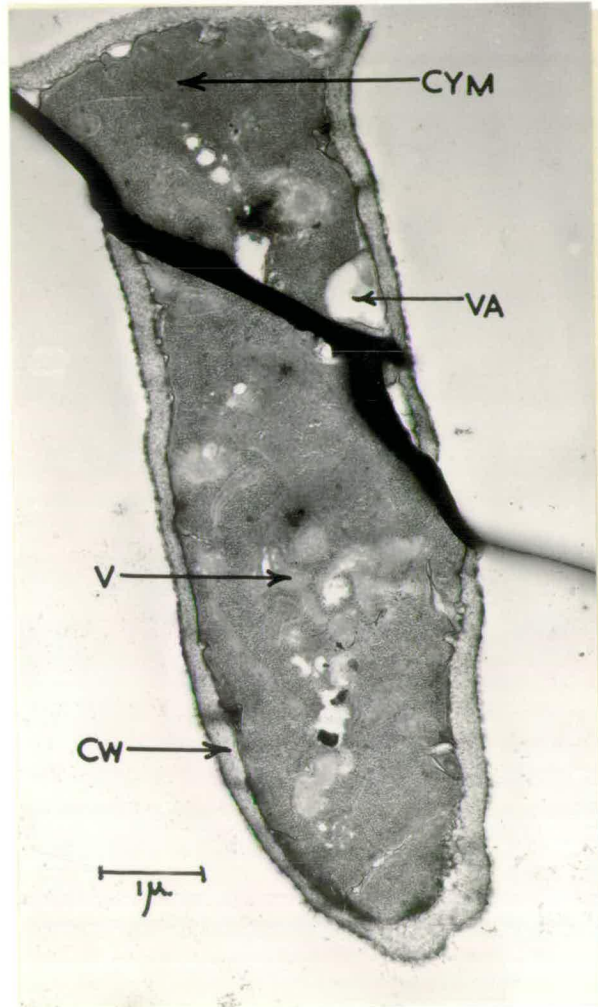


Figure 15. Longitudinal section of cell, fixed in osmium tetroxide, with no staining. CW = cell wall; CYM = cytoplasmic membrane; V = vesicle; VA = vacuole; note ribosomes in the cytoplasm.

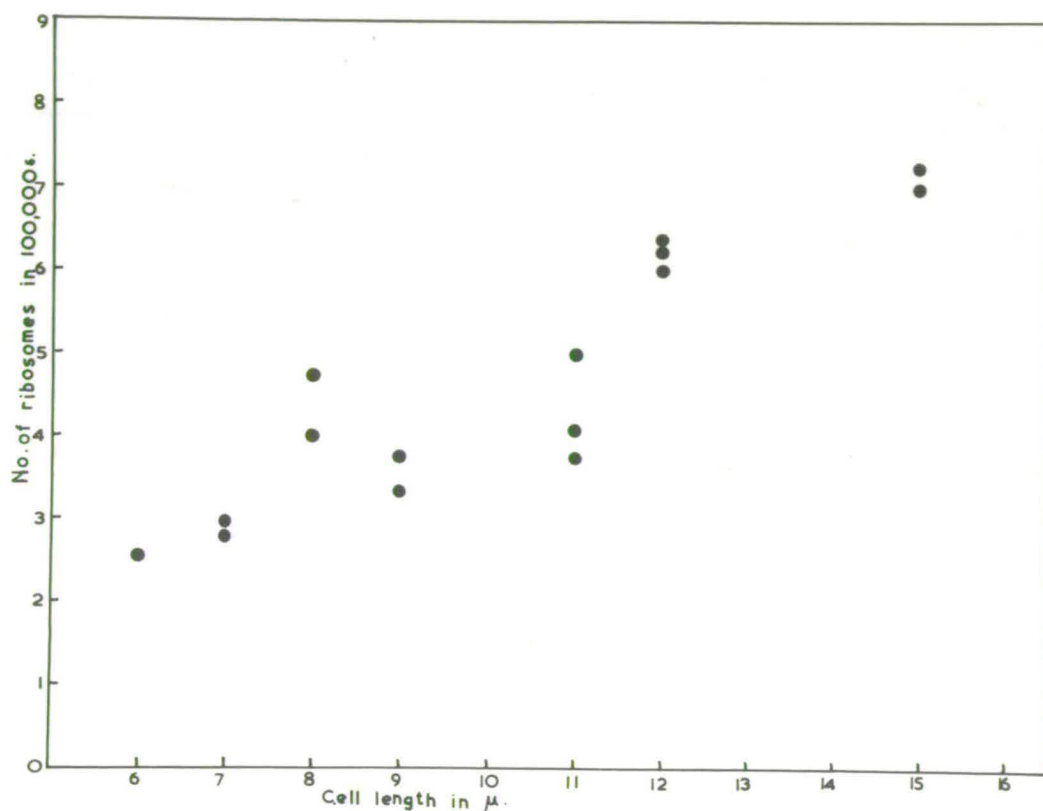


Figure 16. Numbers of ribosomes counted ^{FROM} ~~in~~ longitudinal sections of S. pombe cells, plotted against cell length.

PART III

An Attempt to separate nuclei from fission yeast in bulk

Introduction

Since I had been able to calculate the number of ribosomes present in the cytoplasm of S. pombe, I felt that it would be rewarding to isolate the nuclei and find the numbers, if any, of ribosomes present in the nucleus. The electron microscope has demonstrated the nucleus to be oval or amoeboid in shape, of about 1.5 μ in diameter and bounded by a distinct membrane. My attempts to isolate nuclei from this cell were abandoned but a short account of the methods employed is included in this section in order to preserve the continuity of the thesis.

Materials and Methods

Because of the toughness of the cell wall, it was planned to digest away the wall, thus exposing the naked protoplasts, to separate the protoplasts from the cell wall fragments by centrifugation, and then to lyse the protoplasts in a sufficiently gentle manner to leave the nuclei intact. The nuclei, it was hoped, could then be purified by centrifugation.

Yeast protoplasts were made by a modification of the method of Holter and Ottolenghi, 1960, using snail cellulase sold as "Suc digestif d'Helix pomatia" by Industrie Biologique Francaise in 1 ml ampoules. Cells were harvested from early log phase cultures when the cell concentration is between 1 and 2×10^6 cells per ml, washed

twice in 1.2 M sorbitol, and resuspended in 1.2 M sorbitol at a concentration of 50×10^6 cells per ml. This suspension of cells was now incubated at 37°C with 0.4 mls of snail juice/ml of solution added. Incubation was continued for 3 hours, by which time most of the protoplasts were free from the cell walls. After centrifuging 10 mls of the protoplast suspension for 2 minutes at 1000 r.p.m., the sorbitol was removed and the pellet of protoplasts and broken cell walls resuspended in 2 mls of 0.25 M sucrose containing 0.004 M calcium chloride. This material was now layered on to a 10 ml sucrose gradient from 12-80% sucrose, and spun for 90 minutes at 39,000 r.p.m. in the SW 39 Rotor of a Spinco ultracentrifuge. Following the spin, the centrifuge tube contents were dripped out into tubes, 0.2 mls per tube. Protoplasts, distorted, but still apparently intact, were collected from tubes 7-11 free from visible cell wall contamination. Details of sucrose gradient preparation and drip-out procedure is given in Part V Methods section.

Attempts to isolate nuclei from these protoplast preparations were all unsuccessful, one of the main obstacles being the difficulty of seeing or recognising the nuclei by microscopy. Experiments were carried out in which the protoplasts were incubated for 3 minutes with C^{14} adenine in order to introduce a nuclear label, shocked osmotically by the addition of distilled water, and then spun on sucrose density gradients in the hope of obtaining a hot nuclear fraction. Counts suggested that the nuclei invariably burst with the osmotic rupture of the protoplasts.

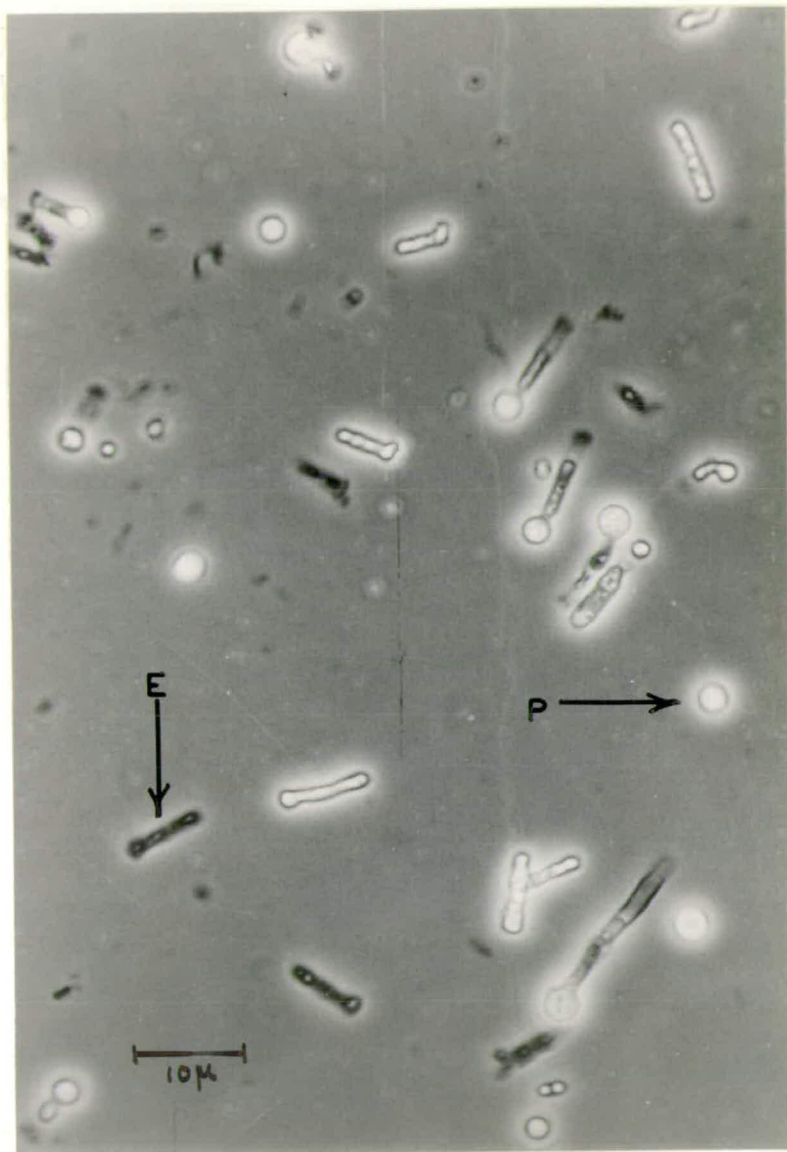


Figure 17. Cells of *S. pombe* after incubation with snail juice, showing the emergence of the protoplasts from the ends of the cells. E = empty cell wall; P = naked protoplast.

PART IV

Isolation of Tetrahymena nuclei

Introduction

Bulk fractions of nuclei have been isolated from many different tissues and a number of reviews of methods of nuclear isolation are to be found in the literature (Dounce, 1952; Brachet, 1957; Roodyn, 1959; Allfrey, 1959; and Roodyn, 1962). Since many papers have been published since the last review, it seems helpful to enumerate the tissues from which bulk nuclear fractions have now been isolated. Bacterial preparations do not, of course, yield true nuclei, but the methods are included for completeness.

<u>Bacillus megatherium</u>	- Spiegelman, Aronson <u>et al.</u> , 1958
Do.	- Godson and Butler, 1962
Seven different plant tissues both Monocotyledon and Dicotyledon	- McLeish, 1963
Tobacco leaves	- Flanim, Birnstiel and Filner, 1963
Pea embryos	- Rho and Bonner, 1961
Sea urchin eggs	- Hinegarden ^{nev} , 1962
Amphibian oocytes	- Baffin, 1959
Hela cells	- Harris and Watts, 1962
Ascites tumour cells	- Hudach and Baker, 1961
Rat liver	- Anderson, 1953
Guinea pig liver	- Maggio, Siekevity and Palade, 1963
Calf thymus	- Kodama and Tedeschi, 1963
Rat prostate	- Hancock, Zelis <u>et al.</u> , 1962
Rat brain	- Sporn, Wanko and Dingran, 1962

Methods of isolation are equally varied and include hand homogenisers (Dounce, 1943), propellers (Harris and Watts, 1962), metal rams (McLeish, 1963) and rollers (Rho and Chipchase, 1962), used in conjunction with a variety of solutions such as detergents (Harris and Watts, 1962), citric acid (Dounce, 1943), 2.2 M sucrose (Chaveau et al., 1957), 0.25 M sucrose (Schneider, 1948), glycerol (Schneider, 1954), 2% formaldehyde (McLeish, 1963), gum arabic (Dounce, 1952), and benzene (Siebert, 1961). There are no accounts in the literature of bulk nuclear isolation techniques applied to Protozoa other than a preliminary report by Britten, 1960, with *Tetrahymena*.

Now the Protozoa do possess certain advantages as a source of nuclei. A number of species of Protozoa can be grown in axenic culture to high yield in short periods of time, the harvested cells represent an homogeneous cell population in terms of cell type, and centrifuged pellets of cells do not include extracellular contaminants, as tends to occur with certain tissue preparations. Since I had become interested in the distribution of ribosomes in cells, but had abandoned the attempt to isolate nuclei from yeast, I began to consider the suitability of Protozoan cells for nuclear preparations and a study of nuclear ribosomes.

Tetrahymena pyriformis, strain W, was selected for the following reasons: (1) *Tetrahymena* are large cells with a conspicuous macronucleus; (2) they can be grown quickly and easily in axenic culture on peptone media; (3) the pellicles of *Tetrahymena* are relatively fragile compared with the walls of yeast and bacteria, and this would facilitate the extraction of undamaged nuclei. The strain W does not possess a micronucleus but only a single macronucleus. Of course

the cell is not without its disadvantages, the chief of which is the questionable analogy between the *Tetrahymena* macronucleus and the nuclei of higher cells. But as far as cell metabolism and the distribution of RNA are concerned, it is likely that the macronuclear function is similar to more normal nuclei from higher cells. Britten, 1960, had reported some preliminary experiments on isolating nuclei from *Tetrahymena pyriformis* strain G.L. which encouraged me to choose *Tetrahymena* cells as a source of nuclei.

Materials and Methods

Tetrahymena pyriformis strain W is a holotrichous ciliate with an oval shape and dimensions of about 80 x 40 μ . It possesses a spherical macronucleus of about 15 μ diameter and no micronuclei. The absence of micronuclei apparently prevents sexual processes and in their absence the macronucleus divides just prior to the time of cell division. Chromosomes have never been observed in the macronuclei, nor in any other *Tetrahymena* strains which are amiconucleate.

Culture medium consisted of 2% Mycological Peptone (Oxo Ltd., London) kept at 28°C as 300 ml aliquots in 1 litre conical flasks. No shaking nor aeration was employed. The growth rate of the *Tetrahymena* under these conditions was plotted, showing an average mean generation time of 8 hours, and cells were normally harvested four days after inoculation, when the concentration approached 200,000 cells/ml. At this stage of growth 100 mls of medium yields approximately 0.8 mls of packed cells on centrifugation. Cells

were harvested by centrifugation in slightly unusual centrifuge bottles - MSE. 69322, 100 ml A.S.T.M. oil fractionation bottles, which permit packing of swimming cells at slow centrifuge speeds, i.e. 750 r.p.m. (120 G.) for 2 minutes. Media was removed by aspirator, cells were washed once in 25 mls of distilled water at 20°C, and resuspended in 10 mls of ice cold Medium A. The Tetrahymena are rapidly shaken up in this medium and then decanted into the 3°C precooled piece of rubber tubing held between the rollers as described below.

Breakage medium. The breakage medium, referred to in the text as Medium A, is 0.25 M sucrose, 0.065 M K_2HPO_4 , 0.0005 M $CaCl_2$, and 0.001 M $MgCl_2$ in distilled water. Since, with time, magnesium phosphate crystallises out from such a solution to form extremely insoluble crystals, the medium was made up immediately prior to use from two parent solutions, one containing the sucrose and $CaCl_2$ and $MgCl_2$, and the other containing the phosphate. All solutions were stored at 3°C. Medium B was the same as Medium A but the sucrose molarity was 0.5 M, while Medium C was as for A but no sucrose was present. The pH of these media is 8.0.

Breaking machine. A slightly adapted clothes wringer was used for breaking cells (Figure 18), first suggested by Dr P.M.B. Walker, and consisting of two 1½ inch rubber rollers, turned via cog wheels by a large handle, and with screw adjustment provided at the roller ends to permit variation in roller clearance and pressure. The wringer was mounted on a stand and kept in a cold room at 3°C.

Red rubber tubing of 18 mm internal bore and 26 mm external diameter was used as a container for the cell suspension during the

roller treatment. Many other types of tubing were tried, polythene, silicone, and rubber of various dimensions, but none were as satisfactory as the type described. Rubber tubing is supplied dusted with French chalk, and thorough cleaning is necessary to remove this prior to use. A special clamp was constructed from $\frac{1}{4}$ inch duralumin, operated by two $1\frac{1}{2}$ inch hexagon headed screws, permitting the clamp to be opened or closed with an Allen key. The mated surfaces of the clamp were knurled and of 2 inch width, and an extremely tight clamp was necessary to prevent escape of the cell suspension during the roller treatment. At the moment prior to passage of the cell suspension through the tubing between the rollers, the internal pressure is high, causing the rubber tubing to swell. This swollen section of the tubing is not suitable for use again, and in practice about one inch of tubing was cut from the top end of the breakage tube after each run through. About six runs could be obtained from a piece of rubber 12 inches long.

Breaking procedure. When the cell suspension has been poured into the precooled rubber tubing, the top of the tubing is quickly clamped by the clamp nuts, the bottom end having been previously secured between the rollers of the breaking machine. The handle of the machine is turned slowly until the suspension forms a bleb in the tubing under pressure. Rather more pressure now forces the suspended cells through the rubber tubing between the rollers and the resulting brie is run into a glass tube, precooled and kept in ice. The brie is left for 10 minutes and then passed once through a cold 20 ml syringe fitted with a size II serum needle, resulting in a suspension of nuclei in a mitochondrial soup, with

a small number of unbroken whole cells remaining.

The trick involved in the breakage procedure is as follows. Immediately following roller treatment, if the roller pressure has been correct, the cells are non-mobile but appear intact under the microscope, although many of the cells have lost their normal refractile appearance. If the brie is sampled 5 minutes after roller treatment, the cell pellicles are seen to be leaking, mitochondria and ^a few nuclei streaming out through the cracks. After 10 minutes almost all cells have lost most of their contents and the syringe treatment breaks down the remainder of the leaky pellicles to leave only cell contents and completely unbroken cells. If the roller pressure is too great, the brie on initial examination after breakage contains many nuclei and smashed cells, but a high proportion of these nuclei are damaged and the ultimate nuclear yield is poor. The ideal appearance after roller treatment is for all cells to be non-mobile, non-refractile, and swollen, with a few beginning to leak mitochondria into the medium.

These breakage conditions yield fairly repeatable results but it has proved impossible so far to completely eliminate the presence of some whole cells in the final brie.

Following syringe treatment, the cell brie was poured into a centrifuge tube and spun for 2 mins. exactly at 500 r.p.m. (40 G), resulting in separation into three distinct layers, a bottom pellet of whole cells and nuclei, a middle layer of mitochondria and nuclei, and a clearer supernatant of mitochondria and smaller particulate matter. The supernatant is sucked off by aspiration and the middle layer, about 2 mls, is carefully removed by a wide

bore pasteur pipette and made up to 10 mls with fresh cold Medium A, whilst the pellet of whole cells and nuclei is discarded. This pellet varies in size with the percentage of whole cells surviving breakage - if the breakage has been exceptionally good and no whole cells remain, then no pellet will appear, but it is always safest to discard the bottom 0.2 mls of the centrifuge fraction, despite the fact that some 10% of the nuclei are to be found in this region.

In order to separate nuclei from mitochondria, the 10 mls of Medium A with the mitochondria and nuclear fraction suspended in it is now divided into two 5 ml aliquots, and each layered carefully on to 5 mls of cold Medium B. Centrifugation for 2 mins. at 1000 r.p.m. (120 G) gives a nuclear pellet and leaves most of the mitochondria still layered on the surface of Medium B. The upper layer is aspirated off and the lower 2 mls of centrifuge fraction made up to Medium A composition by the addition of 2 mls of cold Medium C. A yield of between 50 and 80% of the original total cell number is now harvested as nuclei; further washing by a repeat layering on Medium B and centrifugation leads to less mitochondrial contamination but reduces the nuclear yield by about 10%.

Results and Discussion

The method of breaking cells prior to nuclear isolation.

Numerous methods are available for breaking cells - Hughes pres French pressure cell, sonication, alumina grinding, Waring blender - but few of them are sufficiently gentle to leave the nuclei intact after the breakage procedure. An ideal method would be to specifically digest away the cell membrane and pellicle by enzymic means leaving the cell contents unharmed, but such enzyme preparations are not available. Attempts to achieve digestion of this type on Tetrahymena with trypsin and lipase were unsuccessful. Harris and Watts, 1962, have employed a mild detergent, Tween 80, in dilute solution at 0.1% for lysing HeLa and other cells, combining the detergent treatment with agitation by propellor. Tetrahymena cells will survive and swim actively in 0.1% Tween 80 for a considerable time, and when concentrations high enough to induce lysis are used, the nuclei lyse also. This holds true for all the detergents tried on Tetrahymena cells, i.e. Triton X 100, Sodium deoxycholate, Sodium lauryl sulphate, and Tween 80. Fragile cells such as tissue culture cells may be broken by osmotic shock but Tetrahymena are resistant to this treatment, while rapid freezing and thawing breaks the Tetrahymena nuclei along with the cell pellicle.

Of the numerous mechanical methods for breaking cells, the hand homogeniser has been the most frequently used for isolation of nuclear fractions, and it is an effective way of rupturing Tetrahymena cells. Many kinds of homogenisers have been tried, but most of them were extremely laborious and yielded unreproducible results. The reason

for the lack of reproducibility in many homogenisers is the increase in the cylinder bore caused by frictional wear. Ground glass homogenisers are particularly prone to wear if narrow clearances are used. The most successful homogeniser with *Tetrahymena* consisted of a 6 inch long cylinder of "Veridia" constant bore glass tubing (Chance) of 35 mm bore, closed at the bottom by a rubber gasket and held firmly in an adapted adjustable retort stand, with specially made stainless steel pistons which were moved up and down by a hand-operated screw. Clearance between cylinder and piston was 0.002 inch. Unfortunately, with this clearance, the expectation of life of the glass cylinders was rather low. Four or five strokes of the piston were necessary to accomplish 70% cell breakage in Medium A at 3°C.

Rollers were used by Rho and Chipchase, 1962, for preparing nuclei from ungerminated pea embryos, and probably passage between rollers is a process analagous to the squeeze between piston and cylinder in a homogeniser. However, in the method which I have used in which the cell suspension is contained in a sealed rubber tube during passage between rollers, there is probably the added factor of sudden pressure change. Bacterial cells have been effectively broken by sudden reduction of gas pressure (Fraser, 1951), and when the internal pressure in the constricted rubber tube is sufficient to force the contents through between the rollers, there is a sudden reduction in pressure which may play a part in cracking open the *Tetrahymena* cells. Only one passage through the machine is necessary (a second passage invariably breaks many nuclei), and reproducibility is good once the optimum conditions of pressure imposed by roller clearance have been assessed by trial and error. Cells were assayed

by light microscopy after each breakage to check on the efficiency of breakage. Percentages of broken cells to original cell counts were between 80 and 100%, and frequently better than 90%. As discussed under "Nuclear yield", the percentage of whole nuclei against number of cells broken averaged about 85% immediately following the syringe treatment.

In the work of Britten, 1960, on isolating nuclei from *Tetrahymena*, the cells were first treated with acridine orange, which appears to toughen the nuclear membrane, and then homogenised in a Waring blender, yielding a broken cell brie with over 50% nuclei intact. But the reproducibility of the method seems to be poor, and the yields of nuclei which I obtained by a repeat of Britten's method were low.

Choice of a suspension medium.

Very many different media have been used in nuclear isolation procedures, and nuclei from different sources react differently to the same medium. *Tetrahymena* nuclei lyse in distilled water while liver nuclei remain intact (Harris and Watts, 1962). Methods for finding a suitable medium are largely empirical, but certain limiting factors exist. Nuclei in many media lose the homogeneous appearance which they possess in vivo, becoming coarsely granular. This phenomenon is presumably due to the precipitation of the nucleoprotein and occurs very noticeably in media containing citric acid (Mirsky and Pollister, 1946). But in sucrose solutions most nuclei remain homogeneous (Hogeboom, Schneider and Palade, 1958), and their in vivo appearance may be further preserved by the addition of calcium to the

sucrose. Without calcium, nuclei in sucrose tend to clump together, becoming distorted and broken, presumably due to leakage of DNA (Philpot and Stanier), but the addition of small quantities of calcium largely prevents clumping. Calcium in concentrations above 0.001 M gives nuclei the granular appearance already mentioned, probably due to nucleoprotein aggregation (Figure 20). The work of Kay, Smellie et al., 1956, has demonstrated that nuclei in aqueous media lose protein and probably RNA; the choice of a sucrose calcium chloride medium for nuclear isolation seems to be supported by most of the evidence.

Commencing with this proviso, I experimented with many variants of pH, calcium concentration and sucrose concentration. The composition of Medium A is the result of two separate considerations, one being the preservation of nuclear structure and the other being the efficiency of breakage of the cell pellicles when the Tetrahymena are homogenised in Medium A. Present composition of Medium A is extremely critical, since small deviations in pH, magnesium or calcium concentrations lead to poor breakage and poor nuclear yield. Every effort has been made to keep up the magnesium concentration so that the nuclei isolated in the medium can be used for ribosome extraction experiments.

The method of isolating the nuclear fractions.

Methods of separating nuclei from other components in cell bries are of two main types, filtration or sieving, using cloth, membrane filter or wire screen, and centrifugation. The yield of clean nuclei from Tetrahymena fractions subjected to various kinds of

filters and sieves was invariably rather low, and these preparative procedures were rejected in favour of centrifugation. As will be discussed under the "Nuclear contamination" section, the cleanliness of a nuclear fraction is to a great extent proportional to the number of washing steps employed. But some compromise between nuclear yield and nuclear purity must be accepted since the yield is reduced by each centrifugation and change of medium. The centrifugation scheme outlined in the methods section has the chief merit of being simple and speedy: more sophisticated methods involving density gradients require longer centrifugation and render difficult the preparation of large quantities of nuclei. A higher percentage yield of nuclei would be harvested if the removal of the whole cells did not involve also the loss of a large number of nuclei, but the initial centrifugation step was used because of its simplicity and no attempt was made to salvage the nuclei which came down with the whole cell fraction.

There is evidence that exposure to sucrose in concentrations above 0.4 Molar causes loss of function in nuclei (Allfrey et al., 1957), but the brief exposure to 0.5 M sucrose during the layering procedure may not be too serious. Since I did not envisage using the nuclei for incorporation experiments, I felt justified in including this step. Dilution of the nuclear/mitochondrial fraction with Medium A prior to layering on to Medium B was done in order to prevent streaming during centrifugation, i.e. the movement of groups of particles together when high concentrations of material are layered.

I have carried out some preliminary experiments on nuclear

isolation procedures using high speed sucrose gradient centrifugation in a special disc centrifuge made by Dr P.M.B. Walker, but not enough work has been done to assess whether this technique might improve on the one described.

Integrity of the nuclei.

Ideal nuclear preparations contain nuclei which are identical in appearance, chemical composition and biological activity to those in the living cell. However, in this work on *Tetrahymena* nuclei, no attempt has been made to test biological activity, and chemical composition has been tested only so far as nucleic acids are concerned. This has thrown the emphasis on judging integrity by morphological appearance. Of course microscopical scrutiny can never compensate for the lack of biochemical and functional comparison, and the efficiency and general usefulness of this method of isolating *Tetrahymena* nuclei cannot be properly determined until the nuclei are assessed on other than purely morphological grounds.

Nuclear fractions have been examined by a Baker phase contrast microscope and Figures 24 and 25 illustrate the results. The nuclei remain spherical, and the contents are mainly homogeneous except for the dark spots appearing close to the nuclear membrane. These are presumably the numerous small and scattered nucleoli seen in the electron micrographs of *Tetrahymena* in a position close to the nuclear membrane (Nanney and Rudzinska, 1961). No swelling of the nuclei normally occurs and they will retain their appearance for over 6 hours in cold Medium A. However, if the medium is allowed to warm up to room temperature the nuclei soon become granular in appearance,

eventually swelling and becoming paler prior to breaking completely (Figure 26). But this phenomenon is very variable and some nuclear preparations retained their morphological appearance for two hours at room temperature.

One merit of the method of cell breakage and extraction employed is that the percentage of nuclei damaged by the breaking procedure is low. This is to be expected in a technique which breaks the pellicle mechanically but allows the nucleus to emerge from the cracked cell as the cell contents reach equilibrium with the surrounding medium. When the cell is cracked, the mitochondrial agitation persists and the intense Brownian movement eventually disperses the entire cell contents. Nuclei damaged during cell breakage become misshapen and their contents often leach out through holes torn in the nuclear membrane. Such nuclei represent less than 1% of the preparation immediately following syringe treatment, but the percentage rises to about 3% after the centrifugation procedures of isolation and washing.

It would be profitable to examine these Tetrahymena nuclear preparations in the electron microscope, although the usefulness of the instrument in this work is limited. Many recent accounts of nuclear isolation procedures have included electron micrographs (Maggio et al., 1963; and Kodama and Tedeschi, 1963), and certainly this is an excellent way of measuring contamination. But the procedures used in preparing material for the electron microscope are such that they may well obscure changes which have occurred in nuclei after isolation. It is questionable whether nuclei which appear granular or swollen in phase contrast microscopy would appear

untypical in the electron microscope.

Nuclear contamination.

Preparations of isolated nuclei may be contaminated with any or all of the following - whole cells; cell membranes, walls or pellicles and cilia; cytoplasmic membranes and vesicles; mitochondria; and cytoplasm.

(1) Whole cells. Contamination with whole cells has ^{not} proved a serious difficulty in this work, since the loss of 10% of the nuclei has been sacrificed to removing the whole cells. Tetrahymena cells are easily distinguishable and their occurrence in nuclear fractions is about 1.5% of nuclei.

(2) Pellicle. During breakage, the pellicle of the cell is fractured, and with the leakage of the cell contents it becomes more broken and eventually disintegrates into pieces. It can readily be identified by the attached cilia, many of which continue to beat after the pellicle has shattered into small parts: the oval cone of this ciliate is especially resistant to damage and will continue to function for an hour after detachment from the cell in cold medium A. However, pieces of pellicle are reduced to a small size by the syringe treatment, and sediment with the whole cell fraction on centrifugation. Cilia readily become detached and remain in the mitochondrial fraction and neither they nor pieces of pellicle constitute an important contaminant in this technique.

(3) Cytoplasmic membranes and vesicles. During the preparation I have often observed that cytoplasmic vesicles emerge from broken cells and are sometimes very similar to nuclei in appearance,

especially under phase contrast. Not only do complete vesicles emerge from the cells after breakage, but the cytoplasmic membranes often round up after breakage to form vesicles. These may be empty or may contain some mitochondria and cytoplasm. In certain media, especially those rich in calcium and with sucrose concentrations below 0.25 M, many of these vesicles form and are very difficult to separate from nuclei. The breakage and isolation technique which I have evolved reduces the occurrence of these vesicles and they do not represent a serious contaminant of the nuclear fractions.

(4) Mitochondria. No attempt has been made to estimate mitochondrial contamination biochemically. Hogeboom et al., 1952, have conclusively shown that the small amount of cytochrome oxidase found in the nuclear fraction was entirely derived from mitochondrial contamination. However, mitochondria may easily be counted by phase contrast microscopy, and their contribution to contamination assessed (Shelton, Schneider and Striebich, 1953). In Medium A, the mitochondria of *Tetrahymena* are sausage shaped, do not swell noticeably, and do not apparently disintegrate. They often tend to stick to nuclei and they are a very serious nuclear contaminant. Clumped nuclei especially harbour numerous mitochondria in the clumps (Figure 23). Average nuclear fractions classed as usable had between 2 and 3 times as many mitochondria as nuclei (Figures 24 & 25 since the volume of a mitochondrion is rather less than $\frac{1}{100}$ th of a *Tetrahymena* nucleus, this implies a contamination of probably not more than 4%. It is possible to reduce the mitochondrial contamination of the nuclei by further centrifugation washes, but such steps reduce the yield of nuclei and I felt justified in accepting a

compromise between yield and mitochondrial contamination. Liver nuclei isolated by Maggio et al., 1963, had less than one mitochondrion per ten nuclei, but liver nuclei isolated by Bornig et al., 1960, had 10% of the total mitochondria remaining in the nuclear fraction, and Rotherham et al., 1956, 5%. Hogeboom et al., 1952, counted 1.5 mitochondria per nucleus under light optics. Most workers regrettably fail to give figures for estimated mitochondrial contamination.

(5) Cytoplasm. Probably the most formidable and insidious contaminant of nuclear preparations is cytoplasm, formidable because it is difficult to overcome and insidious because it is difficult to detect. A recent study of Kodama and Tedeschi, 1963, using electron microscopy has revealed that as many as 30% of thymus nuclei isolated in sucrose, are heavily contaminated by a coat of cytoplasm. The problem becomes acute when particles such as ribosomes are claimed to be present both inside and outside the nucleus (Wang, 1962). No obvious biochemical tests are available whereby cytoplasmic molecular components can be recognised, since almost all such components do occur also inside the nucleus. The recent report of a high concentration of Sialic acid (Levin and Thomas, 1961) in the microsomal fraction and its total absence from the nuclear fraction is promising. Probably electron microscopy is at present the most reliable guide to cytoplasmic contamination, although during preparation for the electron microscope, cytoplasmic contamination could be dislodged from the nuclei or become so condensed as to be discernible only in very favourable sections.



Nuclear yield.

This can be determined by direct counts of nuclei at various stages of breakage and isolation, and ultimately of the final nuclear fraction. DNA determinations can also be used as an indication of yield, assuming that the nucleus is the sole intracellular site of DNA: since nuclear fragments may still contain DNA it is important to recognise that DNA determinations are not necessarily a measure of the recovery of intact nuclei.

Counts of nuclei and whole cells have been made in Fuchs Rosenthal haemocytometer slides and compared with the numbers of whole cells in the suspension prior to breakage. In the table shown below figures are percentages of the whole cell number present before breakage.

Counts made immediately following breakage and syringe treatments.

<u>Whole cells prior to breakage</u>	<u>Loss</u>	<u>Whole cells after breakage</u>	<u>Intact nuclei</u>
100	12	15	73
100	14	5	81
100	16	8	76
100	10	14	76
<u>Average 100</u>	<u>13</u>	<u>10.5</u>	<u>76.5</u>

Counts made in final nuclear fraction.

<u>Whole cells prior to breakage</u>	<u>Loss</u>	<u>Whole cells in fraction</u>	<u>Intact nuclei</u>
100	48	0	52
100	35	2	63
100	37	2	61
100	27	1	72
<u>Average 100</u>	<u>37</u>	<u>1</u>	<u>62</u>

It will be seen that there is considerable inherent variation between these experiments, and the spread is actually wider than indicated in that occasional routine samples (about 1 in 20) have been abandoned for experimental purposes because of their unsatisfactory appearance in the microscope, while others counted only as % surviving nuclei without whole cells, gave counts sometimes higher than 72% of the original cells. But the figures presented are of four runs made for the purposes of cell and nuclear counts. The total number of nuclei counted in these runs was over 2000.

As will be discussed in the next section, DNA estimations were made on the nuclear fraction and compared with the total DNA in the cell suspension prior to breakage.

Total DNA in cell suspension = 8.37×10^{-4} gms

Total DNA in nuclear fraction = 4.01×10^{-4} gms

This is a 48% recovery of DNA, not all of which may represent intact nuclei. But the accuracy of the diphenylamine reaction is probably not very high in this experiment, since the amounts of nuclear material used in the test are rather small. In the excellent review of isolation techniques of rat and mouse liver nuclei by Roodyn, 1963 a table of nuclear yield is included. The DNA recovery values run from 5-100% and average 76%. Other workers give yield in terms of % nuclei counted and these run from 47-95% and average 64%. Maggio et al., 1963, recovered 25-35% of the DNA in the nuclear fraction.

Nucleic acid content of the nuclei.

Samples of the Tetrahymena whole cells and nuclear fractions have been assayed for total RNA and DNA. The nucleic acids were extracted

by the modified Schmitt Tannhauser technique described by Hutchison and Munro, 1961, and the quantity of DNA determined by Burton's (1956) modification of the diphenylamine reaction and RNA by the Orcinol reaction (Albaum and Unbriet, 1947).

DNA content of whole cell = 16×10^{-12} gms

RNA " " " " = 108×10^{-12} gms

RNA " " nuclear fraction = 1.3×10^{-4} gms.

Using the DNA value for the nuclear fraction as a measure for the number of nuclei present, the estimated RNA content of the single nucleus is 5.2×10^{-12} gms.

These figures give a cell ratio of RNA/DNA of 6.7/1

and a nuclear ratio of RNA/DNA of 1/3

There are some rather curious discrepancies in the literature regarding the nucleic acid content of Tetrahymena pyriformis, and a list of determinations is given below.

Figures represent amount per cell in 10^{-12} gms

Cell Strain	RNA	DNA	Reference	Year
MT. 1/1		18.9	Cerroni and Goldstein	1960
MT. 1	148		Lederberg and Mazia	1960
GL	246	13.6	Scherbaum	1957
W	1,280	30	Iverson and Giese	1957
W	13,700	2,070	Roth	1956
W	108	16	Maclean	1963

Now it seems that there is fairly general agreement that the RNA/DNA ratio is about 10/1 (Heinrich et al., 1957; Flavin and

Engelman, 1953; and Sueoka, 1961) and it is curious that the ratio of Roth's figures is about right despite the great quantitative difference between them and those of other authors. If it is accepted that the DNA of the Tetrahymena cell is between 15 and 20×10^{-12} gms, then the RNA value is unlikely to be much in excess of 200×10^{-12} gms. Although my figure for DNA content is in reasonable agreement with that of Scherbaum, there is some discrepancy in the RNA determinations: the direction of the discrepancy is a little surprising in view of the fact that Tetrahymena strain GL is smaller than strain W.

A nuclear ratio of RNA/DNA of $\frac{1}{3}$ is probably acceptable. Ratios for other nuclei have been found as $\frac{1}{3.5}$ for rat liver, $\frac{1}{6}$ for rabbit liver, and $\frac{1}{17}$ for calf thymus (McIndoe and Davidson, 1952). Since the Tetrahymena nucleus has two or three times as much DNA as most mammalian nuclei, being presumably polyploid, then this implies that the nuclear RNA content must be very much higher than in most mammalian tissues. The high nuclear RNA content may be an indication of cytoplasmic contamination, but the whole question of nuclear nucleic acid content, especially regarding RNA, is bedevilled with the twin possibilities of cytoplasmic contamination on the one hand and of the leakage and loss of intranuclear molecules during the isolation procedure, on the other.

General Discussion of Nuclear Isolation

Although the scheme described for preparing Tetrahymena nuclei is not faultless, it is, I believe, promising. With some refinement the breakage procedure would be close to ideal, but improvement of

the nuclear separation scheme would be beneficial, especially by way of reducing mitochondrial contaminants. And it is essential, if this isolation method is to be used for experimental work on nuclei, that the degree of cytoplasmic contamination be checked by electron microscopy and any available biochemical means. Perhaps the most disappointing aspect of the method is its specificity. Other cell types such as calf thymus yield a high percentage of damaged nuclei when subjected to the roller treatment, and when Tetrahymena Strain GL was used instead of Strain W, the percentage breakage was rather low.

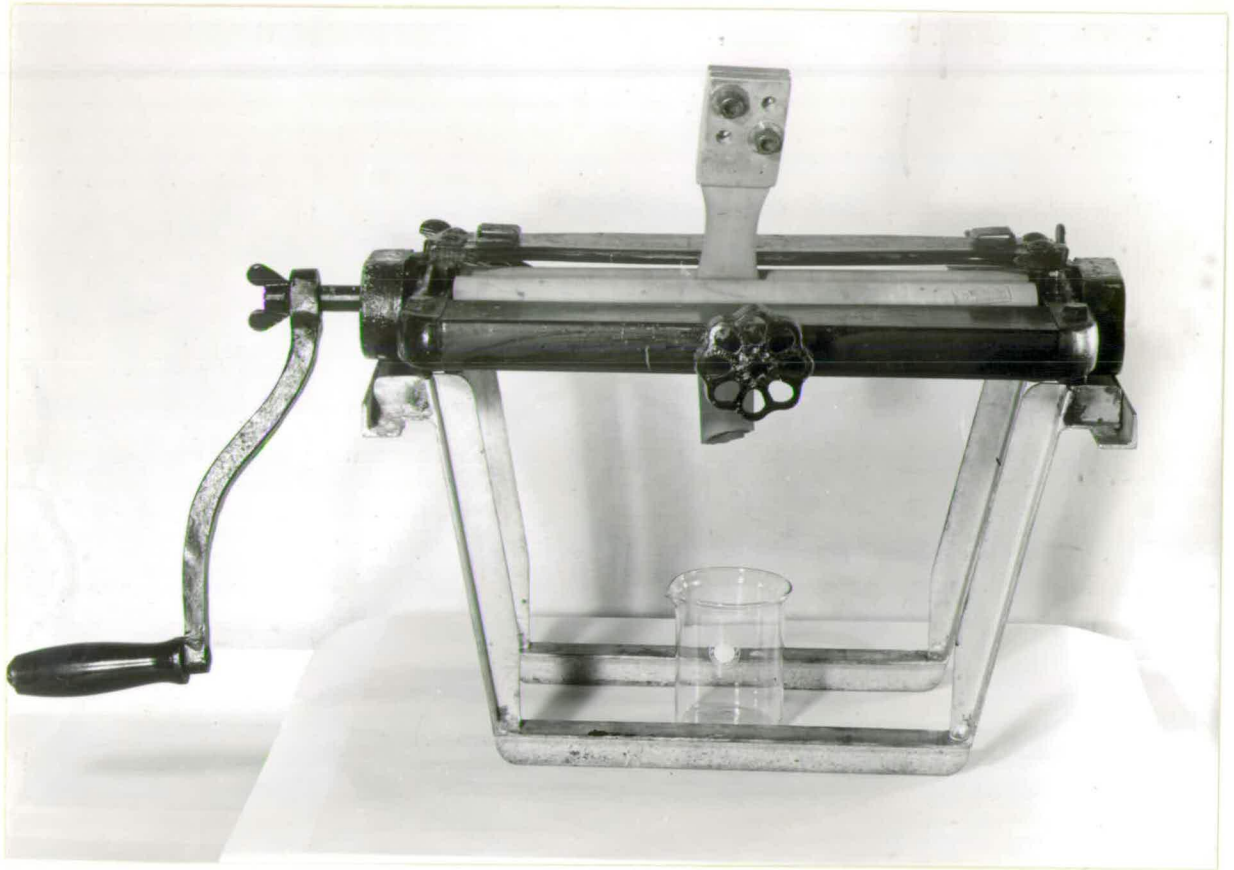


Figure 18. Cell breaking machine.

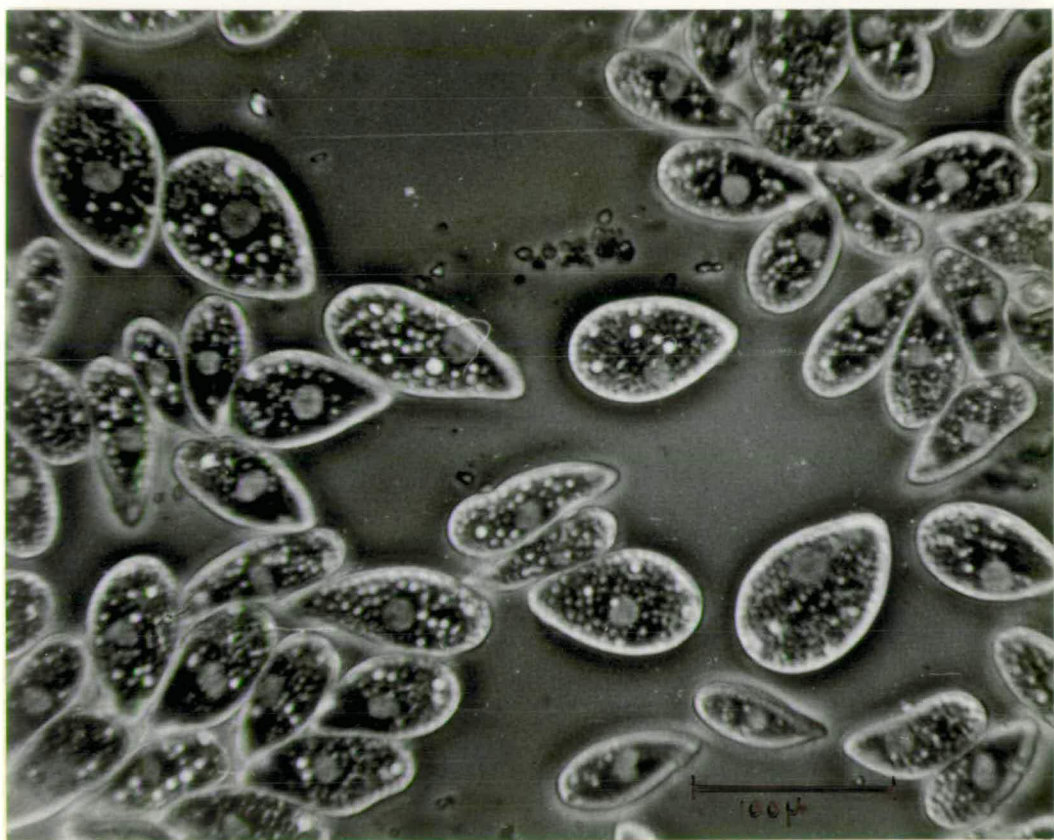


Figure 19. Cells of Tetrahymena pyriformis viewed by phase contrast.
The spherical nucleus can be seen in most of the cells.

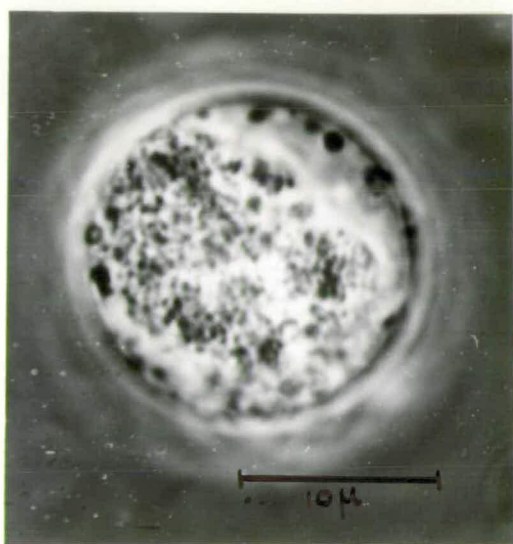


Figure 20. Nucleus of Tetrahymena, isolated in Medium A, and later exposed to a high calcium concentration. Note the aggregated nucleoprotein. Phase contrast.

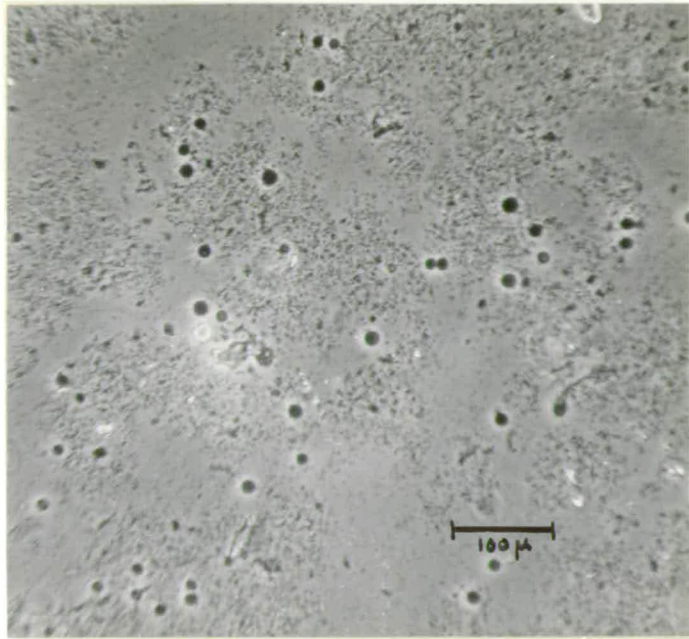


Figure 21. *Tetrahymena* nuclei in the homogenate immediately following the syringe treatment and prior to centrifugation. Phase contrast.

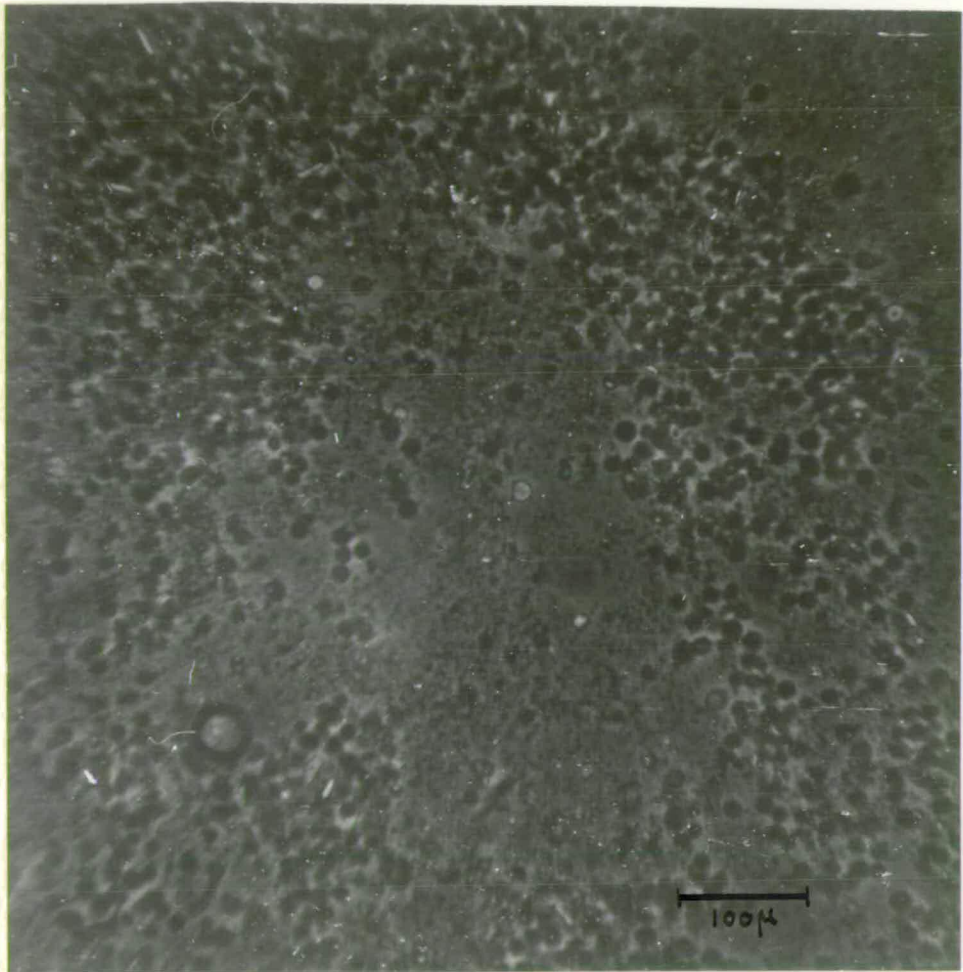


Figure 22. Bulk fraction of nuclei and mitochondria after the first centrifugation. Phase contrast.

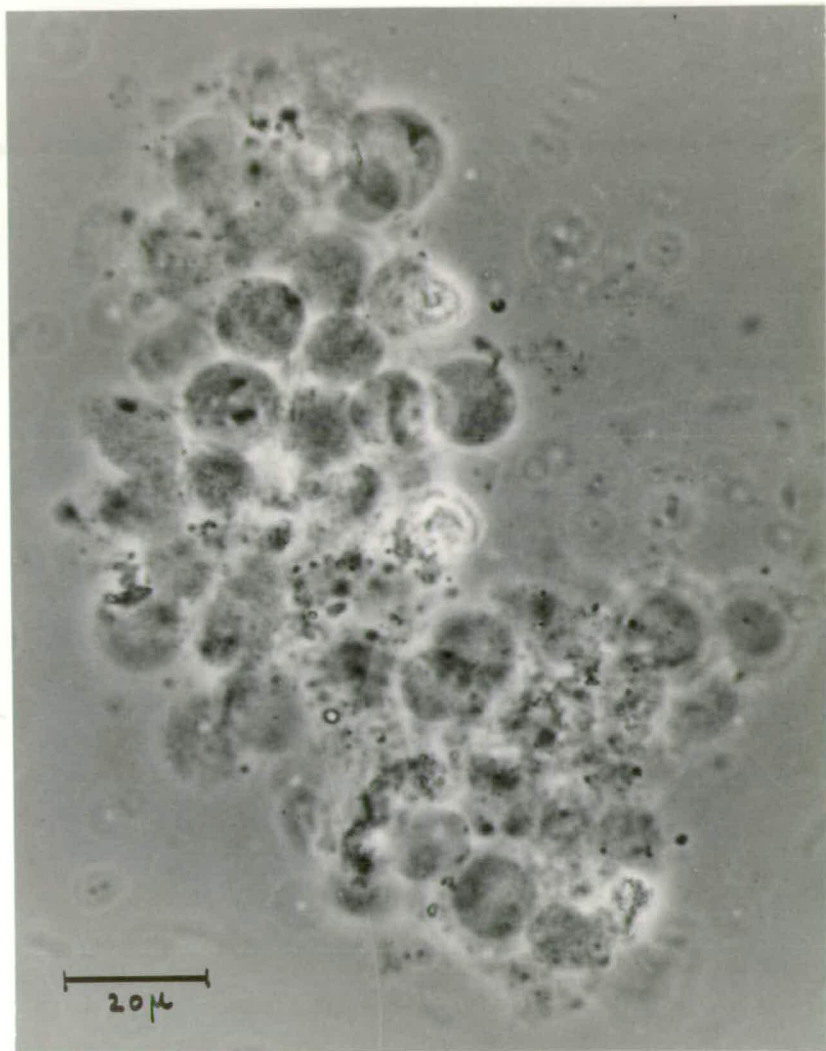


Figure 23. Nuclear clumping of *Tetrahymena* nuclei in Medium A with low calcium content. Note the numerous mitochondria involved in the clump. Phase contrast.

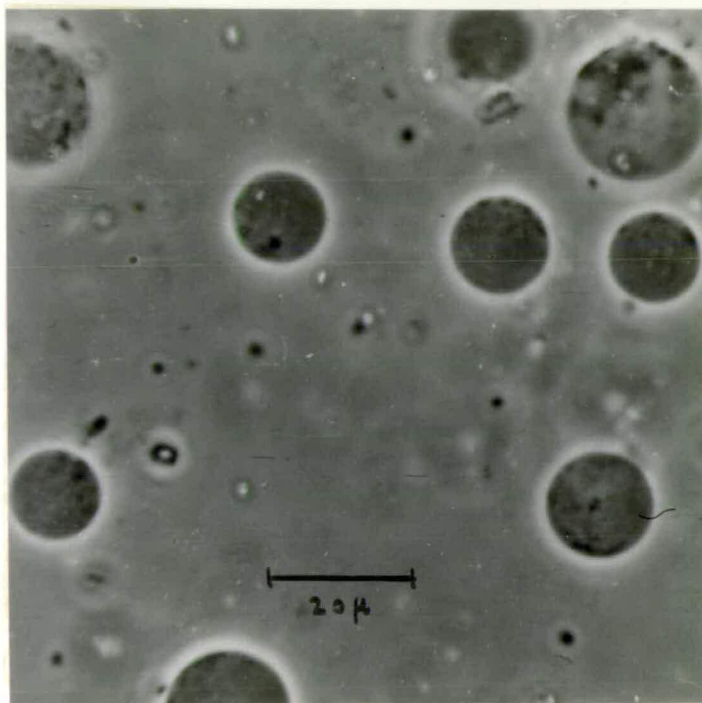


Figure 24. Final nuclear preparation. Small refractile bodies are mitochondria. Phase contrast.

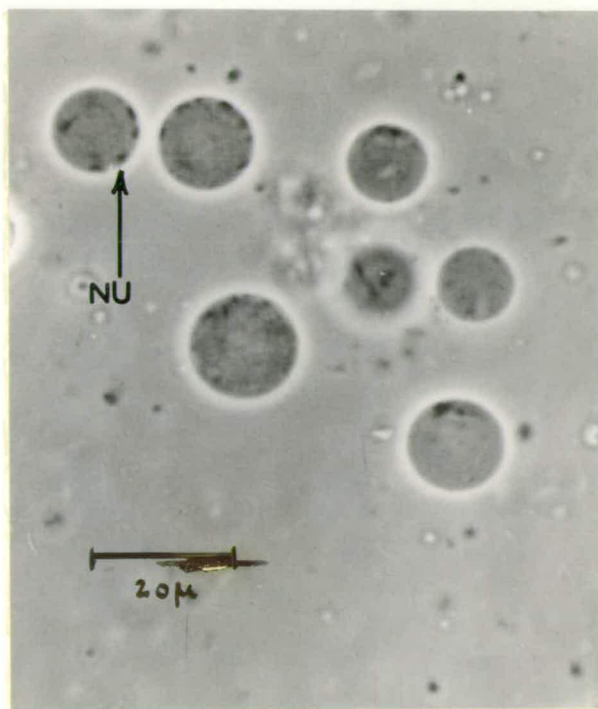


Figure 25. Final nuclear preparation. Peripheral nucleoli (NU) visible in the nuclei. Small refractile bodies are mitochondria. Phase contrast.



Figure 26. Final nuclear preparation, but after 1 hour at room temperature. Note the distorted and pale appearance of some nuclei and the granular chromatin of others. Phase contrast.

PART V

Ribosomes from Tetrahymena cells and nuclei

Introduction

This part of the thesis is concerned with experiments on the separation of ribosomes from whole cell and nuclear fractions of Tetrahymena by centrifugation through sucrose gradients. There are three main methods of study on ribosomal structure and function, electron microscopy, isolation in the preparative ultracentrifuge, ^{including incorporation studies} and behaviour in the analytical ultracentrifuge. Since I did not have access to an analytical centrifuge, I have confined myself to the preparative method. The velocities at which different cell components move in the centrifuge varies widely, and it is helpful to preferentially slow down the faster moving particles as they move down the tube. This is accomplished by constructing a gradient of sucrose such that relatively ^{dilute} ~~weak~~ and therefore non-viscous sucrose lies at the top of the tube and the concentration of sucrose gradually increases down the length of the tube. Such gradients, often inappropriately called density gradients, but better described as viscosity gradients, have been very widely used for the fractionation of ribosomes and other cell components.

The information which can be derived from sucrose gradient centrifugation is limited, but labelling of the cells prior to fractionation considerably increases the usefulness of the method. However the first step in such a project is to run unlabelled material and acquire good reproducibility, and all of the experiments to be described here are with unlabelled cells.

Analyses of centrifuge runs in sucrose gradients yield two classes of information. Firstly, the amount of material appearing in certain centrifuge fractions permits determination of the total quantity of this component in the cell or cell fraction. Secondly, the movement of particles in the sucrose gradient gives some indication of their characteristics, since information on the performance of ribosomes of known sedimentation constant during sucrose gradient centrifugation is available in the literature. But accurate estimation of the sedimentation constant is not possible for this the analytical ultracentrifuge is necessary.

This work was originally undertaken as an attempt to isolate nuclear ribosomes, or at least to compare the distribution of ribosomes in the nucleus with that of the whole cell. But the ribosomes of *Tetrahymena* are of considerable interest in their own right. At a time when the ribosomes were believed to possess encoded genetic information for protein structure, it was thought that a cell would possess ribosomes specific for the production of different proteins, and thus that the RNA composition of ribosomes might vary. Moreover, when it became clear that the base sequence of the DNA constituted genetic information, the ratio of the four bases in the DNA might have been expected to be reflected in the base ratios of the total ribosomal RNA. This proved to be false. Indeed, not only did ribosomes from one cell type have base ratios very different from the DNA, but ribosomes from many different kinds of cells, even from different phyla, proved to have base ratios of remarkable similarity.

At present, knowledge and ideas about ribosome structure and

function are as follows. The DNA of a cell bears information in terms of the sequence of the four bases strung along the sugar-phosphate molecular backbone, but at any one time only a part of this DNA information is likely to be actively involved in dictating protein molecular structure. An RNA quite distinct from ribosomal RNA, and designated the messenger RNA, copies the active sites of the DNA in its own complementary base sequence and conveys this information to the ribosome, with which it becomes associated and on which the protein molecule is synthesised under the direction of the messenger molecule. It seems likely, therefore, that the ribosomes have no genetic specificity, acting only as sites or work benches, and are all similar in structure, having RNA molecules of identical composition. Distinct cistrons for the base sequence of ribosomal RNA are therefore visualised, and good evidence for their existence is now available (Yankofsky and Spiegelman, 1962). Such a scheme for protein synthesis is now widely accepted although certain aspects of it are still open to question (Harris, 1963).

Certain predictions which follow from this scheme have now been verified. It would be expected that the overall base ratios of the messenger RNA fraction would not be identical to the overall DNA base ratios, but would probably mirror them fairly well in a comparatively undifferentiated cell. This indeed is true, and such RNAs have been isolated from bacteria (Astrachan and Volkin, 1958; Gros et al., 1960 and Brenner, 1961). Moreover, if the ribosomal RNA is not a genetic template, evolution would not necessarily have evoked changes in its composition, and therefore it would not be surprising if cells from different organisms, whose DNA base ratios had diverged widely, might

retain the same ribosomal base ratios. This again has proved correct. Cell free protein synthesis occurs when RNA from a plant virus, presumably analagous to messenger RNA, is incubated with E. coli ribosomes (Haselkorn, et al., 1963). And ~~base ratios of~~ ribosomes from almost all plant, animal and bacterial sources have rather similar base ratios despite the great diversity of DNA base ratios. In general, ribosomal RNA has a high G C content and low A U, and in particular the amount of guanine is greater than that of any other base.

One probable exception to this ribosome base ratio pattern is Tetrahymena. Ribosomal RNA has not been examined in this organism, but the base ratios of the total RNA show a uniquely low guanine content: adenine is the major base present. Now Tetrahymena has a very low G C content in the DNA, around 25%, and therefore it would be expected that the complementary messenger RNA would contribute some adenine and very little guanine to the total RNA. But it is unlikely that the messenger fraction would constitute more than 1-3% of the total RNA and the base ratios of the transfer RNA are not strong in adenine (Brown, 1963). One is left with the conclusion that, unlike all other known ribosomal RNAs, which are rather homogeneous and have a high guanine content, Tetrahymena ribosomal RNA has a low guanine content and a high adenine content.

	A	T, U	G	C	
DNA	35.4	35.4	14.5	14.7	- Jones and Thompson, 1963
Total RNA	30.3	26.7	22.2	20.6	- Cummins and Plaut, 1962
Transfer RNA	18.6	18.2	30.2	28.9	- Brown, 1963

Since the ribosomal RNA of *Tetrahymena* is unusual, it is interesting to discover whether *Tetrahymena* ribosomes behave in other ways similarly to ribosomes from other cell types.

Separation of ribosomes from nuclear preparations has been carried out by a number of groups. Frenster, Allfrey and Mirsky, 1960, have separated ribosomes from calf thymus nuclei and Wang, 1960 and 1961, from liver nuclei, while Birnstiel, Chipchase and Hyde, 1963, have isolated ribosomes from pea nucleolar fractions. An exhaustive article on the work of the group at the Rockefeller Institute has been published by Pogo et al., 1962, and all of these authors have verified the appearance of the ribonucleoprotein particles by electron microscopy. Unfortunately, there is little indication in these papers of the ribosomal yield from the nuclear fraction, and all the tissues, with the possible exception of the pea tissue, were differentiated and probably manufacturing few, if any, new ribosomes.

Ribosomes have been separated from *Tetrahymena* and spun in caesium chloride density gradients by Britten, 1960, and by Plesner, 1961. Britten determined that the main ribosomal peak constituted 90S particles while Plesner, working with synchronised cells, calculated the presence of 70S, 80S and 100S ribosomes.

The work to be described here will show that in the conditions of growth and extraction employed, only one chief ribosomal peak occurs and that the behaviour of nuclear ribosomes under the conditions of centrifugation differs from that of the whole cell ribosomes.

Materials and Methods

Cell fractions.

Tetrahymena pyriformis strain W cells were grown and harvested and subjected to roller treatment as described in Part IV. Fractions designated 'whole cells' consisted of post-roller homogenates in Medium A while nuclear fractions were prepared in Medium A, also as described in Part IV. All samples were kept in ice and except where otherwise mentioned, sodium deoxycholate was added to a concentration of 0.5%. After 5 minutes incubation with the deoxycholate, 6 ml aliquots were spun in the cold at 5,000 g for 15 minutes, and the supernatant carefully decanted, the pellet of debris being discarded.

Preparation of gradients.

Linear sucrose gradients were made using a gradient machine constructed in this department after the pattern used by Britten, Roberts et al., 1959, but with a glass spiral stirrer turned at about 500 r.p.m. The sucrose solutions were made up to concentrations of 10% and 30% w/v in Tris magnesium buffer at pH 7.4 - 0.01 M magnesium acetate, and 0.005 Tris. The magnesium concentration of a medium designed for ribosomal isolation and fractionation is of great importance, since ribosomes will aggregate and disaggregate as the concentration is raised or lowered. The choice of 0.01 M magnesium was made since it has been used by the majority of workers for fractionating ribosomes from a great variety of cells, and the greatest range of ribosomal peaks from 20S to 100S can be obtained from E. coli cells using 0.01 M magnesium (McQuillen, 1962).

Gradients took about half an hour each to drip out into the Lusteroid cellulose centrifuge tubes to a total volume of 24 mls. All gradients were prepared shortly before use in a cold room at 3°C and with tubes already inserted in the centrifuge buckets to prevent unnecessary handling.

Ultracentrifugation.

One ml of the supernatants resulting from the 5,000 g spin of the cell fractions was carefully layered on to the top of the sucrose gradients via a syringe needle, and the gradients spun for three hours at 25,000 r.p.m. in the SW-25 Rotor of a Model L Preparative Spinco. During a spin, the brake of the instrument was switched off when the rotor had slowed to about 5,000 r.p.m. and the rotor was watched during initial acceleration and final deceleration to ensure the smooth rise and fall of the buckets. On two occasions, gradients were discarded because of the failure of the bucket to fall until the centrifuge had almost stopped.

Fraction collection.

Centrifuge tubes were removed from the buckets and supported in a clamp, when a needle mounted in a brass block was used to punch the bottom of the tube and the contents dripped out into numbered glass sample tubes. Due to the variation in drop size, sample size was not standardized by number of drops, but by the level of the fraction in the glass sample tubes, since this gave much more reproducible collection. On average, 30 samples were collected from each centrifuge tube.

Analysis of fractions.

From each sample tube two drops were removed and used for measurement of sucrose refractive index in an Abbe Refractometer. The remainder of the tube contents were diluted with 0.5 ml of Tris magnesium buffer and their absorption at 2,600 Å read in a Unicam S.P. 500 Spectrophotometer.

Results

Firstly, runs were made to assay the recovery of ribosomes in whole cells broken in Medium A, Medium A minus magnesium plus deoxycholate, and Medium A plus deoxycholate. As shown in Figures 28, 29 and 30, no ribosomal recovery occurred without magnesium, but when normal Medium A was employed without deoxycholate a rather broad peak was obtained. Figure 30 is the recovery obtained with Medium A plus deoxycholate and here the resolution is better and the yield good. Presumably the mild detergent improves the profile by preventing ribosomes being contaminated by proteinaceous material from membranes or partly synthesised proteins.

The ribosomal peak occurs in tubes 15, 16 and 17, which suggests a sedimentation constant of around 80S from data extrapolated from characterised ribosomes run on sucrose gradients by other workers (Gros et al., 1961). Three runs of nuclei in Medium A plus deoxycholate have been made and the results are shown in Figures 32, 33 and 34. Addition of deoxycholate to the nuclear fraction brings about lysis of the nuclei immediately. It is a somewhat complex matter to compare the whole cell and nuclear fractions. An attempt

can be made as follows. Assuming a cell breakage and ribosomal extraction of 100% from whole cells, and noting that only one tenth of the total 10 ml supernatant from the whole cell fraction was used, we can assume that the whole cell ribosomal peak represents certainly no more than one tenth of the total cell ribosomes. Now the nuclear volume of a cell of Tetrahymena pyriformis is about one twentieth of the volume of the whole cell (Summers, 1963), and therefore assuming a nuclear yield of 50% and noting that all of the nuclear sample from the 10 ml aliquot of cells was used, we can deduce that the nuclear fraction might contain ~~half~~^{1/4} of the ribosomal yield of the whole cell aliquot. This assumes that the concentration of ribosome in nuclei and whole cells is the same. In fact, as seen in the figures, the yield of ribosomes in this peak is very low. Each nuclear run gave a small peak in parallel with the ribosomal peak of the whole cell fraction, but the maximum reading in each case was 0.07 O.D. units, compared with the broad ribosomal peak of the whole cell fraction with three readings at or above 0.6 O.D. units. Taking the sum of tubes 12 to 18, the whole cell ribosomes give a total O.D. reading of 3.19 compared with a maximal reading of tubes 12 to 18 from Figure 32 of 0.37. Since undoubtedly there is a certain base line reading which contributes to both, it is clear that the nuclear ribosomal yield is considerably less than one tenth and probably much nearer one hundredth of the whole cell ribosomal yield at this peak.

Some comment is necessary regarding Figure 34, one of the nuclear fraction runs. The peculiar profile from tube 17 upwards, I interpret as resulting from mixing of the top contents of the tube,

probably caused by a sudden jolt of the centrifuge bucket during acceleration or deceleration.

One very important observation made by all of the workers on nuclear ribosomes is the fact that the particles aggregate strongly at concentrations of magnesium normally suitable for the isolation of cytoplasmic ribosomes. To quote Wang, 1961, "At 0.01 M magnesium chloride, precipitation of nuclear ribonucleoprotein has reached its maximum, while less than 10% of liver ribosomal ribonucleoprotein is precipitated at this concentration of magnesium." Now one of the conspicuous aspects of all three runs of *Tetrahymena* nuclear fraction is the quantity of absorbing material which appears at or near the bottom of the tube. It does seem that these profiles confirm the findings of others in that the nuclear ribonucleoprotein form large aggregates at this magnesium molarity, whereas the cytoplasmic ribosomes, or, as in these experiments, the ribosomes from whole cell homogenates, show a peak of particles around 80S and do not generally aggregate to form larger groups. A considerable number of runs of whole cell material have been made, and only in one of them is there accumulation of much absorbing material at the bottom of the tube. This profile is shown in Figure 31, and it will be seen that in this run not only is there a large mass at the bottom of the tube, but there are two other peaks of material heavier than the main peak, one appearing in tube 6 and another as a shoulder on the heavy side of the main 80S peak.

Discussion

The results of the experiments described here do not throw much light on the question which they were intended to answer - how many and what sort of ribosomes are to be found in the Tetrahymena nucleus. Quantities of absorbing material at the base of the nuclear fraction gradient suggest that some material which may in the cell nucleus be organised as ribosomes, has aggregated together under the influence of the magnesium concentration of the sucrose. On the other hand, it seems at least possible that within the living nucleus these particles are aggregated together to form the nucleolus and that under conditions of low magnesium the nucleolar organisation may be broken down. Not only have Birnstiel, Chipchase and Hyde, 1963, shown that ribosomes are recoverable from pea nucleoli under conditions of low magnesium, but Chipchase and Birnstiel, 1963, have used the DNA/RNA hybridisation technique to demonstrate that cytoplasmic ribosomal RNA and nucleolar RNA compete for the same hybridisation sites on the DNA and therefore presumably are coded by the same cistrons.

There are then two possible alternative explanations to the nuclear ribosome evidence so far accumulated. On the one hand, the nucleolus may consist of ribosomes or ribosome-like particles which aggregate together more rapidly and easily than cytoplasmic ribosomes at magnesium concentrations in excess of 0.01 M. If this is so, and this is taken to explain the aggregation phenomenon of isolated nuclear ribosomes, then it appears that apart from the nucleolus, nuclei are possessed of few, if any, ribosomes. On the

other hand, it may be that nuclei possess considerable numbers of ribosomes quite apart from the nucleolus and that these ribosomes are distinct from cytoplasmic ribosomes in that their response to the magnesium ion concentration is very different.

Tetrahymena nuclear ribosomes share with ribosomes from nuclei of liver, thymus and peas a greater tendency to aggregate in the presence of magnesium than is found with cytoplasmic ribosomes. This suggests that there is something distinct about the nuclear organisation and that it may be possible to differentiate between nuclear and cytoplasmic ribosomes by their reaction to high magnesium concentration. Perhaps the small peaks of 80S ribosomes appearing in the Tetrahymena nuclear fraction profiles represents cytoplasmic contamination; if this is so, it is at least satisfactory to note that the degree of contamination is rather low.

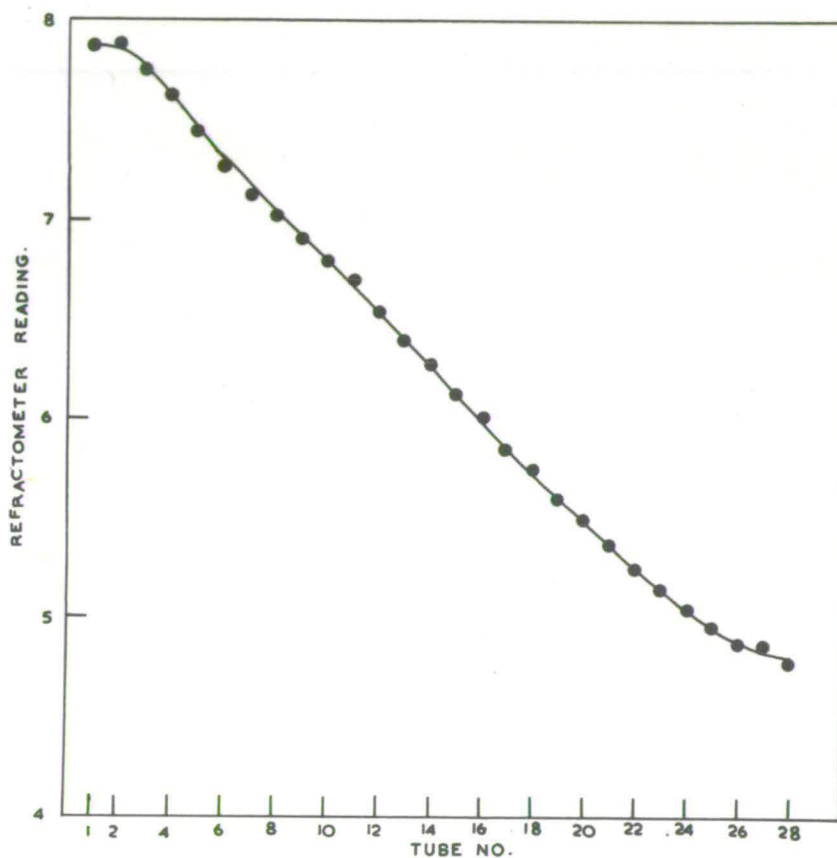


Figure 27. Linear gradient of sucrose in Tris magnesium buffer, running from 10% to 30% sucrose, dripped out after 3 hour spin in SW 25 at 25,000 r.p.m. and sample drops measured in an Abbe refractometer.

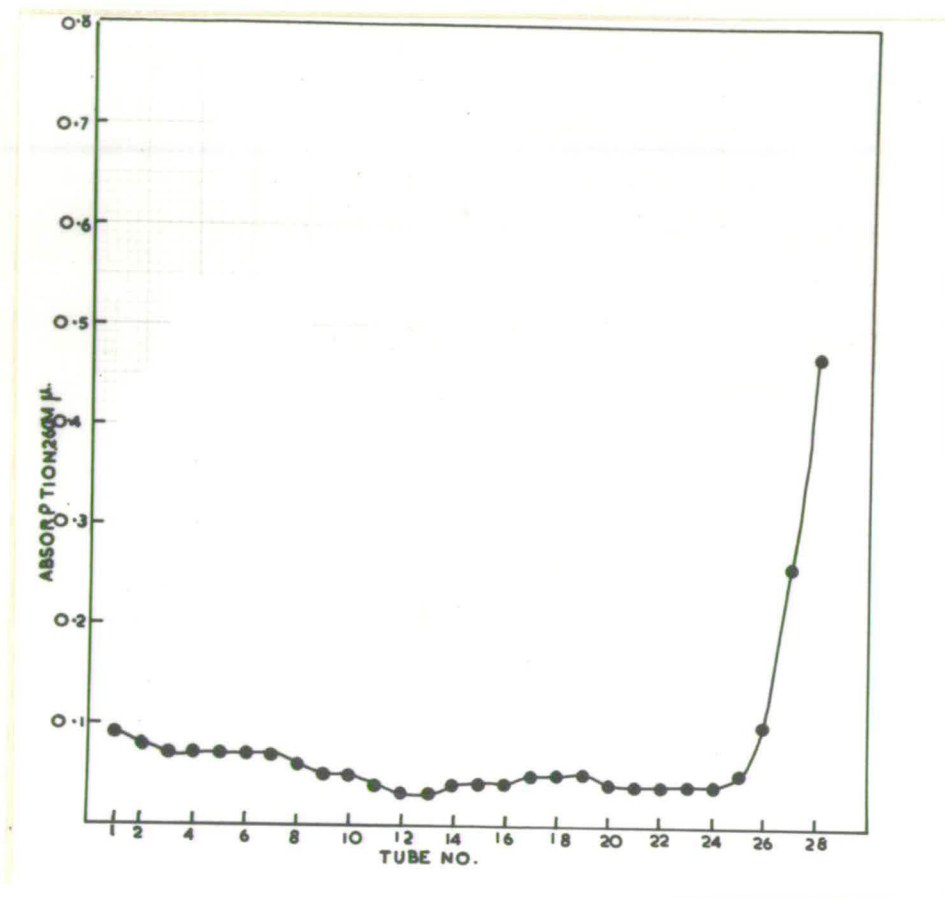


Figure 28. Sucrose gradient analysis of crushed whole cells in Medium A minus Magnesium, with deoxycholate added. 3 hour spin at 25,000 r.p.m.

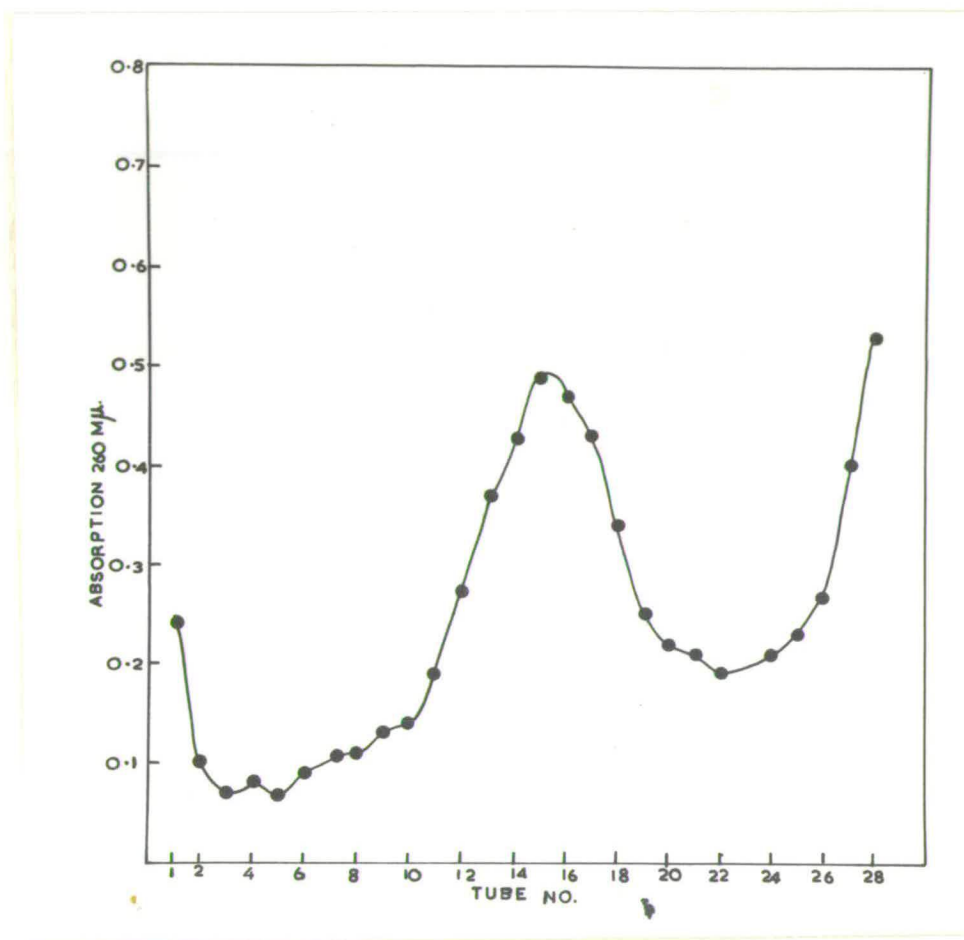


Figure 29. Sucrose gradient analysis of crushed whole cells in Medium A. 3 hour spin at 25,000 r.p.m.

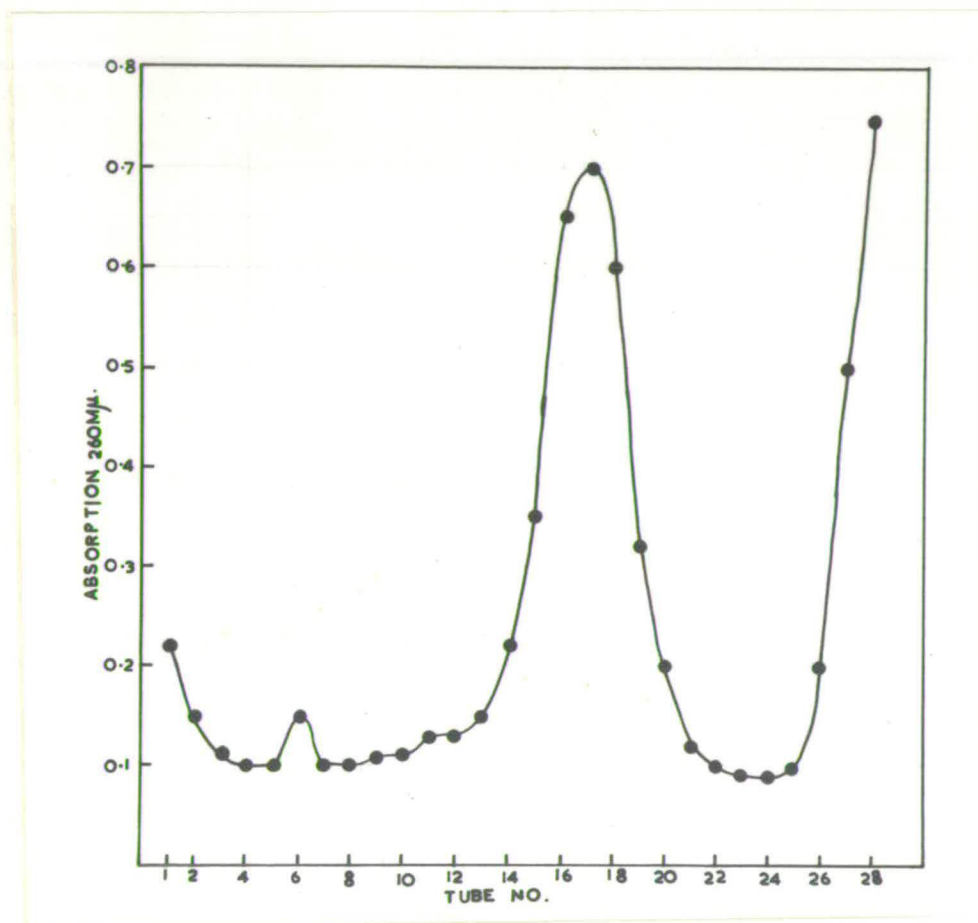


Figure 30. Sucrose gradient analysis of crushed whole cells in Medium A with deoxycholate added. 3 hour spin at 25,000 r.p.m.

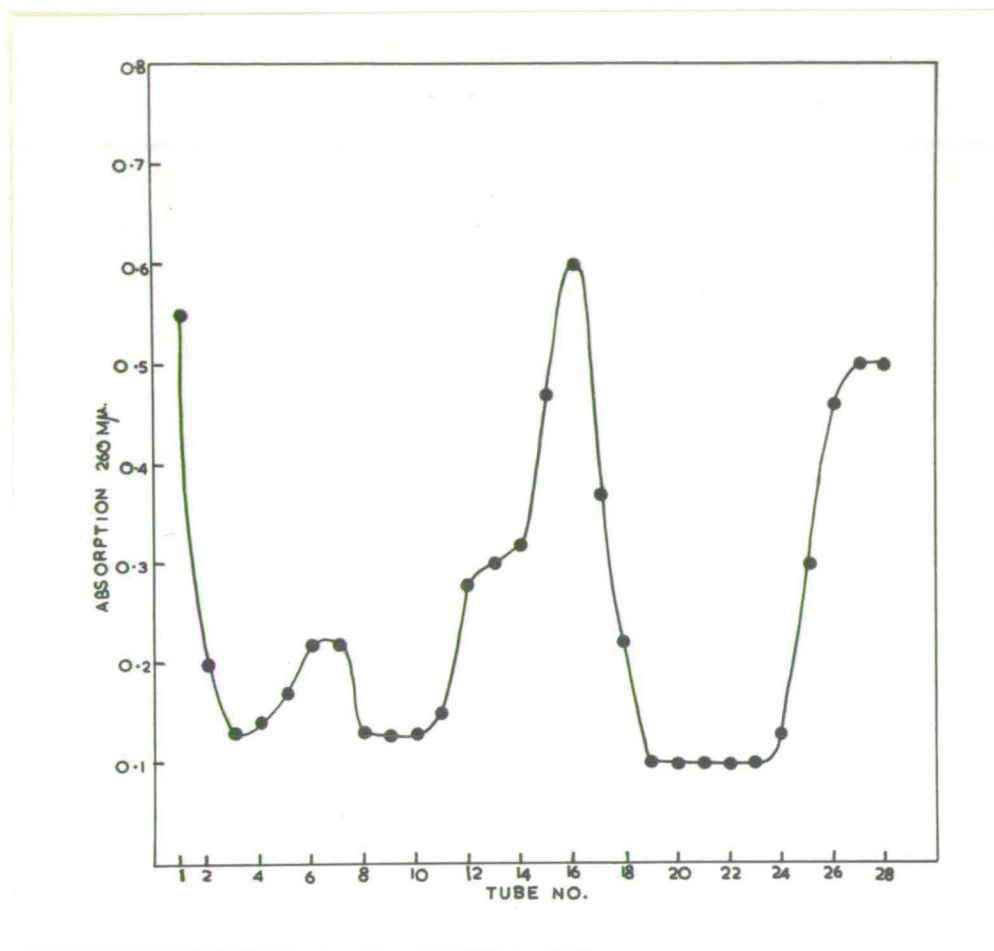


Figure 31. Sucrose gradient analysis of crushed whole cells in Medium A with deoxycholate added. Note the heavy material forming peaks nearer to the bottom of the tube than the normal 80S peak. 3 hour spin at 25,000 r.p.m.

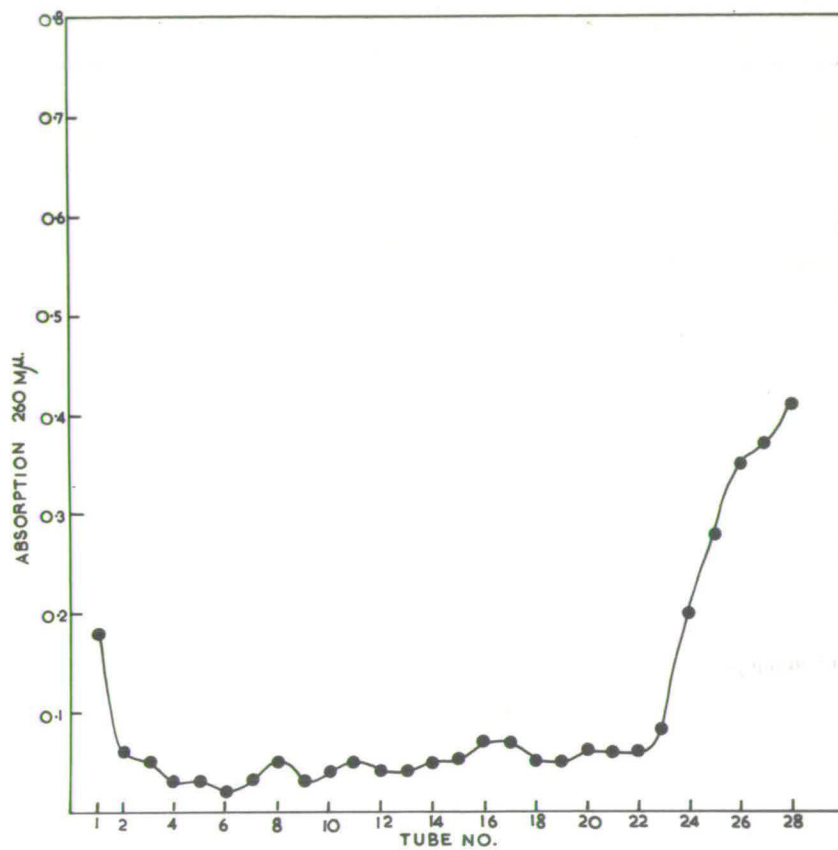


Figure 32. Sucrose gradient analysis of *Tetrahymena* nuclear fraction. 3 hour spin at 25,000 r.p.m.

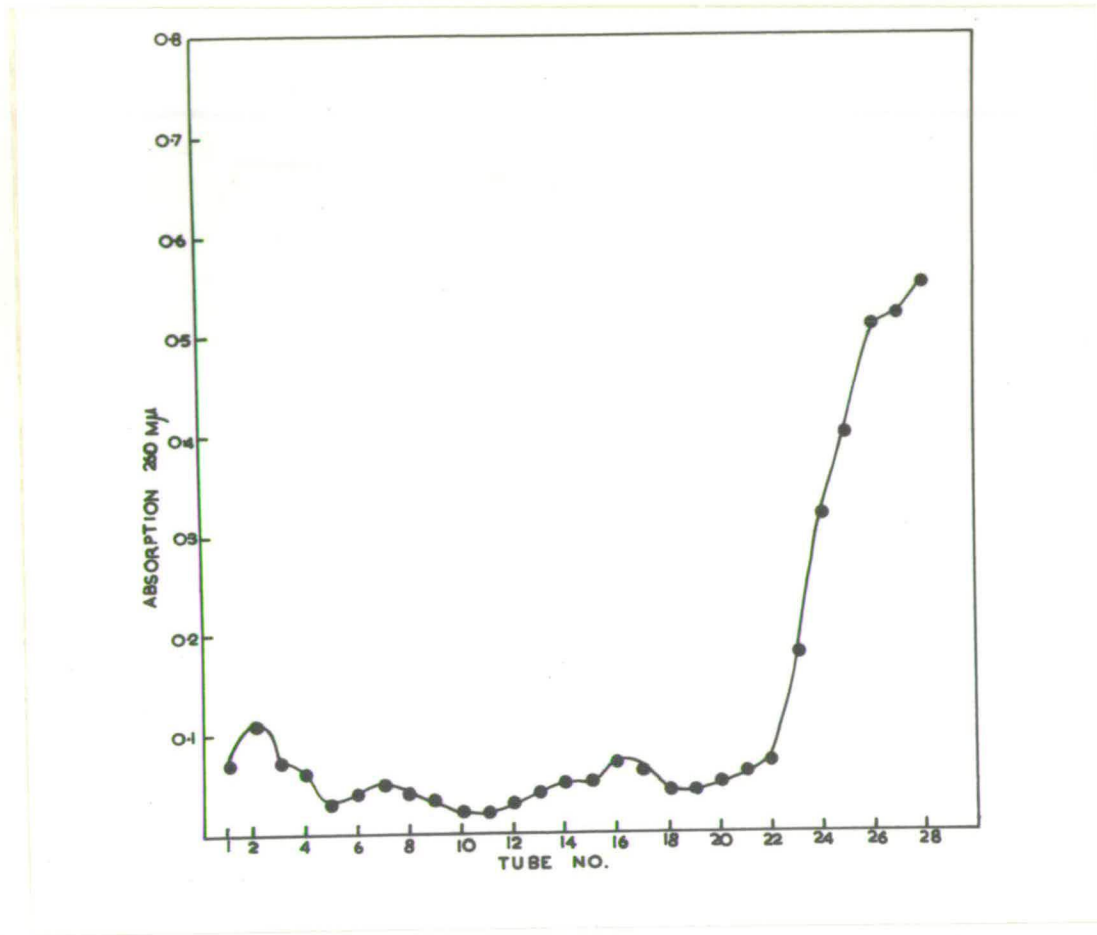


Figure 33. Sucrose gradient analysis of Tetrahymena nuclear fraction. 3 hour spin at 25,000 r.p.m.

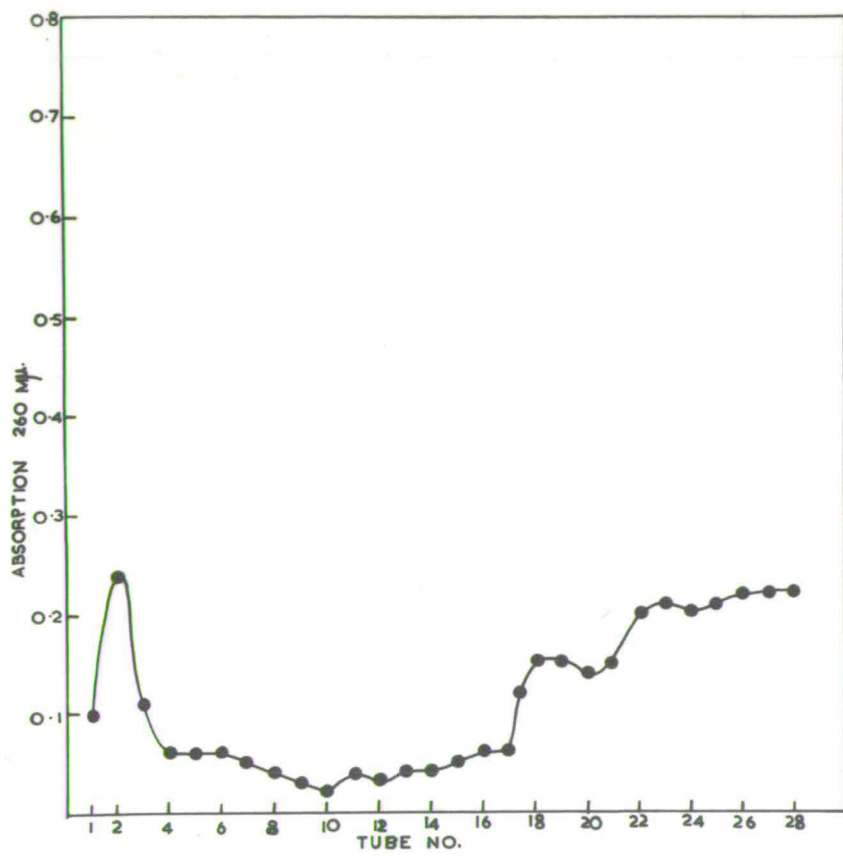


Figure 34. Sucrose gradient analysis of Tetrahymena nuclear fraction. Note the high readings of tube 18 upwards, ascribed to accidental bumping of the tube. 3 hour spin at 25,000 r.p.m.

PART VI - APPENDIX

Denaturation of Tetrahymena DNA

Introduction

One of the predictions which follow from Watson and Crick's model of DNA structure is that under certain conditions the two anti-parallel chains of the molecule, normally interwoven as a double helix, would separate to become two single chains without specific linkages between them. Such a partial decomposition of a molecule is often referred to as denaturation, and its occurrence in DNA presupposes that forces exist which would break the bonds holding the two chains together, without breaking the bonds holding together the groups which comprise the single chain structure. Evidence for strand separation in vivo comes from the experiments of Meselson and Stahl, 1958, and Cairns, 1962, on bacterial and phage DNA, and the semi-conservative model for DNA replication supported by most of the present evidence, involves the separation of the DNA double-helix during the normal DNA replication of the cell.

Numerous techniques are available which can be employed to measure the denaturation of extracted DNA and these have been exhaustively discussed in the excellent review by Marmur, Rownd and Schildkrut, 1963. Of all these techniques for determination of DNA denaturation, probably the simplest is spectrophotometry. When the 2,600 Å⁰ ultra-violet absorption is measured, it is found that a DNA sample gives a significantly higher reading after it has been exposed to denaturing conditions. This phenomenon, known as the hyperchromic

effect (Magasanik and Chargaff, 1951), was first observed with RNA before and after ribonuclease digestion, but it is now recognised that the effect can be brought about by a variety of gentler methods which do not result in the complete degradation of the molecule. Although not indisputably established, it seems likely that when the double-stranded molecule has been denatured to yield two single strands, the 2,600 Å absorption rises by about 30% of its original value. This rise in absorption, as measured in the spectrophotometer, can thus be used as an indication of denaturation.

A variety of conditions can induce denaturation, but one of the easiest and most reproducible is increased temperature. In standard conditions the temperature at which the hyperchromic effect occurs is known as the melting temperature, designated ^{at} T_m and the average melting temperature at which half of the total hyperchromic effect of a sample occurs, designated $T_{\frac{1}{2}m}$. Since the G-C base linkage of DNA appears to melt at a higher temperature than the A-T linkage, perhaps because of the extra hydrogen bond involved, the $T_{\frac{1}{2}m}$ of a DNA sample can be used as an index of average base composition in terms of %GC and AT. Marmur and Doty, 1962, have exploited this method for determination of base composition of DNA from a very large number of organisms.

For many years it was believed that denaturation was an irreversible reaction, but it is now clear that this is not so. Renaturation of denatured DNA has been verified by a number of different criteria, including a return to the native absorption value, renewed biological activity, and return to the previously

high viscosity of the undenatured polymer (Geiduschek, 1962). The renaturation of denatured or melted DNA is often referred to as annealing, and it is supposed that the single strands present in the melted solution reform the double-helical pattern by cross-pairing between the bases of complementary molecules. However, two distinct types of renaturation occur, only one of which is believed to represent true annealing (Geiduschek, 1961). If denatured DNA from any source is rapidly quenched in ice from the melting temperature, a sharp drop in absorbance occurs, but if the temperature is again raised, the sample melts out at a much lower temperature than the previous T_m . The probable explanation of this phenomenon is that under rapid cooling, the DNA single strands form comparatively random and unspecific bonds between the bases of neighbouring strands; when the temperature is again raised these rapidly formed bonds break easily because of their comparatively unspecific character, and the hyperchromic effect is observed at a temperature much below the true T_m . On the other hand, with DNA from bacterial and viral sources, if denaturation is followed by slow cooling, eventual return to a near native absorbance is again observed, but reheating brings about no effect until the T_m is reached, when a normal hyperchromic rise takes place. This phenomenon is referred to as annealing and is believed to consist of a genuine return to the specifically double stranded form of the molecule (Geiduschek, 1962). These two types of renaturation are termed Type I and Type II reversibility in current literature.

Although Type I reversibility is a universal phenomenon with

every type of DNA tested, Type II reversibility occurs only with DNA from some sources. Many other factors besides source affect Type II reversibility - concentration, temperature, molecular weight, and ionic strength of medium, but all except molecular weight can be controlled and standardized easily. It is logical to believe that in standardized conditions there are two main factors which affect the Type II reversibility phenomenon, molecular weight and intermolecular heterogeneity, the latter depending on source. Marmur and Doty, 1961, have shown that a high molecular weight sample of DNA will anneal better than a low molecular weight sample of the same DNA and suggest that a zipper mechanism occurs in annealing by which a few long molecules will anneal faster than many small ones. The second factor, that of intermolecular heterogeneity, is the one in which I have been chiefly interested in the experiments to be described here.

If a double stranded phage DNA sample is melted and, for the sake of argument, the phage genome is presumed to be a single molecule, the resulting solution will consist of only two types of DNA strands. On slow cooling the chance of any one strand finding its complementary strand is rather high even if it is imagined that complementary strands often make abortive attempts to join up in the wrong places. But if the DNA sample contains many different molecular species, each double stranded, then on melting and attempted annealing, each single strand is presented with the problem of finding its own proper, though not necessarily original, neighbour amongst the numerous adjacent molecules, with no means of recognition

except trial and error pairing along available lengths. It is conceivable that, even with high molecular weight, a certain degree of intermolecular heterogeneity would totally preclude complementary pairing of strands.

Such an argument has been put forward by Marmur and Doty, 1961, in order to explain the effect of source on Type II reversibility of DNA structure. They found that under their experimental conditions, phage DNA anneals rapidly and completely, bacterial DNA anneals slowly and partially, and mammalian DNA does not anneal at all. It follows that, if their line of argument is correct, within standardized conditions the speed and degree of annealing is a measure of DNA intermolecular heterogeneity. There is no account in the literature of the attempted annealing of protozoan DNA, and since there are evolutionary reasons for believing that protozoa may be genetically simpler than higher organisms, it is of interest to test the molecular complexity of their genome by annealing experiments.

There is no direct relationship between this problem and the experiments already described in earlier parts of my thesis but since I was growing *Tetrahymena* in large quantities and had all the necessary equipment at hand, I felt justified in satisfying my curiosity about the behaviour of the *Tetrahymena* DNA.

Materials and Methods

DNA preparation.

Bacterial DNA was prepared from *Bacillus megatherium* by the method of Marmur, 1961, and from *Tetrahymena pyriformis* strain W

by an expanded version of Marmur's technique used by Dr P.M.B. Walker in this department. The purified samples of DNA were finally dissolved in 0.3 M sodium chloride, 0.03 M sodium citrate for the experiments.

Temperature control.

Heating and temperature control of the DNA solutions was carried out in a specially constructed copper block which fitted into the sliding carriage of the Unicam Spectrophotometer. The solution was contained in 3 ml stoppered silica cells, used without the stoppers, but with a layer of Nujol liquid paraffin covering the DNA solution to prevent evaporation. Heating and cooling of the copper block was carried out by water, circulating in channels in the block, hot water being supplied by a thermostatically controlled circotherm. Actual temperatures within the cells were determined by a control run with a thermocouple within the cell, and the temperature readings correlated with the circotherm thermometer readings at minute intervals. Very little change in temperature occurred after three minutes at any one temperature, and during experimental runs readings were taken after three minutes at the desired temperature, and the actual temperature within the cells calculated from the thermocouple calibration graph.

Heating and cooling sequence.

Experimental methods followed as closely as possible those used by Marmur and Doty, 1961, in their thermal renaturation experiments. DNA samples were heated in the cells, and absorption readings at 2,600 Å taken at 5°C intervals up to 75°C after which readings were

taken at 2°C intervals. When the temperature reached 100°C the temperature was kept constant for ten minutes after which both the experimental cell containing DNA solution and control cell containing saline citrate were rapidly quenched in ice for 5 minutes. On returning to the copper block, now pre-cooled to 67°C, absorption readings were taken every two minutes for the first ten minutes and thereafter at 5 minute intervals.

Results

As found by Marmur and Doty, 1962, Bacillus megatherium DNA has a T_m of about 87°C and Tetrahymena pyriformis DNA about 82°C in the experimental conditions used, correlating with a GC content of 38% for B. megatherium and 25% for Tetrahymena. Annealing of the B. megatherium DNA was similar to that found by Marmur and Doty, showing a reduction after 90 minutes of 80% of the hyperchromic rise. This sample was then reheated to the T_m to confirm that the annealing was a genuine Type II reversal. When the DNA of Tetrahymena pyriformis was quenched the Type I reversal involved only 50% of the hyperchromic rise and on heating at 67°C the absorption rose again almost to the original hyperchromic reading, and showed no perceptible Type II reversal after 90 minutes.

The graphs illustrating the melting and annealing profiles are shown in Figures 37 and 38. Each experiment was carried out three times with identical results. The absorption curves of the DNA samples are shown in Figures 35 and 36.

Discussion

If the interpretation put on their results by Marmur and Doty, 1962, is correct, then the present results show that the intermolecular heterogeneity of Tetrahymena DNA is appreciably higher than that of bacterial DNA and that it is much more analagous to mammalian DNA in its complexity.

Some doubt has been cast on the assertion regarding strand separation during denaturation by light scattering studies (Cavaliere et al., 1961, and Peacocke and Walker, 1962). And certainly the evidence does not run all one way. For example, the ability to hybridise and anneal mammalian DNA on Agar columns when the molecular weight is sufficiently reduced (McCarthy, to be published) does not agree with Marmur and Doty's (1961) findings that annealing was more efficient at high molecular weights. But although some present evidence regarding strand separation and molecular weight reduction during melting is apparently contradictory it seems that the main weight of evidence argues in favour of strand separation. The phenomenon of a Type I reversibility on rapid cooling of all melted double stranded DNA establishes that even if the strands are not technically apart, the relationship between paired strands is sufficiently disordered to prevent the immediate resumption of the complementary double stranded form. Furthermore, the fact that under identical conditions, phage DNA anneals completely, bacterial DNA partially, and mammalian DNA not at all, suggests that intermolecular complexity is an important factor irrespective of the base composition of the DNA.

Every effort has been made during the DNA preparative methods to prevent reduction of molecular weight, but the molecular weight characteristics of the bacterial and tetrahymena DNAs used in the experiment are not known, and it is possible that the results are considerably affected by this factor.

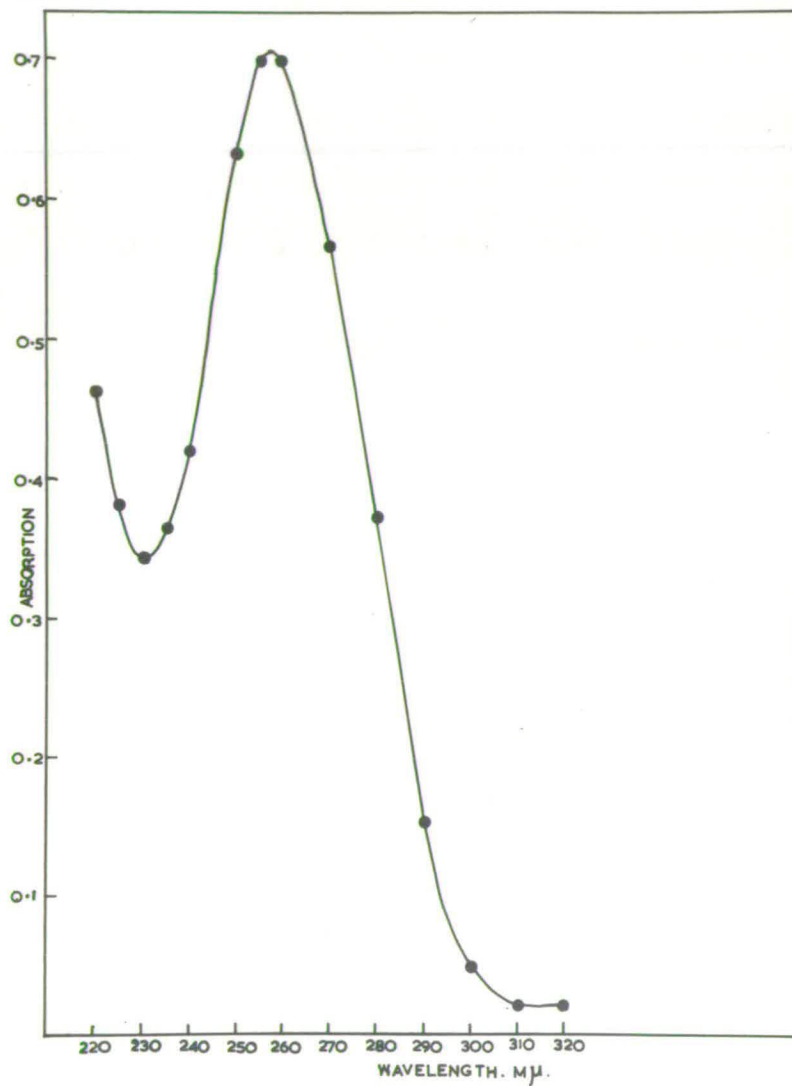


Figure 35. Absorption spectrum of DNA extracted from Bacillus megatherium and purified by the method of Marmur.

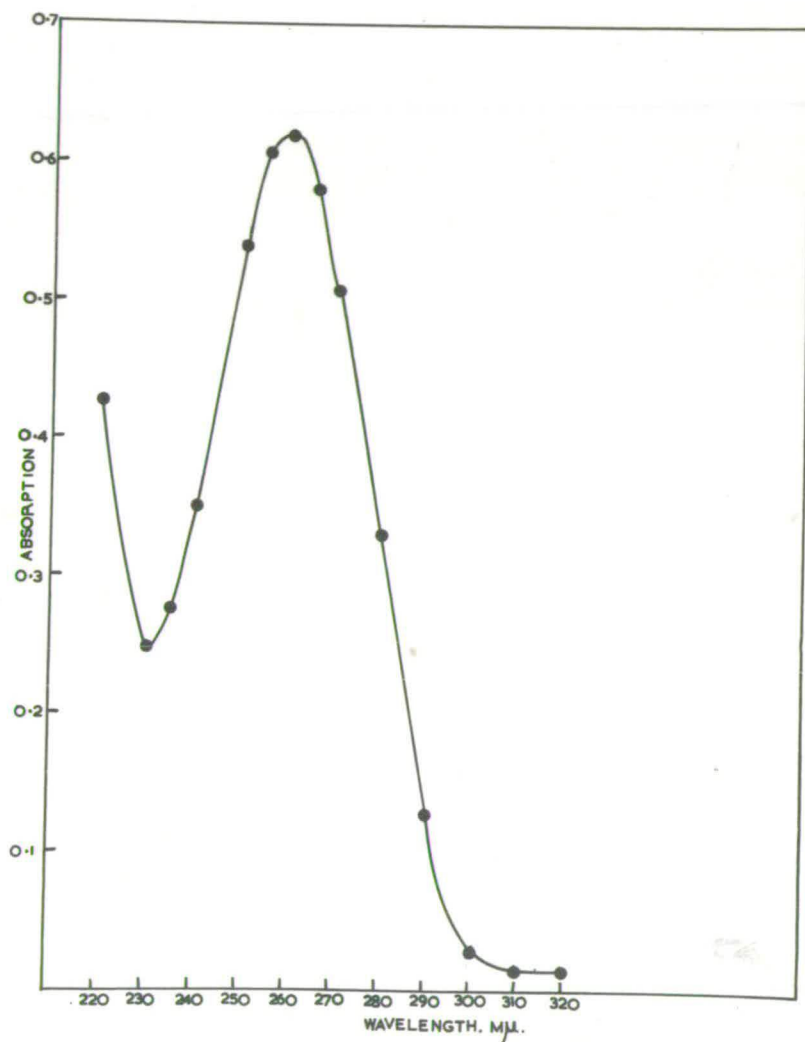


Figure 36. Absorption spectrum of DNA extracted from Tetrahymena pyriformis strain W, and purified by an expanded version of Marmur's method.

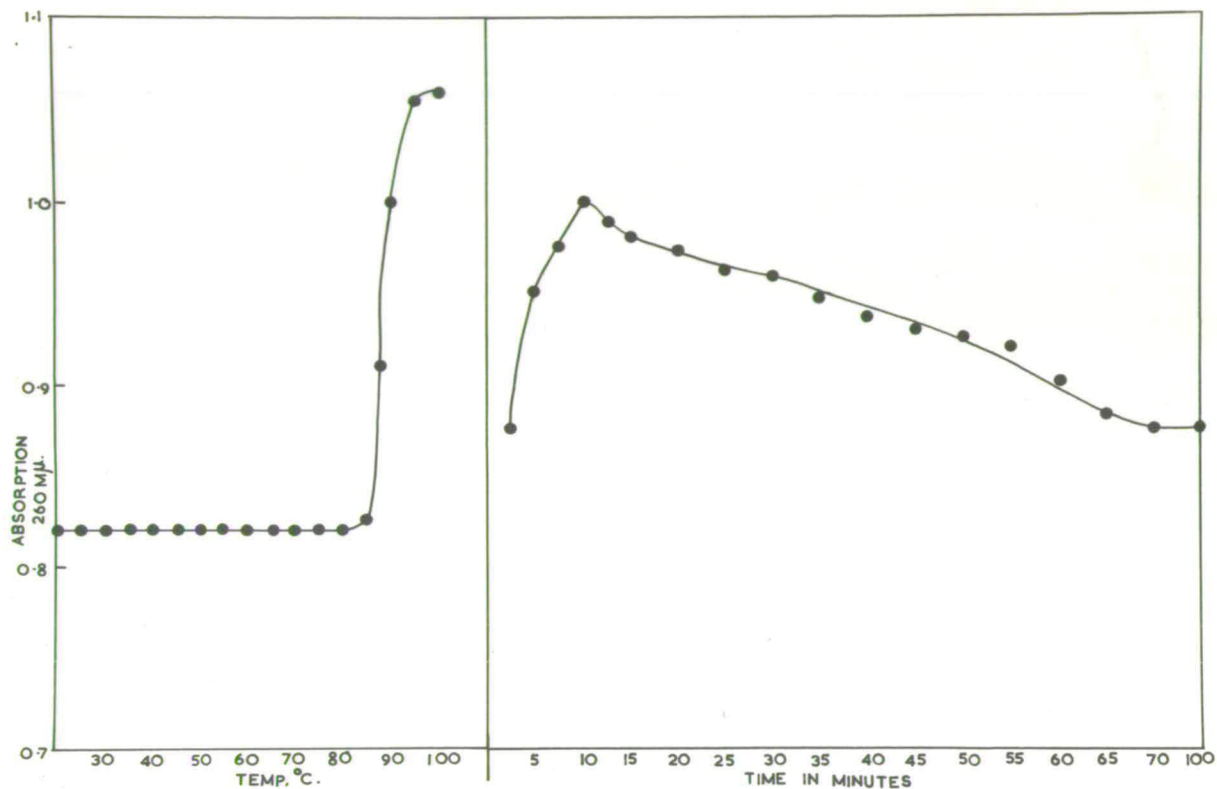


Figure 37. Melting and annealing of DNA from B. megatherium: the first part of the graph shows the absorption during heating from 20°C to 100°C. After heating, the sample was kept at 100°C for 10 minutes and then quenched in ice. The second part of the graph shows the absorption of the quenched sample on return to a preheated block at 67°C, readings taken at 2 minute intervals at first, later at 5 minute intervals.

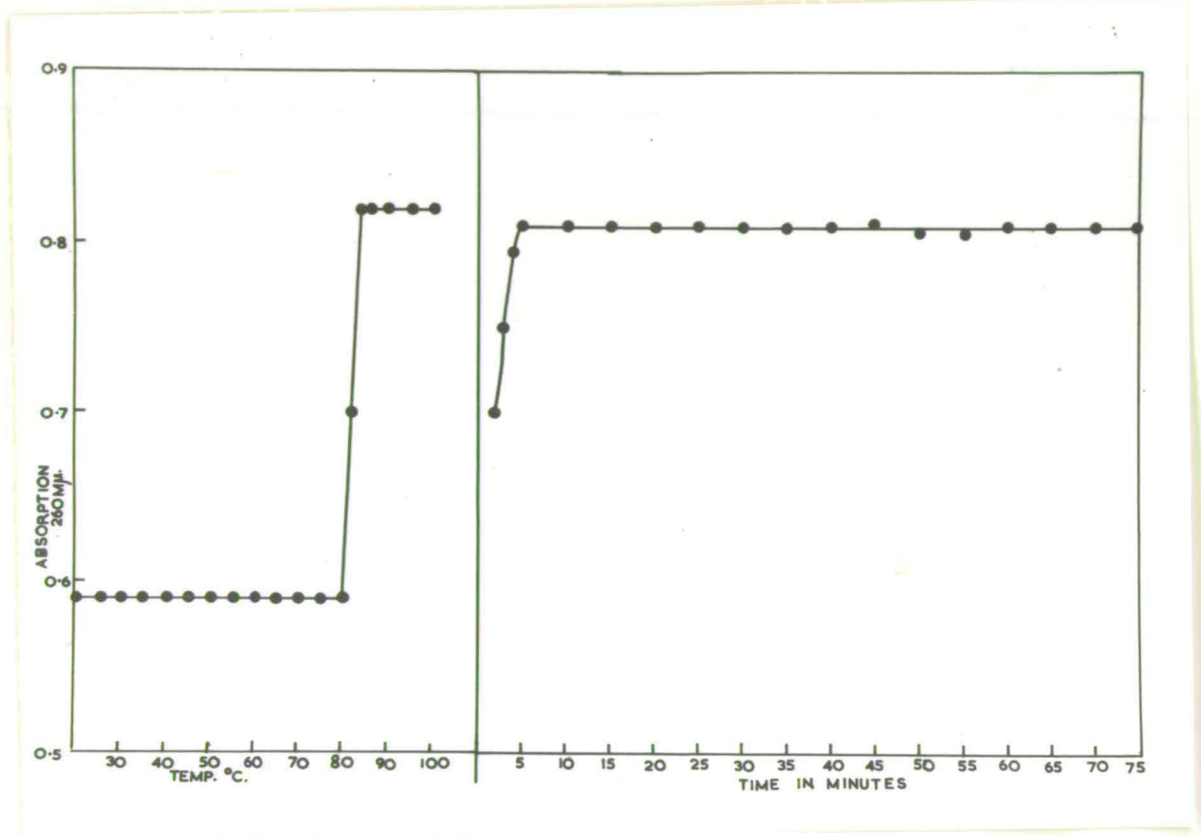


Figure 38. Melting and attempted annealing of DNA from Tetrahymena pyriformis. The first part of the graph shows the absorption during heating from 20°C to 100°C. After heating, the sample was kept at 100°C for 10 minutes and then quenched in ice. The second part of the graph shows the absorption of the quenched sample on return to a preheated block at 67°C, readings taken at 2 minute intervals at first, later at 5 minute interval N.B. A similar fraction was kept at 57°C for 2 hours and 60°C for another 3 hours after quenching, with no detectable decrease in absorption.

ABBREVIATIONS

DNA	-	Deoxyribonucleic acid
RNA	-	Ribonucleic acid
DNase	-	Deoxyribonuclease
RNase	-	Ribonuclease
C	-	Cytidylic acid
A	-	Adenylic acid
G	-	Guanylic acid
U	-	Uridylic acid
T	-	Thymidylic acid

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