

GENETIC AND BIOCHEMICAL STUDIES ON CYTOPLASMIC PARTICLES
IN PARAMECIUM

by

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SUMMARY

1. The mate-killer or mu particles of stock 540 of Paramecium aurelia have been isolated by a method involving fractionation of cell homogenates on a column of cellulose, followed by separation of the particles on gradients of ficoll. The aim of the studies of isolated particles was to attempt to determine their biological nature, and to try to gain an insight into the relationship of protozoa and particle.

2. Isolated mu particles were characterised in several ways. Electron microscopy showed that there was only a small amount of contamination (5% or less of dry weight), and that the main contaminants were cilia and their fragments, mitochondria and some other small debris. A.aerogenes, the Paramecium food bacterium, was very rare in the preparations, and tests by plating of suspensions of mu particles on nutrient agar and by using antiserum against the A.aerogenes showed that this contamination was indeed negligible.

3. Earlier work on the fine structure of mu particles was confirmed. Thin sections of mu particles showed a marked resemblance to those of bacteria, but there were differences, the most marked being the lack of a differentiated nuclear structure in the mu particle. Mu particles were also stained and observed in the light microscope, they were Gram negative, not acid-fast and did not possess a capsule or flagella.

4. Mu particles contained DNA, RNA, protein and probably carbohydrate and lipid. With all further studies, control preparations were made from animals not bearing mu particles but genotypically identical to stock 540 bearing mu. Such control preparations contained very little material (5% or less in dry weight terms), and this did not contribute significantly to the results.

5. On the basis of these and earlier studies, the mu particle might most closely be compared to a bacterium. This hypothesis was tested by an attempt to show the presence in the mu particle of two compounds found only in bacterial cell-walls, α - ϵ diaminopimelic acid (DAP) and muramic acid. It was clearly established that DAP was present in the mu particle, using paper chromatography and analysis in an amino-acid analyser. The results of tests for muramic acid were not so definite, but its presence was indicated. This result, combined with the results of electron microscopy shows that the mu particle is indeed a bacterium, an intracellular symbiont in the Paramecium.

6. The DNA present in the mu particle was isolated and characterised by two methods, density gradient centrifugation in CsCl and the determination of the temperature of melting (T_m), which is the midpoint of the increase in absorbancy that occurs when DNA solutions are heated to a temperature at which the two strands of the molecule separate. DNA from isolated macronuclei of Paramecium was similarly characterised. It was found that the DNA of the mu particle was very similar in these characters to the DNA of the macronucleus. Mu particle DNA had a density of 1.694 gm/cm^3 and a T_m of 82° against values of 1.693 gm/cm^3 and 81.8° for macronuclear DNA. This leads to an estimate of 31-32 mole % guanine + cytosine for both types of DNA. This value for macronuclear DNA is in agreement with earlier work. The mu particle DNA result is likely to be genuine, as no such DNA could be found in control preparations from sensitive cells, and treatment of isolated mu particles with deoxyribonuclease before lysis did not destroy the DNA, indicating that it is membrane protected, i.e. inside the particle.

7. Isolated mu particles were found to be capable of incorporating radioactive ATP, GTP or UTP into an acid-insoluble product by a reaction that possessed the properties of DNA-dependent RNA synthesis. For optimal activity the presence of ATP, CTP, GTP and UTP was required and there was an absolute dependence on Mg^{++} . The reaction was inhibited by Actinomycin D, known to inhibit DNA-dependent RNA synthesis, and a ribonuclease-sensitive product was synthesised. The ratio of incorporation of ATP to GTP was consistent with the synthesis of RNA complementary to DNA of the composition found for that of the mu particle.

8. In the light of these results, the nature and possible relationships of the mu particle are discussed, with particular reference to the possible mode of evolution of cell organelles, in particular the chloroplast and mitochondrion.

9. In an appendix, methods for the fractionation of P.aurelia homogenates are discussed and methods for the isolation of macronuclei, mitochondria and ribosomal monomers are described. These methods may be of value for future biochemical studies using P.aurelia.

INTRODUCTION

Several stocks of Paramecium aurelia have been observed to show the phenomenon of mate-killing. Conjugation of any of these stocks with a stock sensitive to mate-killing results in the death of the exconjugant animal deriving its cytoplasm from the sensitive stock, while the exconjugant deriving its cytoplasm from the mate-killer stock survives and grows normally. Crossing of two different mate-killer stocks results in mutual mate-killing, i.e. death of both exconjugants, but all mate-killer stocks are self-immune.

Beale (1957) showed that the mate-killer stock 540 (syngen 1) contained rod-shaped particles in the cytoplasm, visible in the phase-contrast microscope which were similar to the particles described by Siegel (1953) in three stocks of syngen 8 that he found to be mate-killers. Beale and Jurand (1966) demonstrated that the mate-killer stocks 548 and 551 (both syngen 1) also contained rod-shaped particles, and that the particles were distinct morphologically and in mate-killing ability. The presence of the particles is responsible for the mate-killing effect, for the particles may be lost in culture, or removed by various treatments, and mate-killing ability is then lost.

Other types of cytoplasmic particle have been described in P.aurelia. Of these the best known is the kappa particle (Sonneborn 1943, Preer 1948, Preer & Stark 1953, Sonneborn, 1959), but there are numerous others, for example lambda, sigma and nu (Sonneborn, Mueller & Schneller, 1959) and pi (Hanson 1954). Many of the other particles have been described in syngen 4, the other extensively studied syngen of P.aurelia. However, although these particles are distinct in various ways, and some of them are similar

in appearance to the mate-killer particles of syngen 1, none of them cause a mate-killing effect, appearing instead to produce a lethal agent which is exuded into the culture fluid and then assimilated by the sensitive cell. Stock 51 (syngen 4) bearing kappa particles, and stock 299 (syngen 4), bearing lambda particles, have been extensively studied by T.M. Sonneborn, J.R. Preer, and W.J. van Wagtendonk and collaborators.

The phylogenetic status of these particles has been the subject of much experiment and speculation over a period of many years. Sonneborn (1959), in an exhaustive review, concluded that the particles were endosymbiotes, probably bacterial in nature, reproducing independently of the host cell, though their presence or absence was determined by host genes, and of course they altered the relationship of the bearer animal to other paramecia. This view was reached on the basis of work carried out mainly on kappa of stock 51. Beale and Jurand (1960) studied the mu particle of stock 540 by cytochemical and electron microscopic methods, and their conclusions were generally in agreement with those reached from work on kappa of stock 51 (Dippell, 1958; Hamilton & Gettner 1958).

Further work with kappa of stock 51 and lambda of stock 299 has tended to confirm this view. Smith-Sonneborn and van Wagtendonk (1964), using kappa purified by passage through the ion-exchange resin ECTEOLA showed that purified preparations contained DNA, RNA, protein and possibly carbohydrate and phospholipid, while Smith-Sonneborn, Green and Marmur (1963) found a species of DNA of density 1.696 gm/cc in animals carrying kappa; this DNA was not present in sensitive animals. Behme (1964) found various specific DNA's in the particle-bearing stocks 138 (mu), 540 (mu), 239

(lambda) and 114 (sigma); these results were all obtained from crude lysates of animals centrifuged in caesium chloride gradients. The lambda particle of stock 299 was studied by van Wagtenonk and Tanguay (1963), also using purified preparations. The results showed the presence of DNA, RNA and protein, carbohydrate and phospholipid. Stock 299 bearing lambda has an advantage in that it will grow and maintain its particles in axenic medium, and van Wagtenonk, Clark and Godoy (1963) succeeded in culturing the lambda particles in a complex medium, after extracting them from axenically grown paramecia. The isolated particles retained the characteristics associated with intra-cellular lambda particles, and were capable of re-infecting stock 299 animals which had lost their lambda particles due to treatment with antibiotics.

The success of van Wagtenonk, Clark and Godoy (1963) in culturing the lambda particle outside the host cell is the most suggestive evidence for the bacterial nature of this particle, but they also found that the vitamin folic acid was not a requirement for the lambda particles in culture, nor for stock 299 when it bears lambda; however this vitamin is essential for stock 299 when it does not bear lambda (Soldo, 1963). Hence it seems that lambda can synthesise folic acid, not only for itself but for the host paramecium as well.

Some recent work on the kappa particle has tended to obscure the rather simple picture that has been built up. Preer and Stark (1953) showed that kappa of stock 51 was of two kinds - brights and non brights, so named on account of their appearance in the phase-contrast microscope. Bright particles contain a refractile or R body, and are responsible for

while non-bright particles do not cause the killing effect, the killing effect, but are the reproductive body. A similar situation was found in stock 7 (syngen 2) by Preer, Siegel and Stark (1953). Several workers have studied the R body by electron microscopy (Dippell, 1958; Hamilton and Gettner, 1958; Anderson, ^{Preer}Preer and Bray, 1964). Mueller (1962) found that the normally compact R body may change to a long filamentous form, accomplished by unwinding into a long twisted ribbon about 15 μ long (Anderson, et al. 1964). Preer, Hufnagel and Preer (1966) studied these unwound R bodies of kappa of stock 51 and stock 7. These are very singular structures and it is difficult to propose any hypothesis of their nature, but Preer et al. (1966) point out that they resemble the trichocysts of the flagellated protozoans Chilomonas and Cryptomonas. However, no known bacterial structures are able to undergo such extensive and reversible changes in form. The phylogenetic position of the kappa particle thus seems less clear than formerly, and other particles have not been investigated from the point of view of their phylogenetic status.

It was therefore decided to carry out an investigation of the mu particle of stock 540 to see if the hypothesis of bacterial symbiosis would be substantiated, and to find out something of the biochemical capabilities of the mu particle, if any, with the aim of trying to gain some insight into the relationship of protozoan and particle. The mu particle of stock 540 was chosen because it had not been studied in this way previously, but was still well-understood, having been the subject of much previous work concerned with its genetic control, and because it is clearly distinct in form and effect from any other particle, particularly the kappa and lambda particles.

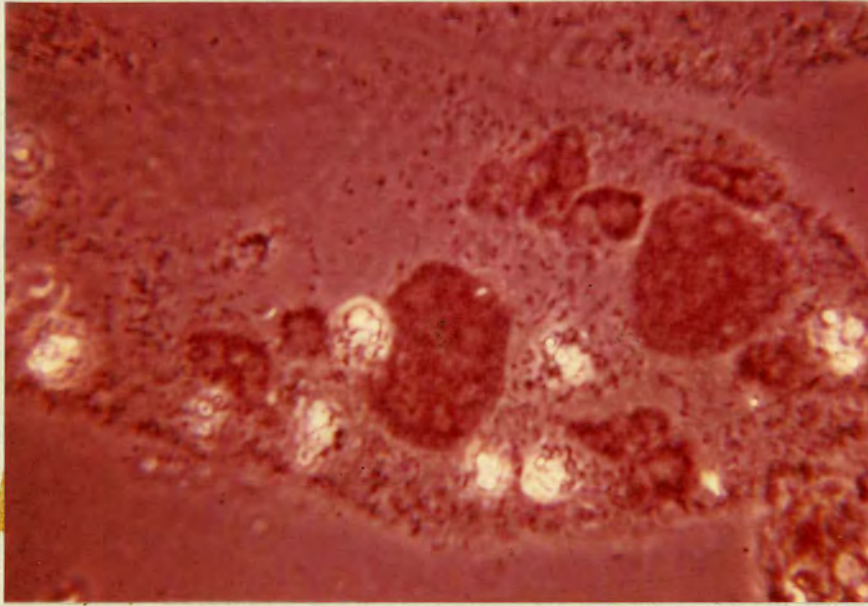


FIGURE 1

Photomicrograph of stock 540 animal bearing mu particles, prepared and stained as described in the text. Mu particles are evident throughout the cytoplasm. The animal is also in autogamy. Phase-contrast, X800.

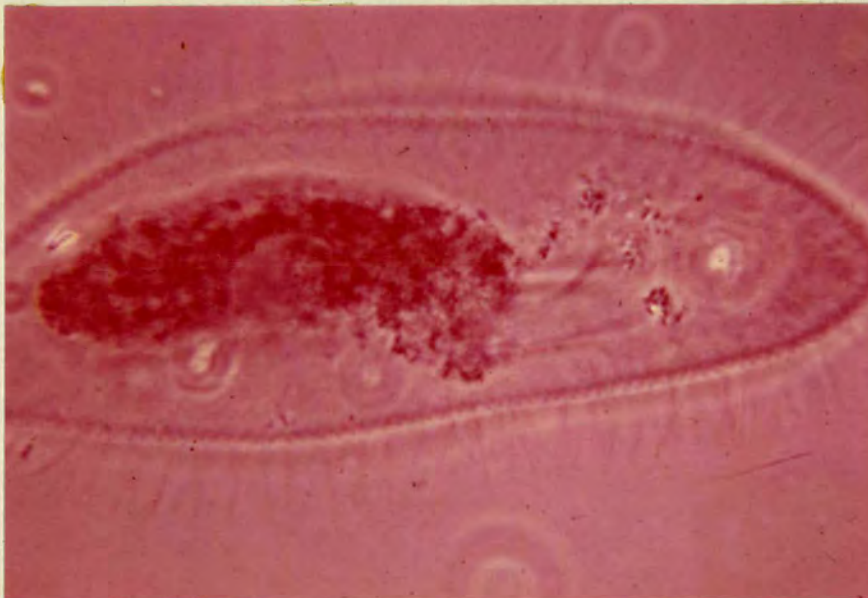


FIGURE 2

Photomicrograph of stock 540 animal not bearing mu particles, prepared and stained as described in the text. No mu particles are visible, but this animal has discharged many tridocysts. Some difficulty was experienced in obtaining consistent reproduction of the red colour of stained animals, as is evident in the photographs. Phase-contrast, X800.

MATERIALS AND METHODSStocks

The following stocks of P.aurelia were used in this study:-

Stock 540, bearing mu particles (mate-killer).

Stock 540, not bearing mu particles (sensitive). Derived from stock 540 bearing mu particles by prolonged culture at 31°.

The presence or absence of mu particles is the sole difference between the mate-killer and sensitive branches of stock 540. See figures 1 and 2. Genetically they are identical.

Stock 513 (a sensitive stock) was also used for a few control experiments.

(1) Maintenance of stocks

Stocks were maintained in bacterised lettuce extract (obtained by boiling 1.8 gms. dried lettuce in 1 litre of distilled water for 15 minutes, filtering, and autoclaving the filtrate at 15 psi for 15 minutes. After cooling, Aerobacter aerogenes was inoculated as food organism). A rough strain of A.aerogenes, strain A3(0), which is of stable serotype was used as food organism for the Paramecium. This was obtained from Dr. J. Wilkinson, Department of Bacteriology, University of Edinburgh. The A.aerogenes was kept on nutrient agar slants in the refrigerator, and subcultured at monthly intervals.

Animals were maintained at 18° in 1 litre batches of lettuce extract (pH 6.8, adjusted with $N/5$ NaOH if necessary), in 3-litre Thomson culture flasks. They were subcultured every 3 weeks. This method was found to be more reliable with regard to particle maintenance than the slide-culture method recommended by Sonneborn (1950), and also greatly reduced the labour involved in setting up mass cultures (50 or more litres). Lettuce extract was used as it was found to be most reliable for prolonged particle maintenance. Autogamy was induced every 3-6 months, using the methods described by Sonneborn (1950), and fresh maintenance cultures were set up from the ex-autogamous clones.

Animals were checked for presence or absence of particles at subculture and autogamy. The method is described in section 4.

(2) Growth of mass cultures

Mass cultures were grown in bacterised grass medium. Dried grass was extracted with water (120 gms/litre) by boiling for 15 minutes. The extract was filtered through muslin and then cleared by centrifugation at 7000 g. for 5 minutes. It was then autoclaved at 15 psi for 15 minutes. Working strength medium was prepared by diluting 1 litre of concentrate to 20 litres with distilled water, adjusting the pH to 6.8 with Na₂HPO₄. 1 litre aliquots were dispensed to 3 litre Thomson flasks and the medium autoclaved at 15 psi for 15 minutes.

Seed cultures were prepared from maintenance cultures by adding 100 ml of maintenance culture aseptically to 1 litre portions of grass medium. After 3 days growth at 25°-26°, a mass culture was set up by dispensing 75 ml. portions of seed culture into bottles containing 1 litre of grass medium. Generally about 50 litres of mass culture were set up; this required 3-4 litres of seed culture. Mass cultures were harvested after 5 days growth at 25-26°. This method is modified from that of Jones (1965). Animals were checked for presence or absence of particles at all stages of growth of mass cultures, using the method given in section 4.

(3) Collection of organisms

Cultures were filtered through muslin to remove the large pieces of flocculent material produced by Paramecium in the course of its growth, and were then passed through an Alfa-Laval cream separator at a rate of 500-600 ml/^{min}. The concentrated paste of Paramecium was removed from the rotor after about 25 litres had been passed through, in order to avoid

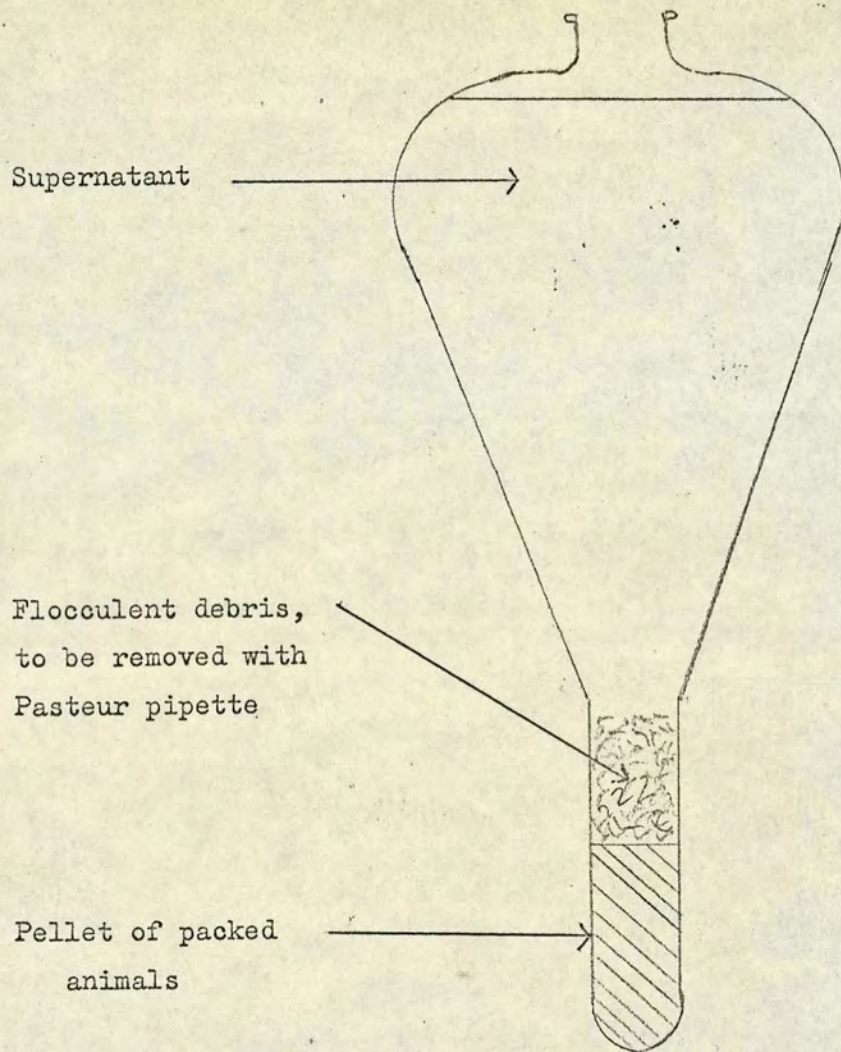


FIGURE 3. Appearance of oil-testing centrifuge tube after centrifugation of suspension of Paramecium.

W

death of the organisms. The rotor was washed out using the solution described by Dryl (1959), which is:-

0.002 M Na citrate
 0.001 M NaH_2PO_4
 0.001 M Na_2HPO_4
 0.0015 M CaCl_2

final pH 7.1

The CaCl_2 must be added last. This solution is a good non-nutritive solution which does not cause death of the animals. Many buffers and physiological solutions are lethal to paramecia.

The animals were centrifuged down at 500 g. for 1.5 minutes in an oil-testing centrifuge, using the pear shaped tubes type IP 75/57. The supernatant was poured away, and any further floccules of debris removed with a pipette. The sediment consisted of packed Paramecium (figure 3). The yield was usually about 1 ml. of packed cells from 4-6 litres of culture. The cells were washed twice with Dryl's solution in the oil-testing centrifuge.

(4) Observation of mu particles

Mu particles were observed in the animals using the method described by Beale and Jurand (1966). The results obtained with this method are far superior to those obtained with the original squash method (Beale, 1957) which was used early in the work. Mu particles were observed using a Zeiss phase contrast microscope at x 800 magnification. This microscope was also used to monitor the stages of preparation.

(5) Isolation of mu particles

Animals harvested and washed as described in section (3) were resuspended in 4 volumes of ice-cold Dryl's solution. They were homogenised using a "Tri-R" stirring motor (Tri-R Instruments, Jamaica, New York, U.S.A.), using 20-30 strokes of a teflon pestle at about 3500 rpm. Homogenisation was checked under a binocular microscope; it was considered complete when no whole animals or large fragments were left. The homogenate was then centrifuged at 35000 g. for 7 minutes (MSE HS18 centrifuge, MSE Ltd., London) and the pellet was resuspended in 40 ml of 0.01 M Na phosphate buffer of pH 6.8. The supernatant was discarded.

Body and cell-wall fragments, trichocysts and many cilia were removed by passing the resuspended pellet through a column of cellulose (Preer, Hufnagel and Preer, 1966). During the preparation and centrifugation of the homogenate, 15-20 gms. of cellulose (Whatman cellulose powder CF 11) were stirred into 200 ml. of 0.01 M Na phosphate pH 6.8 and then poured into a 40 cm. long x 2.5 cm. chromatography column. The column of cellulose was washed with 200 ml. of the phosphate buffer. After draining the washed column to bed level the resuspended 35000 g. pellet was rapidly applied using positive air pressure, then fresh buffer was added above the cellulose, and the column was washed with further buffer, using a peristaltic pump to speed the flow. The eluate was collected until it was no longer visibly turbid. On examination under the phase-contrast microscope the eluate was seen to contain mitochondria (generally rounded and vesiculate), a small proportion of the cilia, a few A.aerogenes and also the mu particles. Up to 5×10^8 mu particles per ml. were present in the eluate, with a similar or rather larger number of mitochondria.

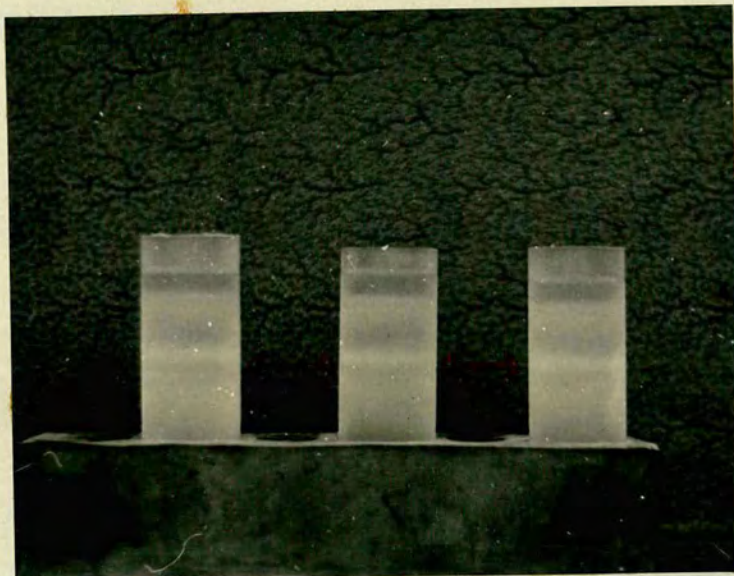


FIGURE 4

Result of ficoll density gradient centrifugation of cellulose column eluate of a homogenate of stock 540 animals bearing mu particles. The band indicated in the photograph consists of mu particles.

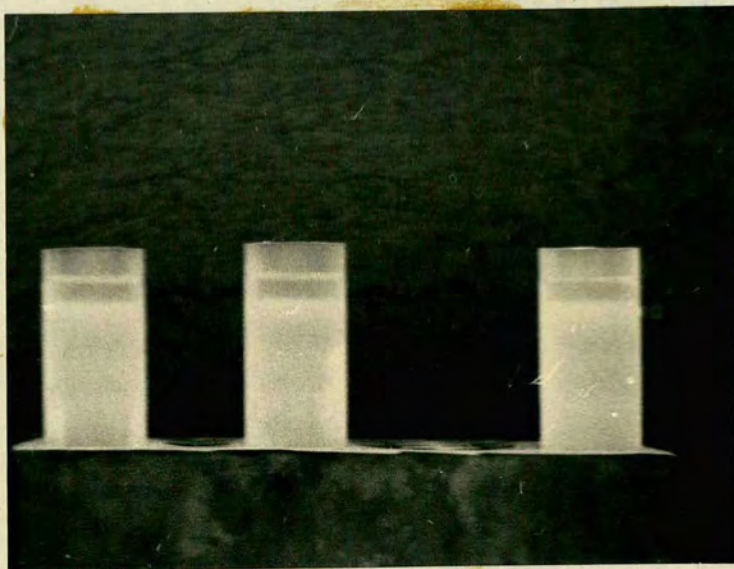


FIGURE 5

Result of ficoll density gradient centrifugation of cellulose column eluate of a homogenate of stock 540 animals not bearing mu particles. No band of the kind shown in figure 4 is evident.

The eluate was centrifuged at 22000 g. for 15 minutes and the pellet was resuspended in 9 ml. of 0.01 M phosphate, pH 6.8. Mu particles were now purified by centrifugation through a gradient of FICOLL (Pharmacia Ltd., London), a high molecular weight polymer of sucrose.

Gradients were prepared by layering 10%, 20%, 30% and 40% solutions of ficoll (in phosphate buffer) into thin walled 40 ml. polythene centrifuge tubes 18-24 hours before use. Gradients were 32 ml., and 8 ml. of each concentration was used. They were stood at 4° before use. Owing to the high viscosity of concentrated ficoll solutions such a gradient is probably discontinuous. 3 ml. of the resuspended pellet was layered over each gradient (normally 3 were run), the gradients were loaded into the 3 x 40 ml. swingout rotor of the MSE "Superspeed 50" ultracentrifuge, and spun at 10000 rpm (10500 g. average) for 45 minutes. After centrifugation the mu particles could be seen as a distinct band in the gradient (figures 4 and 5). This band was only present in preparations from stock 540 mate-killers (figure 4); preparations from stock 540 sensitives or (stock 513 did not contain this band (figure 5).

Generally, gradients were fractionated using a 5 ml. syringe and blunt-tipped 18G needle 2 inches long. ^{Six} 6 fractions of 3 ml. each were removed. Although this method is rather crude, it has several advantages in that it is quick, economical of centrifuge tubes and, with practice, reasonably accurate fractions can be obtained. Occasionally gradients were fractionated using a specially designed tube piercer (MSE Ltd.) and displacing the gradient upwards by pumping 60% sucrose solution in at the bottom. Fractions were then collected with a drop-counting fraction collector.

The gradient fractions were then examined at 800 x under phase-contrast. Mu particles, if present, were very largely in fraction 4 from the top, taking 3 ml. fractions. Mu particles can be easily identified in the phase-contrast microscope. The fractions bearing mu particles were centrifuged at 38000 g. for 10 minutes, the high RCF being necessary to separate the mu particles from the viscous ficoll (about 20% ficoll solution) at this level of the gradient. The pellet of isolated mu particles was washed once with 0.01 M phosphate pH 6.8, followed by centrifugation at 20000 g. for 10 minutes. Finally the pellet was washed with distilled water or a buffer appropriate to the experiment for which the mu particles were to be used.

With the exception of the cellulose column step, which was carried out at room temperature, all steps in the isolation procedure were at 0-4°.

(6) Staining of isolated mu particles

Heat-fixed smears of mu particles were prepared and stained by various standard techniques. These were the Gram stain, acid fast stain, Leifson's flagella stain, and negative staining with nigrosin. Simple staining with crystal violet ~~and~~^{or} carbol fuchsin was also done sometimes.

(7) Examination of mu particles in the electron microscope

Mu particles were fixed and prepared for electron microscopy by the methods given by Kellenberger, Ryter and Séchaud (1958). Mu particles were fixed in veronal-acetate buffered O_3O_4 and $CaCl_2$ to preserve nuclear material, as recommended by these authors. They were embedded in 2% agar,

washed in the veronal-acetate buffer and uranyl acetate, dehydrated through ethyl alcohol, and embedded in araldite. Sections were cut at 500-800 Å with a Porter-Blum Servall microtome. Sections were stained with lead citrate (Reynolds, 1963) + 2% uranyl acetate, and then examined in either a Philips EM 75 or an AEI EM 6 electron microscope.

The electron microscopy was carried out by Mr. R. Sinden.

(8) Chemical techniques for estimation of composition of isolated mu particles

Preparations were tested for the presence of various constituents of cells. DNA was estimated by the diphenylamine reaction as modified by Burton (1956). RNA was estimated by the orcinol reaction (Ogur and Rosen, 1950) and protein by the method of Lowry, Rosebrough, Farr and Randall (1951). Carbohydrate was estimated by the anthrone reaction (~~Fairbairn~~, ^{Trevelyan and Hamison} 1953) and lipid by the silver dichromate method (Bloor, 1947). Assays were carried out using lyophilised mu particles or control preparations.

(9) Hydrolysis and amino-acid composition determination

Isolated mu particles on control sensitive preparations were lyophilised and then 2-3 mg. dry weight of mu (or an entire control preparation) was mixed with 1 ml. of 5 N HCl in a soda-glass tube, and the tube was evacuated, sealed, and then treated for 12-18 hours at 104°. The tube was opened, any debris was centrifuged off at 5,000 g. for 5 minutes, and the supernatant was then dried in vacuo, using an infra-red lamp to speed the process. The residue was washed three times with water, and the final dry residue was dissolved in 0.05 ml. water (for paper chromatography) or in 0.8 ml. water (for analysis by the Technicon amino-acid analyser).

Two

2 dimensional paper chromatography was carried out on Whatmen 3 MM paper using descending phenol-NH₃ in an HCN atmosphere in the first dimension (Dent, 1948), followed by drying the paper at 50° for several hours, then washing out the phenol with ether, and re-drying. Butanol-acetic acid-water (4:1:5) was the second dimension solvent (Partridge, 1948) also descending. Chromatograms were dried as before and then dipped in 0.25% ninhydrin in 9:1 acetone:water (v/v) and treated at 100° for 15 minutes. They were then sprayed with 1% Cu(NO₃)₂ in ethanol, to render the spots permanent. The load of each chromatogram was equivalent to about 1.5 mg. dry wt. of ml, or half an entire control preparation.

For analysis in the amino-acid analyser, the hydrolysate was made 0.5N in HCl, 0.1 μM norleucine was added as an internal standard, and the final volume adjusted to 1 ml. The hydrolysate was kept at 100° for 30 minutes, and then applied to the "Technicon" amino-acid analyser. Elution was by a gradient of pH from pH 2.875 to pH 5.0, 0.02 to 0.6 M Na⁺ ion, as sodium citrate.

(10) Preparation of DNA

DNA, for use as a standard, was prepared from spray-dried Micrococcus lysodeikticus (Cambrian Chemical Co., London S.E. 16), from live A.aerogenes A3(0), and from isolated macronuclei of P.aurelia. Macronuclei were isolated as described in the appendix to this thesis.

To prepare DNA from M.lysodeikticus, 10 gms. of dried cells were suspended in 45 mls. cold 0.15 M NaCl⁻ - 0.015M sodium citrate (SSC) by stirring for 4 hours at 4°. 5 mls. of lysozyme solution (1 mg./ml.) (Sigma Chemical Co.) was added and the mixture incubated at 37° for 45 minutes. This produced a thick, viscous lysate. 3 mls. of 10% sodium lauryl sulphate (SLS) was added and the mixture was kept at 60° for 10

minutes. After cooling, 12 mls. of pronase solution (1 mg./ml. Calbiochem) was added and the digest was incubated at 37° for 18 hours. The pronase was self-digested at 37° for 2 hours before use. The digest was then extracted with an equal volume of 9:1 (v/v) chloroform: n-octanol for 45 minutes, followed by centrifugation at 15,000g. for 10 minutes to break the emulsion. The aqueous layer was removed with a broad-mouthed pipette and then the large interphase layer was removed and re-extracted with 50 mls. SSC for 45 minutes to extract DNA occluded into it. This extract was centrifuged at 10,000 g. for 10 minutes, and the supernatant was added to the earlier extract. Deproteinisation with chloroform: n-octanol and precipitation with ethanol, as described by Marmur (1961) was then repeated 8-10 times. Treatment with RNase (Koch-Light) was included after about 5 deproteinisations. The final preparation was dissolved in 50 mls. SSC, and stored over chloroform in the refrigerator.

DNA from A.aerogenes A3(0) was prepared from fresh cells. Cells were grown in the following medium:

	gm./litre final concentration
KH_2PO_4	6.8
$(\text{NH}_4)_2\text{SO}_4$	2.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5×10^{-4}
+ NaOH to bring pH to 6.8 if necessary	
+ 0.1% w/v glucose added aseptically after separate autoclaving.	

100 ml. of this medium was made up and inoculated with bacteria from a slant. After overnight growth at 31° with aeration a cell suspension with OD 650 m μ

of about 1.3 was produced. This was used as the inoculum for a further 1500 mls. of medium, and the cells were grown with aeration till an OD 650 μ of 1.0 was produced. They were harvested by centrifugation at 8000 g. for 10 minutes at 3°. The pellet was washed once with 250 ml. 0.15 M NaCl - 0.1M EDTA pH 8.0, and then centrifuged again, at the same speed for the same time. The pellet was resuspended in 25 ml. of 0.15 M NaCl - 0.1 M EDTA and 2 ml. of 25% SLS was added. This caused lysis. The lysate was kept at 65° for 15 minutes, cooled and then extracted with 9:1 chloroform: n-octanol, following again the method of Marmur (1961). About 8 deproteinisations with chloroform: n-octanol were carried out; the RNase treatment was given after 4 or 5 deproteinisations. The final preparation was dissolved in 10 ml. SSC, and stored over chloroform in the refrigerator.

DNA was prepared from isolated macronuclei as follows:

the macronuclei were suspended in SSC, to a volume equal to that of the original packed cells from which they were prepared, and 10% SLS was added to a final concentration of 0.1%. The lysate was treated at 65° for 20 minutes, allowed to cool, and then pronase was added to 1 mg./ml. After digestion for 18 hours at 37°, the extract was deproteinised twice with 9:1 chloroform: n-octanol as above, treated with RNase, and then put onto a CsCl gradient.

Purification of DNA from μ particles by pronase followed by chloroform-n-octanol deproteinisations proved to be impracticable, and it was found better to suspend the isolated μ particles (or control preparation from sensitive cells) in 1.5 ml. of 4 x SSC (0.6 M NaCl,

0.06 M Na citrate) and freeze at -20° overnight. After thawing, 0.5 ml. of 10% SLS was added, followed by treatment at 65° for 20 minutes. After cooling, the volume was made up to 3 ml. with 4 x SSC, and this lysate was used for preparative caesium chloride density gradient centrifugation.

Caesium chloride density gradient centrifugation was used to separate and analyse DNA preparations. Analar CsCl was first repurified as follows:- 100 gms. CsCl were dissolved in 80 ml. distilled water, and the solution was filtered through a P3 sinter. 400 ml. ethanol was added, causing precipitation of the CsCl. The solution was stirred at 4° for 2 hours, the CsCl was filtered off and washed with ethanol and ether on the filter. It was dried in vacuo overnight, and then kept at 200° for 48 hours. If there was any charring, the purification was repeated. If not, the material was reduced to a powder, and stored in an air-tight bottle. By this treatment, the absorption at 260 m μ of a solution of CsCl at the strength used in density gradients is reduced to less than 0.05, and is usually less than 0.02.

To run gradients, 3.40 gms. of CsCl were dissolved in 2.5 ml. of the DNA solution or lysate in a small beaker, with gentle shaking. The solution was then poured into a 5 ml. thin-walled polypropylene centrifuge tubes and 1 ml. of liquid paraffin was carefully overlaid. The tubes were loaded into the 3 x 5 ml. swing-out rotor of the MSE "Superspeed 50" centrifuge, and centrifuged at 32000 rpm (100000 g. average) for 65-75 hours at 18° - 20° . The rotor was stopped without brake, and the tubes were carefully removed. Gradients were fractionated by collecting drops, using the MSE tube-piercer. With different gradients, various sizes of fraction were taken, varying from 4 to 8 drops. Of course, for any one gradient,

fractions taken were all the same size. 0.8 or 1.0 ml. SSC was added to each fraction, and the absorption at 260 m μ was read, using 0.5 cm. light-path cells, in a Beckman DB spectrophotometer. Sometimes, gradient fractions were assayed for DNA content, using the diphenylamine method as modified by Burton (1956). The material that was run in a gradient was always assayed for DNA by this method, so that the quantity of DNA on the gradient was known, 300 μ g was about the maximum which could be run on one gradient of the size used here.

Since CsCl gradients were a preparative step for both macronuclear and mu particle DNA, these DNA's were on occasion rebanded. DNA-containing peak fractions were pooled and dialysed for 24 hours against 3 changes of SSC. If it was necessary to reduce the volume of such a solution after dialysis, it was then dialysed against 50% ficoll in SSC. Such material was then subjected to a second CsCl gradient. Quite frequently, however, a second CsCl gradient was not necessary, the first gradient yielding satisfactory material.

(11) Determination of the temperature of melting of DNA

When DNA solutions are heated, a sharp increase in the extinction coefficient occurs at the point at which the native double-stranded form changes to the denatured single-stranded form due to breakage of the inter-chain bands. This increase takes place over a range of several degrees and the mid-point of this rise is known as the temperature of melting, or T_m . For a given concentration of Na^+ ion, T_m is dependent on the percentage of guanine + cytosine in the DNA. Hence by heating the DNA through this thermal transition, an idea of both extent of double-strandedness, and of the composition, may be obtained. Marmur and Doty (1959; 1962) have made

extensive use of this method, and have demonstrated the relationships mentioned above.

The T_m 's of both macronuclear and mu particle DNA were determined. The method used was very similar to that of Marmur and Doty (1962), but a Beckman DB spectrophotometer was used. Temperatures above 92° could be attained, as heat losses were too great at temperatures above this, because only water was used as the circulating heating fluid.

(12) Assay for RNA synthesis

Isolated mu particles and control preparations were assayed for DNA-dependent RNA synthesis using the assay mixture of Weiss (1960), measuring the incorporation of labelled ribonucleoside triphosphate into acid-insoluble material. The triphosphates used were either (8- C^{14})ATP or (4- C^{14})UTP, (both obtained from the Radiochemical Centre, Amersham, Bucks, England) or GTP- H^3 (obtained from Schwarz Bioresearch Inc., Orangeburg, New York, U.S.A.). 0.25 μ C of C^{14} triphosphate on 1.0 μ C of H^3 triphosphate was used in each assay. Various additions to and omission from the assay mixture were made, and these are detailed in the results. Mu particles lysed by freezing and thawing, or a similarly treated control preparation, were assayed. Mu particle assays contained 0.4-0.6 mg. of protein, measured by the method of Lowry et al (1951); control assays, using material from sensitive cells, of course contained far less. Incubation was usually 15 or 20 minutes at 30° , then ice-cold 1M trichloroacetic acid (TCA) was added to a final concentration of 0.6M, and assay tubes were stored at 4° overnight. The precipitates were filtered off through 'Oxoid' 2 cm membrane filters, and the filters were washed 10 times with 5% TCA, twice with ethanol, once with 1:1 ethanol:ether, and

twice with ether. Filters were dried in a current of warm air, and placed in scintillation vials containing 15 ml. of 0.5% PPO-0.03% dimethyl POPOP in toluene. (PPO = 2, 5 diphenyloxazole, and dimethyl POPOP = 2-p-phenylenebis [4-methyl, 5-phenyloxazole]). Samples were counted in a Nuclear-Chicago 6850 liquid scintillation spectrometer. Efficiency was 45-50% for C^{14} and about 3% for H_3 . It was found that up to 0.8 mg. of protein could be used in any one sample before self-absorption by the material on the filter caused any noticeable reduction of counting efficiency.

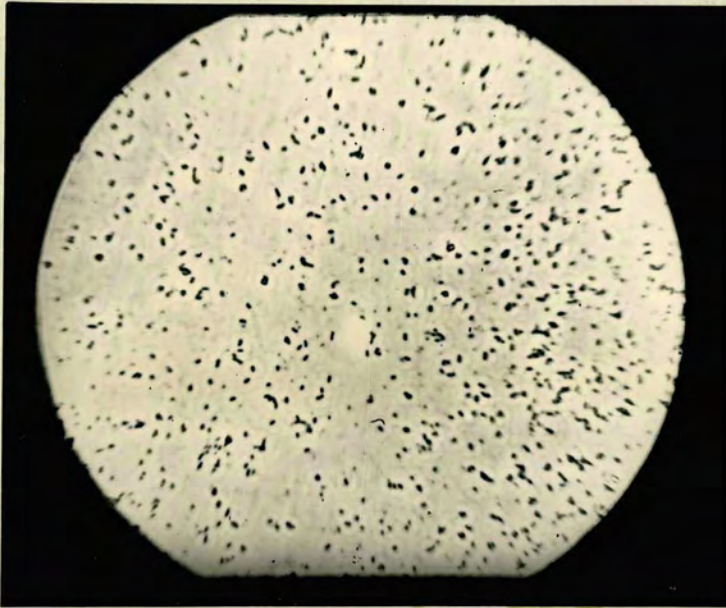


FIGURE 6

Low-power photomicrograph of a preparation of mu particles, showing the predominance of rod-shaped particles. Heat-fixed, phase-contrast, X640.

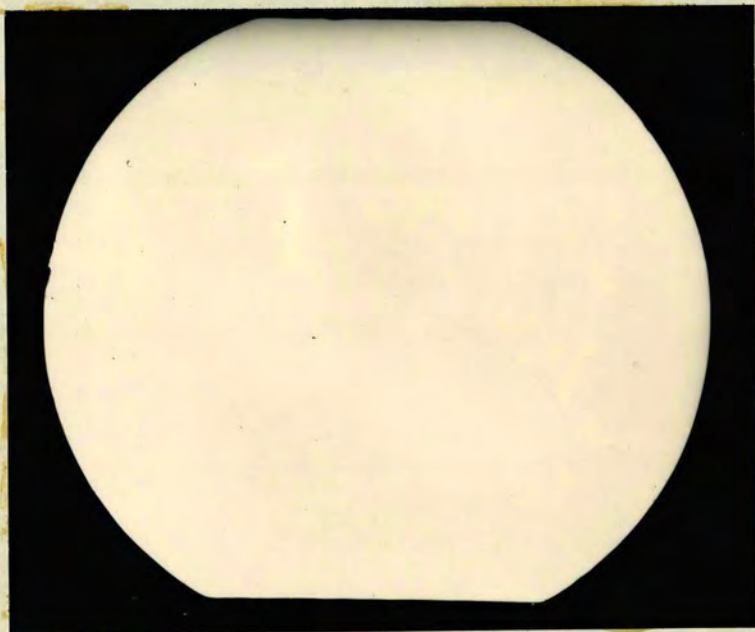


FIGURE 7

Low-power photomicrograph of a preparation derived from an animal not bearing mu particles (a control preparation). No particles are evident. Heat-fixed, phase-contrast, X640.

RESULTS(1) General observations on isolated mu particles

The preparations obtained by following the isolation procedure for mu particles are found to consist of 90% or more rod-shaped particles of size 2 to 5 μ long x 1 μ across when stock 54.0 bearing mu is used. Such particles are not present in preparations obtained from stocks not bearing mu particles. Figure 6 is a photograph of a heat-fixed smear of a preparation from stock 54.0 bearing mu; figure 7 is a similar photograph of a preparation from stock 54.0 not bearing mu. Figure 6 shows the predominance of rod shaped particles in preparations from stock 54.0, though the particles have rather shrunk after heat-fixation. Figure 7 shows how little contaminating material is evident in the phase-contrast microscope. The difference between the preparations is very evident.

Electron microscopy of isolated mu particles showed that the principal contaminants were cilia and fragments of cilia, mitochondria, and various small debris (figure 8). The appearance of the isolated mu particle in the electron microscope compares well with the appearance found by Beale and Jurand (1960; 1966) who observed the mu particles in sectioned whole animals. As these authors found, there is a conspicuous outer double membrane and undifferentiated internal contents (Figures 8 and 9). The capsule reported to surround the mu particle by Beale and Jurand (1960) was not observed in the EM pictures of isolated particles, and neither was there evidence of its presence using nigrosin staining and observation with the light microscope. Beale and Jurand's 1966 paper gives no evidence for the presence of a capsule surrounding the mu particle so it may be that the presence of this cell constituent is variable, or that

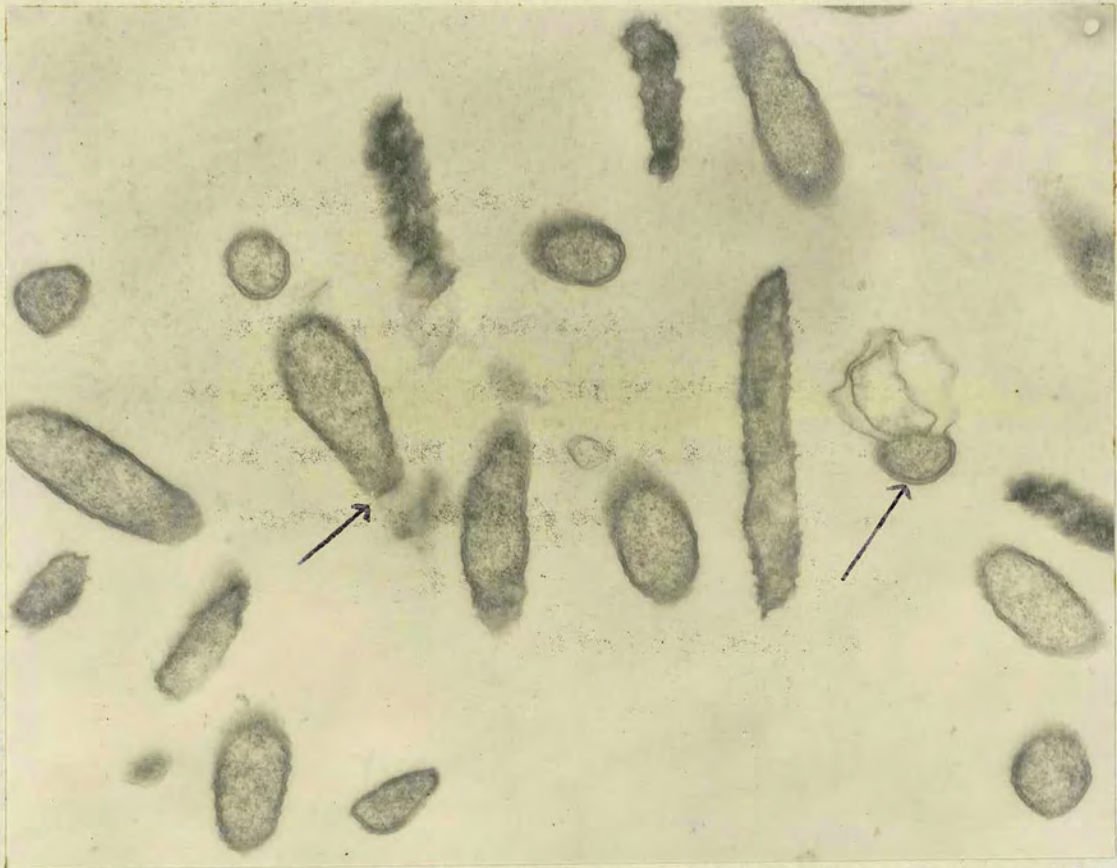


FIGURE 8

Low-power electron micrograph of a preparation of mu particles, showing particles in both longitudinal and cross-section. Some lysis is occurring (arrows) but it is evident that many particles have retained their integrity. Philips EM 75, X7500.

the mu particle has now lost it, or that it was an artefact in the original preparations.

Figure 9 shows a mu particle that has a median constriction. Beale and Jurand (1960) also found mu particles in this form, and interpreted them as being in the course of division. This seems quite likely to be so.

It is also evident from the electron micrographs that some lysis is occurring (figure 8). This may be due to fixation and preparation for observation in the electron microscope, or it may be occurring in the preparation itself, as isolated mu particles eventually lysed irrespective of the media in which they were stored. Isotonic buffers gave somewhat increased protection, but no controlled studies were made. Frozen preparations could be stored for several weeks. There was little lysis after one thawing, but repeated freezing and thawing caused an increasing amount of lysis of the mu particles.

Figure 10 is a higher power electron micrograph of an isolated mu particle. This shows the outer double membrane clearly, but does not show any internal detail. In particular, there is no differentiated nuclear structure, as is observed in certain bacteria, no zone of greater electron density being observed inside the particle. Granules of higher electron density are observed, but although these are of such a size that it is possible that they are ribosomes, the lack of definition makes it impossible to reach a definite conclusion.



FIGURE 9

Similar to figure 8, but shows dividing mu particle (arrowed), and some contaminating debris (labelled).

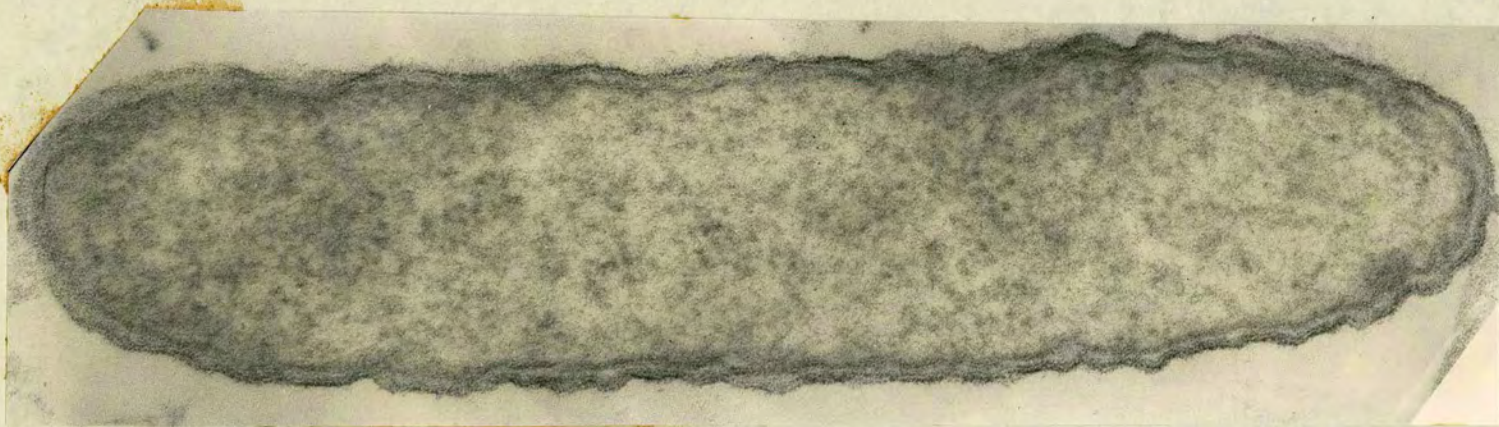


FIGURE 10

Higher power electron micrograph of a single mu particle. This shows the outer double membrane and the granular but undifferentiated internal contents. AET EM 6. X10,000.

Tests for contamination with residual Aerobacter aerogenes, the Paramecium food bacterium, were carried out by two methods, firstly by dilution of the mu particle suspension; and plating on nutrient agar plates, and secondly by treatment of the suspension with serum against the A.aerogenes. The strain of A.aerogenes used was of stable serotype. Table 1 gives the results of typical experiments on the viable counts of both mu and control preparations; the results show the approximate upper and lower limits of contamination with viable A.aerogenes. Table 2 gives the results of agglutination tests on the mu particles, using a serum against the food A.aerogenes. It is evident that the mu is not all agglutinated by this serum, which had a titre of $1/640$ against A.aerogenes A3(0). This is the expected result if the contamination level of the preparations is as low as that found by dilution and plating of suspensions, and examination in the phase-contrast and electron microscopes. Therefore, suspensions are not grossly contaminated with A.aerogenes, and later experiments demonstrated the insignificance of this contamination in other ways (sections 5 and 6).

The actual amount of contaminating material of all kinds was found to vary from preparation to preparation, due principally to the variation in the number of mu particles present in each animal before isolation. P.aurelia stock 54.0 growing in the logarithmic phase contains comparatively few mu particles in each cell; numbers were not counted but were estimated at less than 100 per cell. During the growth of the paramecia the mu particles merely appear to keep pace with the animals, and it is

TABLE 1

CONTAMINATION OF MU AND CONTROL PREPARATIONS WITH VIABLE A. AEROGENES.

Haemocytometer count of no. of particles/ ml suspension	Dilution for plating	Mean no. of col- onies after 60 hrs at 35°.	Viable <u>A. aer-</u> <u>ogenes</u> as % of total particles
1×10^9 /ml	10^{-4}	130/ml	0.130
8×10^8 /ml	10^{-4}	165/ml	0.206
1.5×10^9 /ml	10^{-4}	610/ml	0.406
5×10^7 /ml	10^{-4}	1310/ml	26.2

The left-hand column is a total count of particles of all kinds in a preparation; i.e. mu particles, mitochondria, A. aerogenes and others. Each preparation was made up to 10 ml before the haemocytometer count and all preparations were made from animals that had been grown in 50 litres of medium.

The three upper lines refer to experiments with animals of stock 540 bearing mu particles, while the lower line is a control experiment using stock 513 sensitive animals.

Suspensions were diluted serially to 10^{-4} , and 0.1 ml was plated in triplicate to nutrient agar plates, and the mean number of colonies after 60 hours incubation at 35° taken. Shorter incubation periods did not show the true extent of the contamination.

TABLE 2

CONTAMINATION OF MU PREPARATIONS WITH A. AEROGENES A3(O)

Agglutination of preparations with anti-A3(O) serum

Preparation	Dilution of serum	Agglutination of suspension
Mu particles	1/40	-----
Mu particles	1/80	-----
Mu particles	1/160	-----
Mu particles	no serum	-----
<u>A. aerogenes</u> A3(O)	1/40	+++
<u>A. aerogenes</u> A3(O)	1/80	+++
<u>A. aerogenes</u> A3(O)	1/160	++
<u>A. aerogenes</u> A 3(O)	no serum	-----
No bacteria	1/40	-----

The A. aerogenes were grown up overnight in nutrient broth medium at 37° centrifuged and washed twice with distilled water. The pellet was taken up in Dryl's solution and this suspension was adjusted to an optical density of 1.3 at 650 m μ .

Mu suspension, prepared and washed by the methods described, was adjusted to a similar optical density, also in Dryl's solution.

0.2 ml suspension was mixed with 0.2 ml diluted serum (using Dryl's solution as diluent) in agglutination tubes, and incubated overnight at 37°. Agglutination was then scored on a scale up to 3 +. For the experiments here, the serum had atitre of 1/640.

only after the attainment of the stationary growth phase that the particles begin to proliferate, clearly continuing to grow while the Paramecium does not. After a few days of starvation, each animal may contain 10^4 or 80 particles. In these experiments, animals were grown for 5 days at 25° , and under these conditions the time taken to reach stationary phase was about $3\frac{1}{2}$ days, giving $1\frac{1}{2}$ days starvation. Generally, this resulted in 10^3 or more particles per animal. However, there was considerable variation in this, due to the uncontrolled growth rate, and other factors, such as the time since the animals had been through autogamy. Longer periods of starvation were not satisfactory, for while the number of particles in each animal increased, the number of animals decreased, due to death of the animals in the decline phase of growth. Hence the growth conditions were a rather unsatisfactory compromise between, on the one hand, an attempt to obtain animals containing the maximum number of particles each, and on the other hand, an attempt to obtain the maximum number of animals. As a result, there was rather marked variation between preparations, but generally it was found that, provided there was a yield of animals greater than 4 to 5 ml. of packed cells containing 10^3 particles/cell or more, then a preparation of mu particles containing at least 90% particles could be expected. It was also found that the more contaminated preparations were with cell debris, the poorer the results they gave in other work.

The method of homogenisation of the animals also had some bearing on the purity and yield of mu particles obtained. A teflon pestle type of homogeniser was found to give the most reliable results, other methods such as a Waring blender and a top-drive macerator gave poorer

results, mainly due to greater breakage of cilia, resulting in high contamination with fragments of cilia.

(2) Chemical composition of isolated particles.

Analyses were carried out on the mu particle preparations for the presence of DNA, RNA, protein, carbohydrate and lipid, using the methods detailed in the materials and methods section. Lyophilised material was used. Analyses were also carried out on control preparations from animals not bearing mu particles.

Certain defects in the methods may be noted. The method of Lowry et al. (1951), used for the estimation of protein, in fact estimates the amounts of tryptophan and tyrosine in the material under test. If two proteins differ in tyrosine and tryptophan content then they will give a different result by this method. Certain compounds, for example tris buffer, uric acid or maleate, cause an intensification or reduction of the blue colour obtained in the reaction. It is clear that there is a marked error in this method when applied to both particles and control preparations, for the data in Table 3 imply a 100% protein content if they were genuine. This is a spurious result, because either there is a component present causing intensification of colour, or both mu and control preparations contain more tryptophan and tyrosine per mg. dry weight than the standard protein, bovine serum albumin, fraction V. The first is the more likely of these reasons, as the aminoacid composition data (section 4 of results) do not suggest abnormally high contents of these aminoacids. The carbohydrate estimation is also subject to error, as it is possible that not all the ficoll from the density gradient step of the isolation was washed out during subsequent

TABLE 3

CHEMICAL COMPOSITION OF ISOLATED MU PARTICLES AND OF ISOLATED CONTROL PREPARATIONS

Component Preparation	DNA	RNA	PROTEIN	CARBOHYDRATE	LIPID
Mu particles	51.4 (±9.1) [1.028]	96.0 (±13.8) [1.92]	1035 (±242) [20.70]	45.9 (±9.7) [0.918]	11.3 (±2.0) [0.226]
Control (540 sens)	11.8 (±6.1)	53.8 (±9.3)	1064 (±162)	57.5 (±3.7)	not tested

The upper set of figures for both mu and control preparations is the amount of each component in $\mu\text{g}/\text{mg}$ dry weight, as determined by the methods given. Thus mu particles contain 51.4 μg of DNA per mg dry weight, for example. The results for mu particles are the means of 6 experiments with separate preparations, and the results for control experiments are the means of 3 experiments with separate preparations.

The figures in round brackets are the standard errors of the results above them.

The figures in the square brackets are the approximate figures for the composition of 20 mg dry weight of mu particles, estimated from the values given above them. Thus 20 mg dry weight of mu particles contains 1.028 mg of DNA. Taking the figures for control preparations as given would give an estimate of the contribution of contaminating cell debris to atypical preparation of mu particles, which contained about 20 mg dry weight of mu particles.

centrifugations. As the anthrone method is highly sensitive, this could be a source of error.

Table 3 gives results on the composition of isolated mu particles and isolated control preparations. All results are expressed as μgms of component per mg. dry weight of preparation. The yield of mu preparations and control preparations was extremely different. The yield of mu particles in terms of dry weight was about 1 mg. from 20-25 mg. dry weight of cells, while control preparations gave a yield of about 1 mg. from 500 mg. of cells, again on a dry weight basis. This would suggest that, since the mu preparations contained about 20 times as much material as the control preparations, that the mu particles were about 95% pure, a level of purity substantially in agreement with that estimated by other methods.

The results in Table 3 have been expressed as $\mu\text{g}/\text{mg}$. dry weight because it was felt that on account of the variation between preparations discussed earlier, to present the data as amount of component per preparation was unsatisfactory. However, multiplying the figures given for mu particle preparations by 20 would give an approximation to the total amounts of each component present in a typical preparation of mu. The figures given for control preparations would then represent the contribution of the contaminating cell debris. Figures in square brackets in Table 3 represent the approximate composition of 20 mg. of isolated mu particles, and the contribution of contaminating cell debris may be compared with this by taking the figures for control preparations as given.

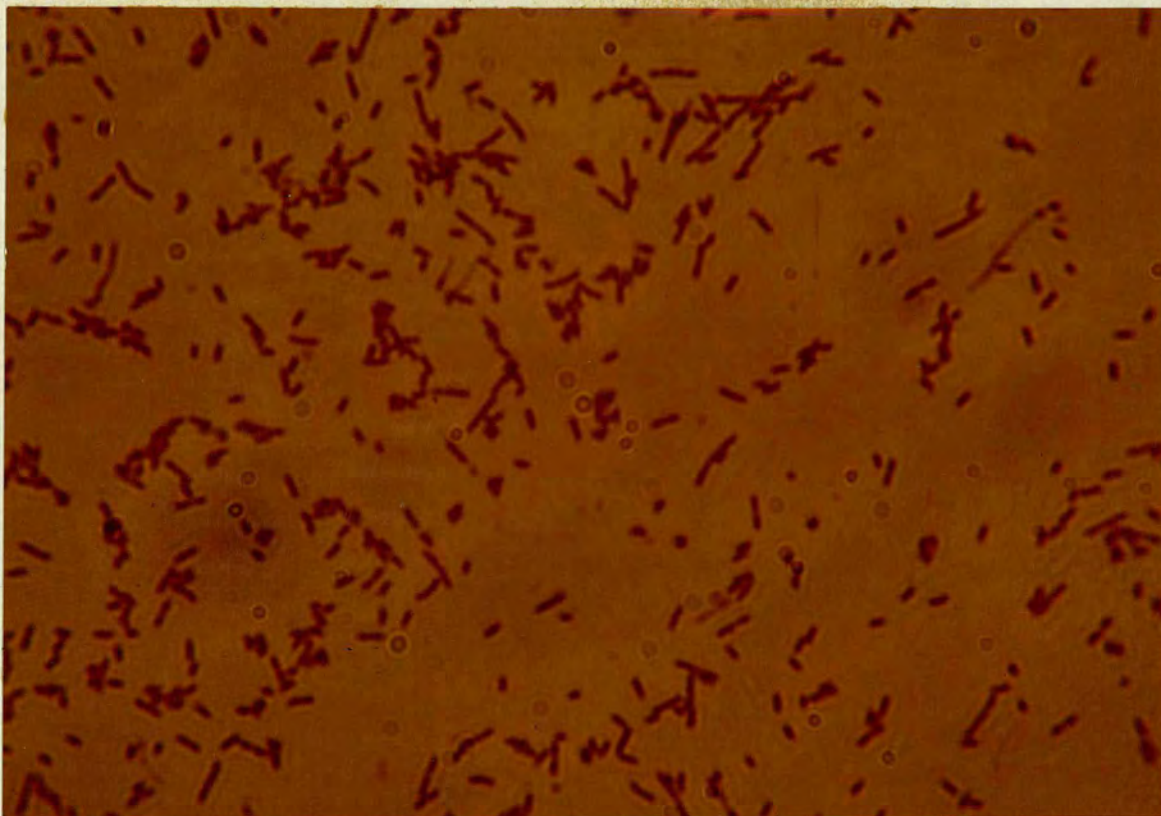


FIGURE 11

Photomicrograph of Gram-stained mu particles. A yellow filter was used, as this improves the clarity of the particles for photography, but it has darkened the red colour of the stained particles. X1600.

From the data in Table 3, it is evident that there are distinct differences between mu particles and control preparations, particularly in DNA and RNA content. Standard errors are given; in some cases these are rather high, but they reflect the variability from preparation to preparation discussed earlier; this is particularly so with the estimations of protein. In one case (DNA estimations on control preparations) the standard error is high because the estimations were at the limit of sensitivity of the method.

It may be concluded that the mu particle contains DNA, RNA, protein and probably carbohydrate and lipid. Later work will confirm this and present results pertaining to the DNA, RNA and protein.

(3) Staining reactions of isolated mu particles

Mu particles gave a negative Gram reaction (figure 11). This was consistent, no preparation tested giving a different result. The mu particle is not acid-fast, and the use of Leifson's flagella stain does not show the presence of flagella. This observation is of course confirmed by electron microscopy. As mentioned previously, nigrosin staining failed to reveal a capsule. Further staining reactions were not carried out as it was felt that little further useful information would be gained.

(4) Aminoacid constituents of mu particles

Earlier evidence (Sonneborn, 1959; Beale & Jurand, 1960) had suggested that the mu particle might be closely compared to a bacterium. It was decided to make a conclusive test of this hypothesis. Salton (1964)

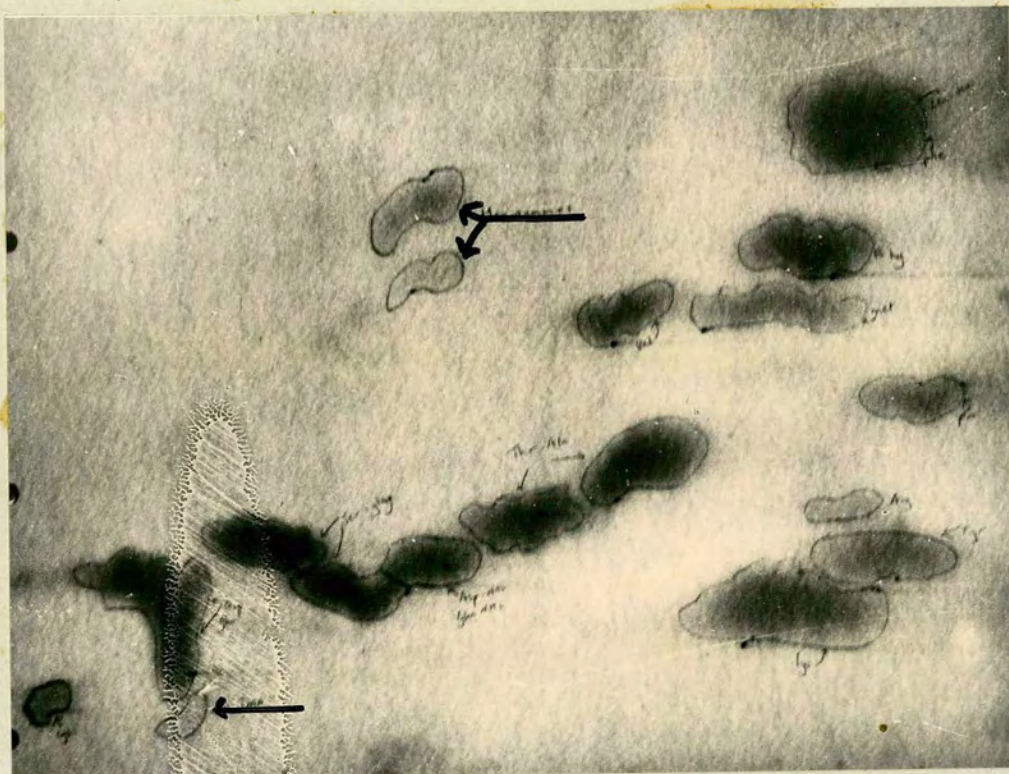


FIGURE 12

Chromatogram of a hydrolysate of mu particles, stained with ninhydrin; the DAP spot is arrowed (lower left) and the 2 hexosamine spots are also arrowed (upper centre). Directions of solvents were as indicated, and the origin was at the lower left hand corner.

and others have pointed out that the bacterial cell-wall has a distinctive structure and contains compounds not found in other parts of the cell or in other organisms. The two best-known and best-characterised of these compounds are the aminoacid α -*e* diaminopimelic acid (DAP) and the hexosamine muramic acid (3-O-carboxyethyl D-glucosamine). So far as can be ascertained these compounds occur exclusively in the glycosaminopeptide of the bacterial cell-wall. Hence a conclusive test for the bacterial nature of the mu particle would be to look for the presence of these two compounds. If a positive result were obtained, showing the presence of these compounds in the mu particle, then this would also suggest that there might be some biochemical capability in the mu particle, for it would seem likely that the mu particle would synthesise its own cell-wall, as this is a specialised synthesis in bacteria (Salton, 1964).

To test for the presence of these compounds, lyophilised mu particles were hydrolysed in a sealed tube with HCl, and the hydrolysate chromatographed both on paper, and in an aminoacid analyser. Results of both methods were in agreement.

Figure 12 is a photograph of a 2-way paper chromatogram of a mu particle hydrolysate, stained with ninhydrin. There are 18 ninhydrin positive spots, some of which are known to correspond to more than one aminoacid. Thus the mu particle probably contains all the aminoacids found in proteins. The arrowed spot (lower left) was found reproducibly in hydrolysates of mu particles; it has an R_f of 0.18 in phenol-NH₃ and R_{gly} of 0.22 in butanol-acetic acid water. (R_f = the distance moved by a compound relative to that moved by the solvent front; R_{gly} = the distance moved by a compound, relative to that moved by glycine.). The values



FIGURE 13

Chromatogram of a hydrolysate of a control preparation, stained with ninhydrin. No DAP or hexosamine spots are evident. Otherwise similar to figure 12.

given for the arrowed spot are very similar to those given by Work and Dewey (1953) for DAP. Figure 13 is a chromatogram of a control hydrolysate, using the material prepared from sensitive cells. There are 10 feeble spots, though several are so feeble that they are not visible on the photograph; however, their positions have been indicated. It is clear that there is no spot comparable to the presumptive DAP spot found in mu particle hydrolysates. Further, the control hydrolysate is of a complete preparation (about 0.4 mg. dry weight in this case), while the mu particle hydrolysate (figure 12) corresponds to approximately one-fifteenth of the total preparation (1.7 mg. dry weight in this case). The insignificance of contamination is thus clearly shown, for it is evident that it cannot have contributed to any of the spots obtained on the chromatogram.

It was possible that the spot found in mu particle hydrolysates was not due to DAP, but to other compounds. The most likely of these are either ethanolamine-0-phosphoric acid or some product of the oxidation of cystine in hydrolysis. It was therefore decided to make use of the aminoacid analyser to determine that DAP really was present.

On analysis of hydrolysates in the aminoacid analyser, essentially the same pattern as was found on paper chromatograms emerged. Hydrolysates of mu particles contained 19 identifiable aminoacids, not counting tryptophan and cystine (figure 14), while hydrolysates of control preparations contained 14 aminoacids, but the amounts were all very small (figure 15). As with the paper chromatograms in figures 12 and 13, there is a similar difference in the amount of material applied to the column. For the mu

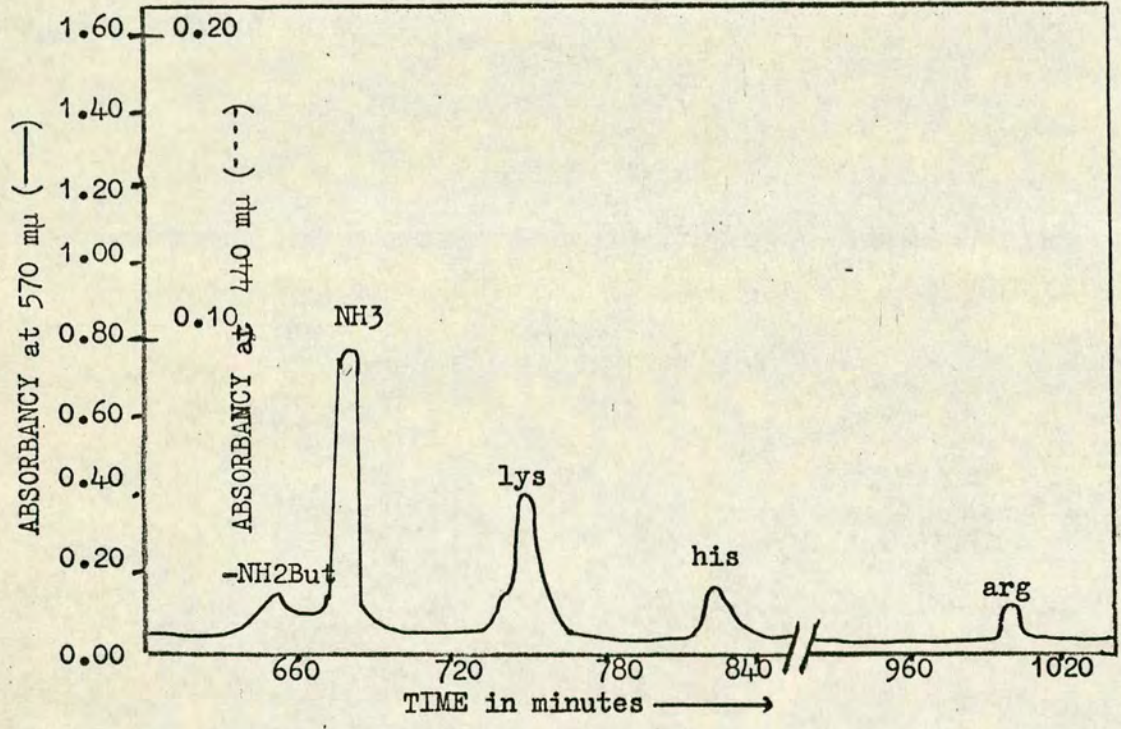
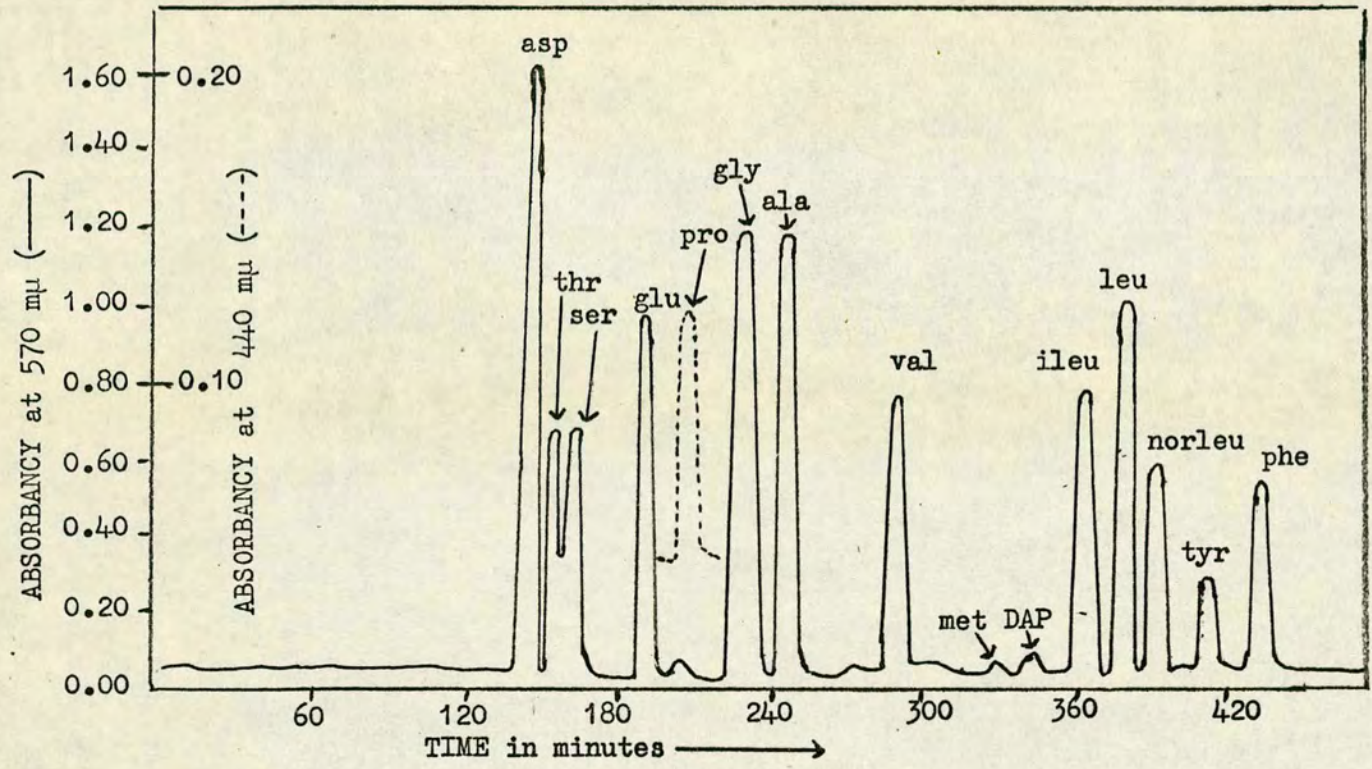


FIGURE 14

Chromatogram of a hydrolysate of mu particles (1.22 mg dry weight) run in a Technicon amino-acid analyser. 0.1 μ M of norleucine was added as an internal standard. Gradient used for elution was modified as described by Burns, Curtis and Kacsner (1965). Identification of peaks is based on Hamilton (1963). Regions of the time axis containing no peaks are omitted.

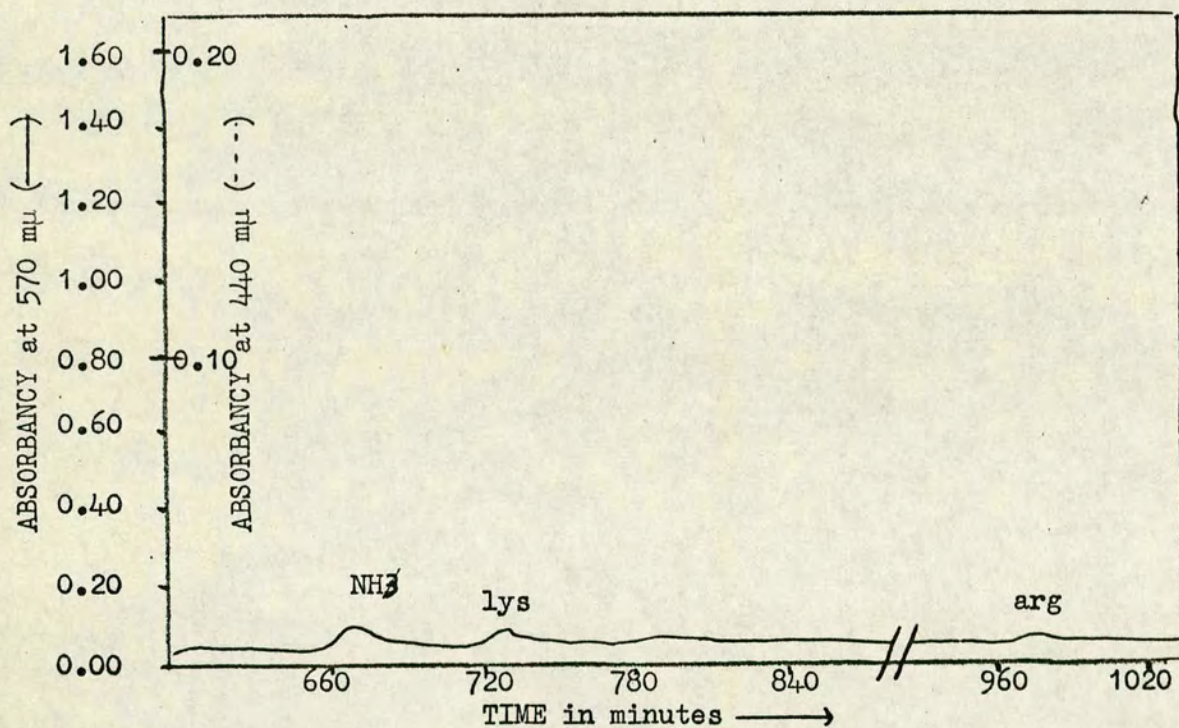
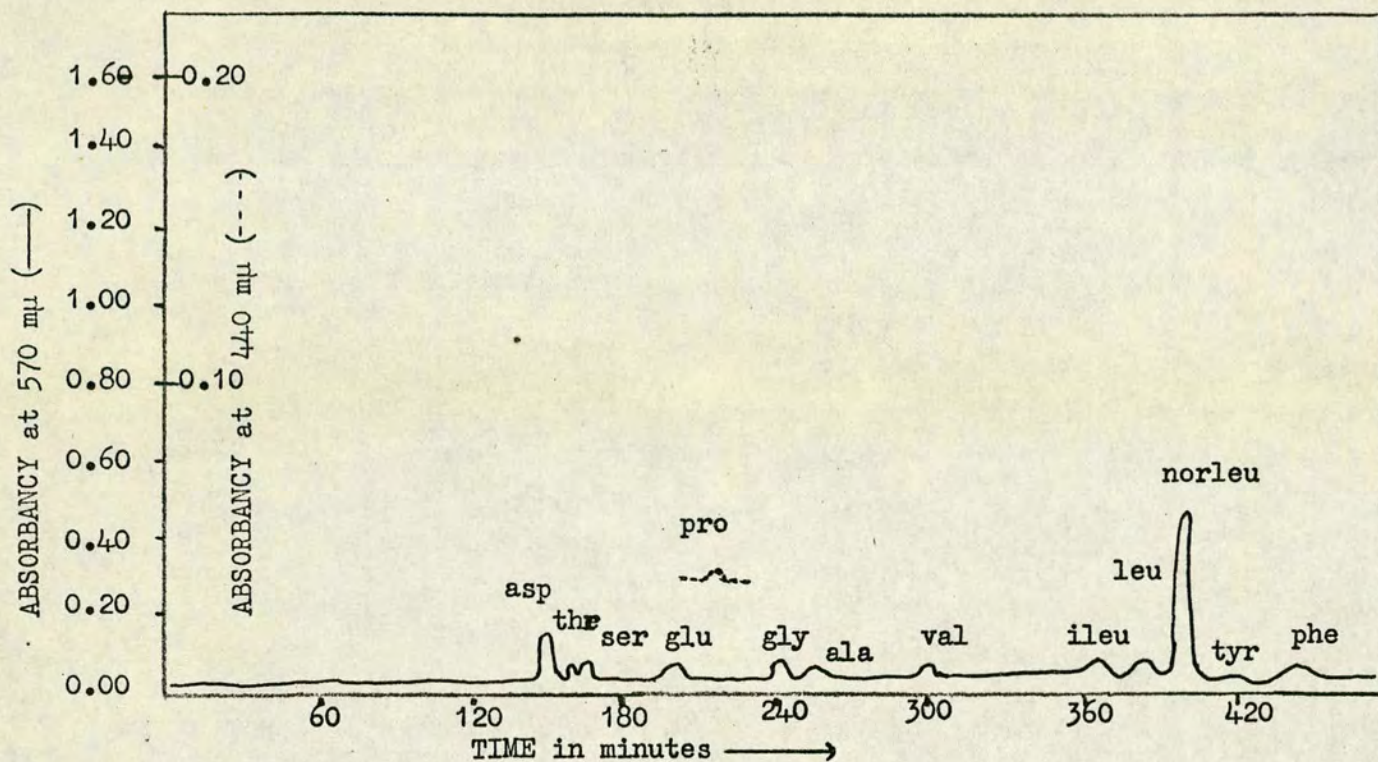


FIGURE 15

Chromatogram of a hydrolysate of a control preparation from stock 540 sensitive (0.13 mg dry weight) run in a Technicon amino-acid analyser. 0.1 μM of norleucine was added as an internal standard. Conditions otherwise as for figure 14. Figure has been drawn to same scale to emphasise difference from figure 14.

particle hydrolysate about one-fifteenth of a preparation was applied: this was 1.22 mg. in this case; for the control hydrolysate half a preparation was applied; this was 0.13 mg. in this case. It is also evident from figures 14 and 15 that there are marked differences in distribution of aminoacids between mu particles and control preparations.

The peaks of interest in figure 14 are the small peak at 343 minutes, which is diaminopimelic acid, and the peak at 652 minutes, which is γ -NH₂-butyric acid, typical of many bacteria, but rarely found in other organisms (Work & Dewey, 1953).

The position of the DAP peak corresponds with the position of elution of authentic DAP, which was eluted after 345 minutes. The identification of peaks is based on the results of Hamilton (1963), but it should be pointed out that a modified gradient of pH and salt concentration was used for elution (Burns, Curtis and Kacser, 1965). This gradient changes the time of elution of some peaks, and the time intervals between them, but the order is unchanged. Hence comparison is straightforward. It is also evident that there was a slight difference between the gradients used in the experiments in figures 14 and 15, as all elution times after phenylalanine are slightly different; however, this error is not sufficient to affect the conclusions in any way.

Table 4 gives the determination of aminoacid composition of mu particles and control preparations. It is evident that differences observed in figures 14 and 15 are perpetuated, and that there is marked difference in composition. Results have not been expressed as quantities

TABLE 4

AMINOACID COMPOSITION OF MU AND CONTROL HYDROLYSATES, DETERMINED BY
AMINOACID ANALYSER

All values are μM of aminoacid per 100 μM of aminoacid total recovery.

Aminoacid	$\mu\text{M}/100\mu\text{M}$ in mu	$\mu\text{M}/100\mu\text{M}$ in control
Aspartic acid	11.18	15.21
Threonine	6.24	6.85
Serine	5.68	6.12
Glutamic acid	10.32	15.36
Proline	6.86	2.35
Glycine	10.50	5.63
Alanine	9.39	5.53
Valine	7.54	3.64
Methionine	0.086	trace
α - ^a -diminopimelic acid	0.278	0.00
Isoleucine	7.66	6.75
Leucine	10.01	10.23
Tyrosine	2.53	0.00
Phenylalanine	5.50	7.00
γ -NH ₂ -butyric acid	2.41	0.00
Lysine	3.21	10.62
Histidine	0.48	1.53
Arginine	0.142	3.16

Total recovery of amino^aacids from the hydrolysates after analysis was about 50% of the dry weight of the original preparation before hydrolysis; this indicates that there was some loss during hydrolysis. With crude material such as this, the amount of loss will vary for each aminoacid. However, it is evident that there are significant differences in aminoacid composition between mu particles and the contaminating cell debris, making it unlikely that components such as DAP are derived from the debris.

of aminoacid/mg. dry weight because it was found that recoveries of aminoacids were rather low, i.e. there were large losses during hydrolysis. This may have been due to poor evacuation before hydrolysis, and with control preparations, to the very small amounts of material handled. Hence data would not be very meaningful. However, as they stand, the data clearly confirm the presence of DAP in mu particles, which was the aim of the experiment.

Attempts to detect muramic acid, the other typical cell-wall component of bacteria, were not so successful. While there were usually 2 hexosamine spots on chromatograms of mu hydrolysates (figure 12), and one of these could be identified as glucosamine, the other could not be identified with any certainty as muramic acid, as ninhydrin and Elson-Morgan reactions were weak. Glucosamine could not be resolved from leucine in the aminoacid analyser; while muramic acid was not resolved from glutamic acid. Not evident in figure 14, but apparent on the original recorder traces is a small shoulder on the glutamic acid peak in mu particle hydrolysates. For the moment, it is probably best to regard the presence of muramic acid as not proven; but the presence of DAP alongside all the other evidence suggests that the mu particle is a bacterium, or at least, most closely comparable to a bacterium.

(5) The DNA of the mu particle.

Smith-Sonneborn, Green and Marmur (1963) showed that animals of stock 51 bearing kappa particles contained a species of DNA of buoyant density 1.696 gm./cm.^3 , which was not present in stock 51 animals not bearing kappa

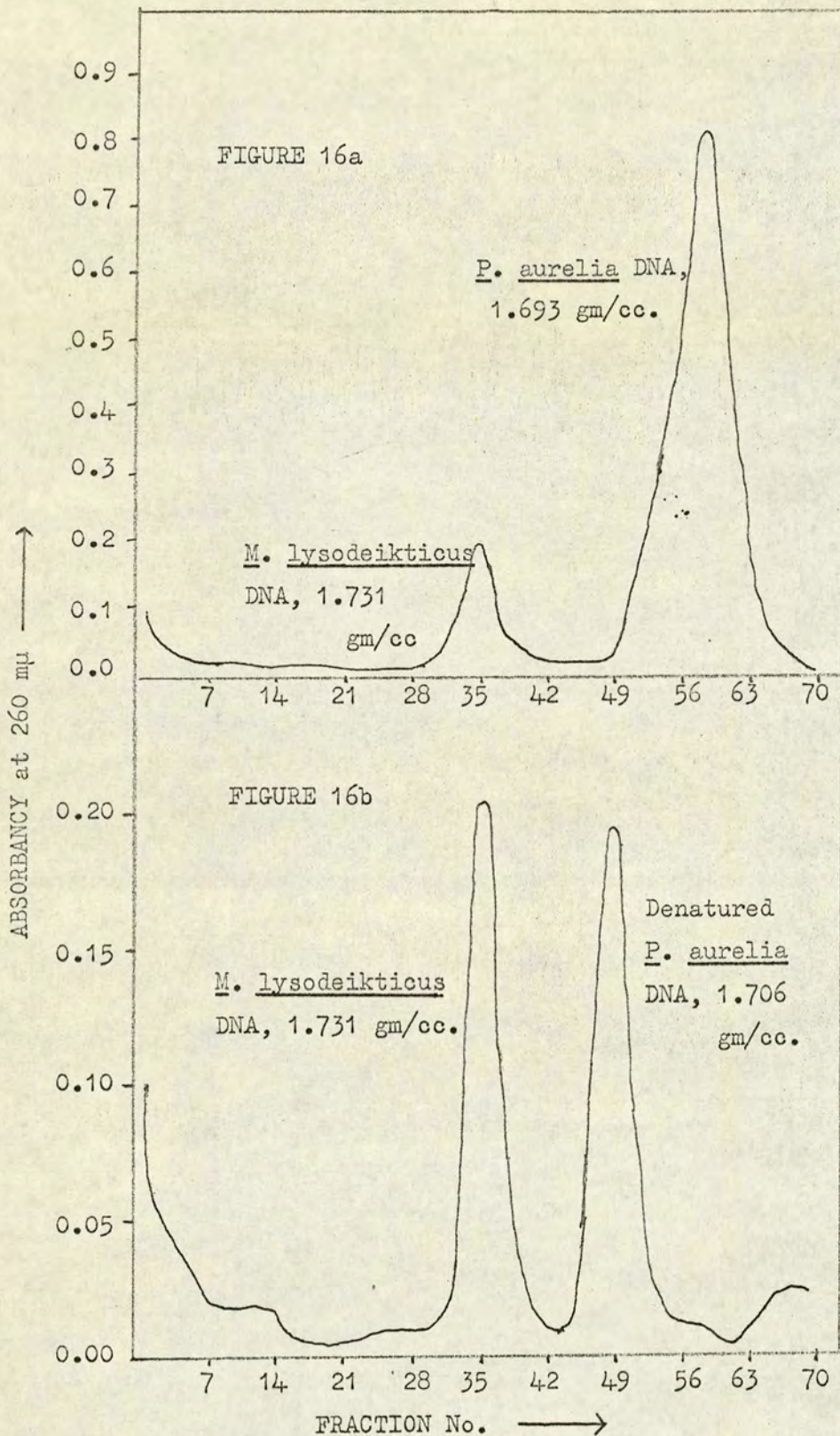


FIGURE 16

CsCl density gradient centrifugation of native (Figure 16a) and denatured (Figure 16b) DNA from isolated macronuclei of stock 540.

Figure 16a. 210 μg of P. aurelia DNA + 58 μg of M. lysodeikticus DNA

Figure 16b. 61 μg of heat-denatured P. aurelia DNA + 58 μg of M. lysodeikticus DNA.

70 hrs. at 32000 rpm at 19°.

4 drop fractions + 1 ml SSC & read at 260 mμ in 0.5 cm. light-path cells.

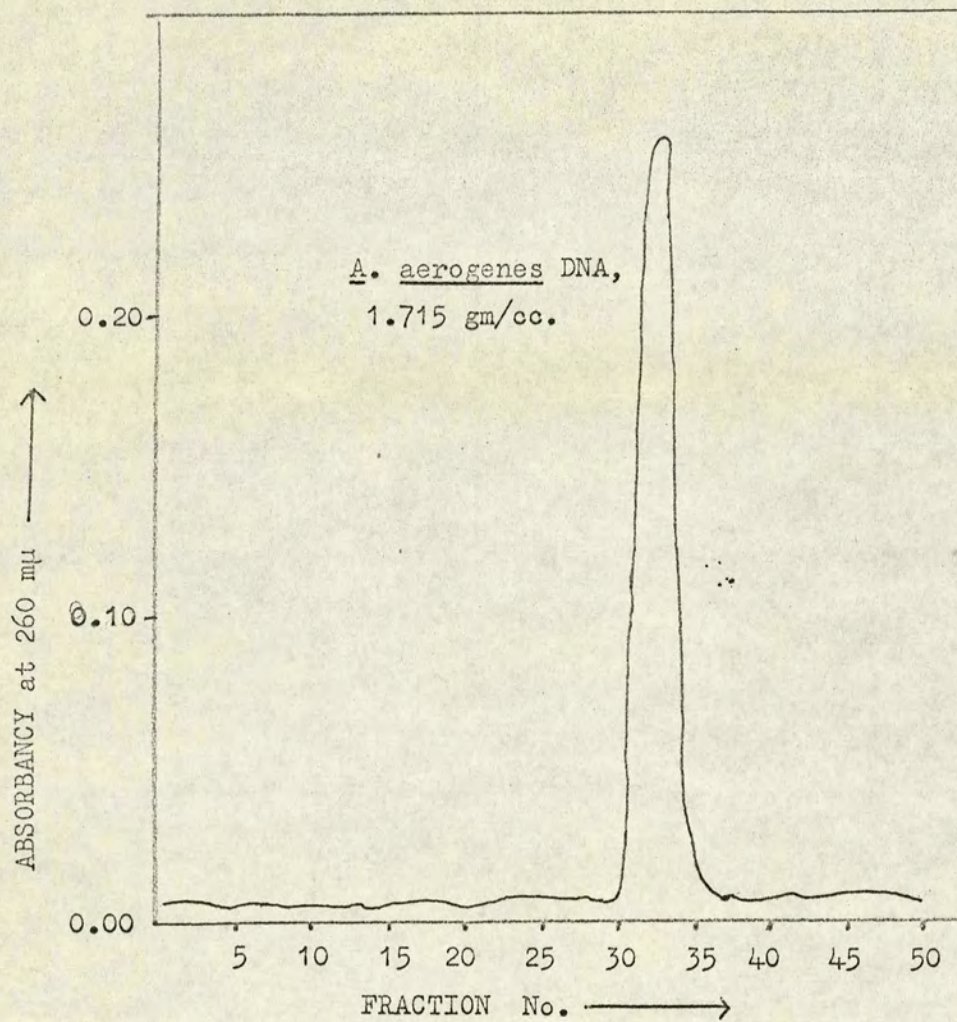


FIGURE 17

CsCl density gradient of A. aerogenes A3(0) DNA. 88 μg.
74 hrs. at 31000 rpm at 18°. 6drop fractions, diluted with 0.8 ml SSC
and read at 260 mμ in 0.5 cm light-path cells.

particles, while Behme (1964) found additional DNA species in several particle-bearing stocks, including 540 mu. These workers centrifuged lysates in CsCl gradient, but did not attempt to prepare DNA. As the results of this study suggested that the mu particle contained DNA (section 2 of Results), it was decided to attempt to prepare and characterise this DNA.

Firstly, however, it was decided to characterise the DNA of stock 540 of P.aurelia by the methods to be used, density gradient centrifugation in CsCl, and the determination of the temperature of melting (T_m). For this work, DNA prepared from isolated macronuclei was used. This was for the following reasons:- first, it was easier to prepare high molecular weight DNA from isolated macronuclei than from whole animals, fewer manipulations being required; and there was greater freedom from nucleases, which are very active in whole animal extracts. Second, the use of DNA from macronuclei avoided any chance of contamination with DNA from residual food bacteria, from mitochondria (DNA was shown to be present in the Paramecium mitochondrion by Suyama and Preer, 1965) or from the DNA claimed to be present in the basal bodies of ciliates (Randall and Disbrey, 1965). Although the amounts of any such contamination would be small, it was felt that it would be better to minimise them.

Macronuclei were isolated as described in the appendix to this thesis, and DNA was prepared as described in Materials and Methods. On centrifugation of such isolated macronuclear DNA in a preparative CsCl gradient a single peak was obtained, with an estimated buoyant density of 1.693 gm/cm^3 (figure 16a). Densities were estimated by reference to marker peaks of M.lysodeikticus DNA and A.aerogenes A3(0) DNA. DNA

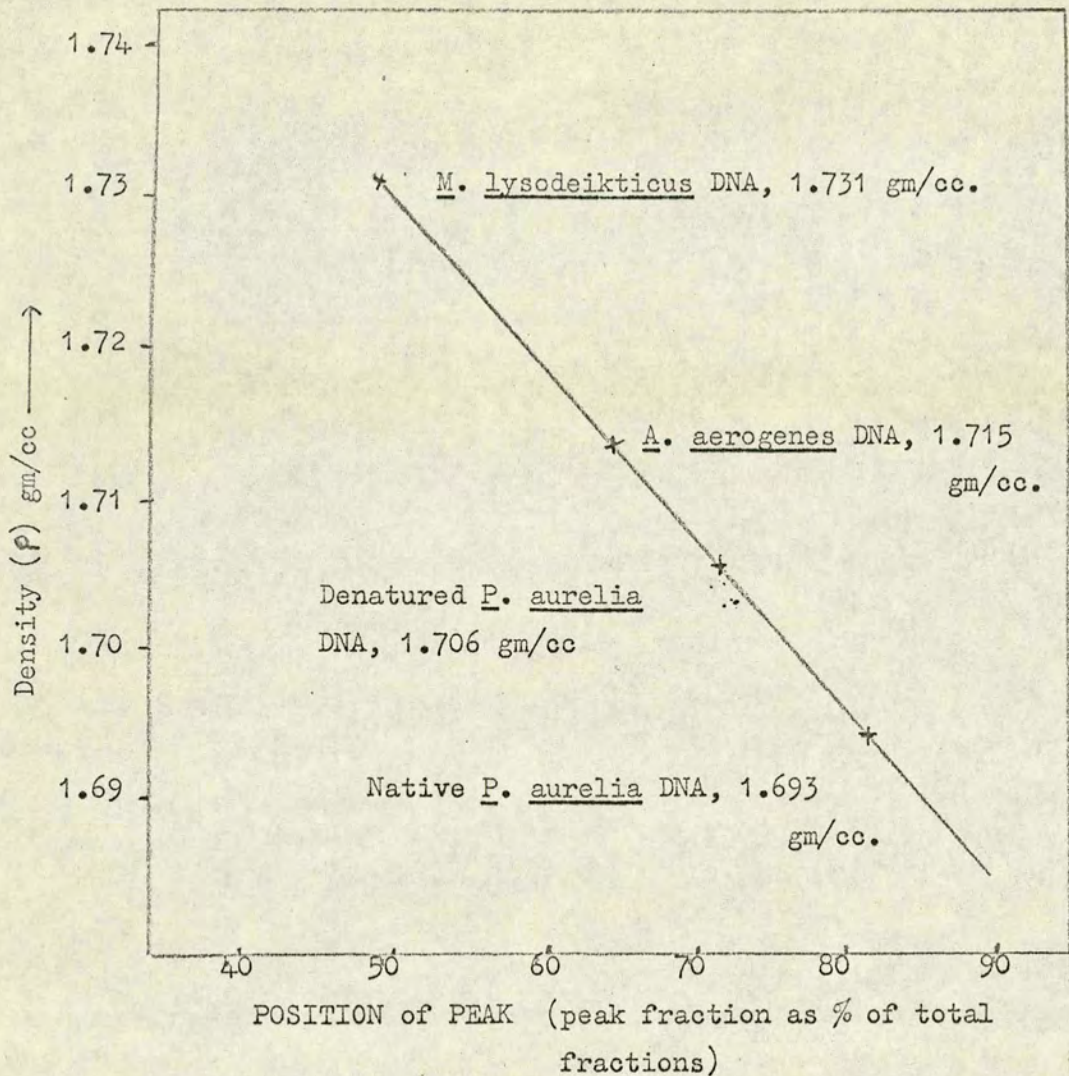


FIGURE 18

Density of DNA against peak position in CsCl gradients used in this study. The densities of M. lysodeikticus and A. aerogenes DNA's are those given by Schildkraut, Marmur and Doty, (1962).

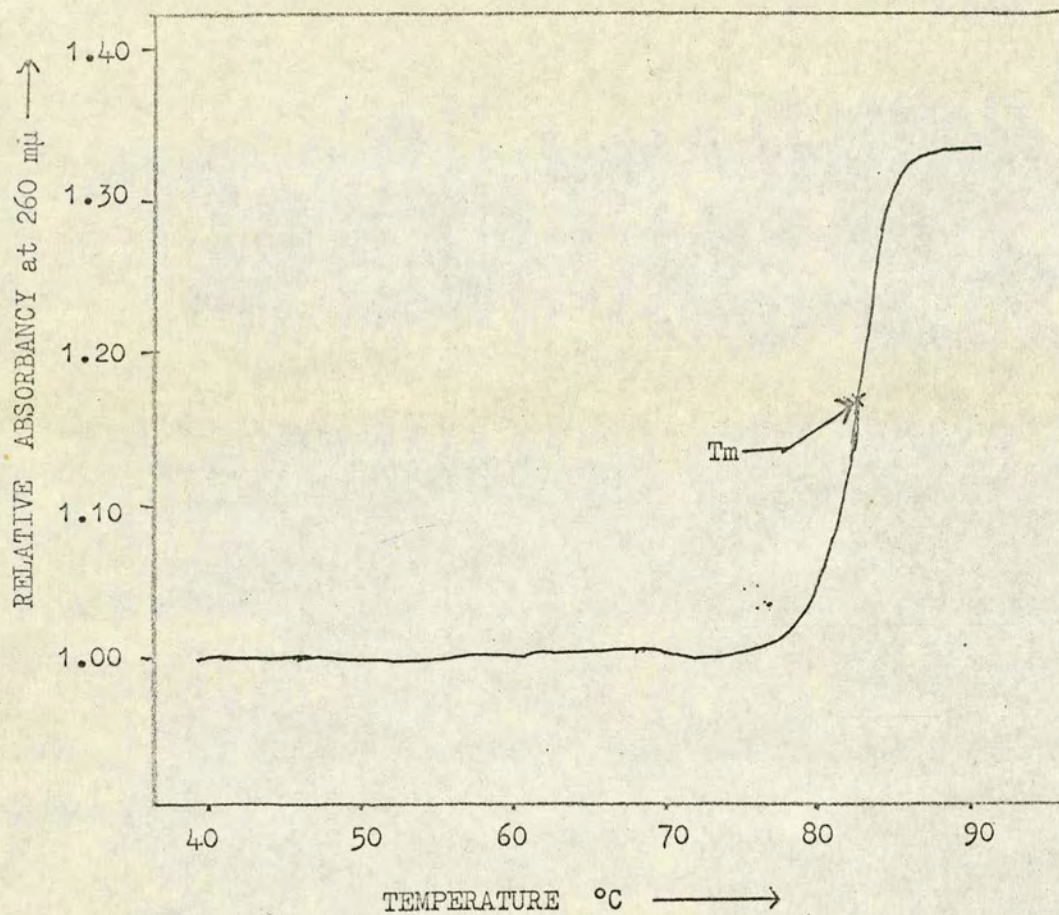


FIGURE 19

Melting curve of P. aurelia macronuclear DNA. Solvent:- SSC.
Mid-point of absorbance increase = 81.8° = Tm.

from these organisms has buoyant densities of 1.731 gm/cm^3 and 1.715 gm/cm^3 , respectively (Schildkraut, Marmur & Doty, 1962). Figure 16a has a marker peak of M.lysodeikticus DNA, while figure 17 shows the result of gradient centrifugation of a sample of A.aerogenes DNA. Figure 18 is a plot of DNA buoyant density against position of peak in a CsCl gradient; effectively of course the position of the peak is a measure of the distance from the centre of rotation. The relationship has been assumed to be linear, but Ifft, Voet and Vinograd (1961) have shown that there are departures from linearity at both ends of the centrifuge cell. However, for these experiments using a swing-out preparative rotor, which has a low resolution of DNA's of different densities, this error can be neglected.

The density of 1.693 gm/cm^3 corresponds to a guanine + cytosine content of 32 mole % (Schildkraut, Marmur & Doty, 1962), for P.aurelia macronuclear DNA. This DNA is double-stranded because on heating a solution to 100° for 5 minutes, and then fast-cooling in an ice-bath, thus preventing renaturation of the separated strands, the buoyant density increased to 1.706 gm/cm^3 (figure 16b). This increase is the expected amount for a double-stranded DNA. The pattern of change of absorbancy at 260 m μ on heating is also as expected for double-stranded DNA (figure 19). Most samples showed a 30-35% increase of absorbancy over a range of 8° - 10° , suggesting a quite homogeneous, high molecular weight DNA. T_m (mean of 6 determinations on different preparations) = 81.8° . Using the relationship of Marmur & Doty (1962) in which

$$T_m = 69.3 + 0.41 (\%GC)$$

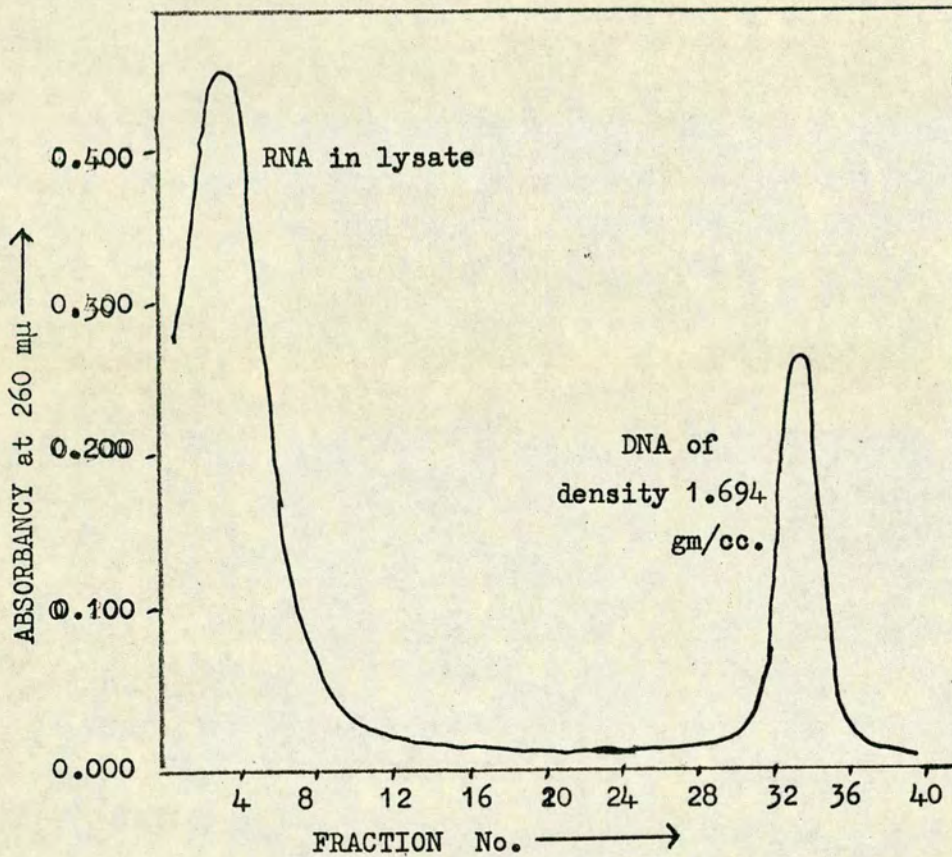


FIGURE 20

CsCl density gradient centrifugation of a 2.5% SLS lysate of mu particles. No marker DNA added. The lysate contained 91 μg of DNA.

67 hours at 32000 rpm at 19°.

7 drop fractions, diluted with 1 ml of SSC, and read at 260 mμ in 0.5 cm light-path cells.

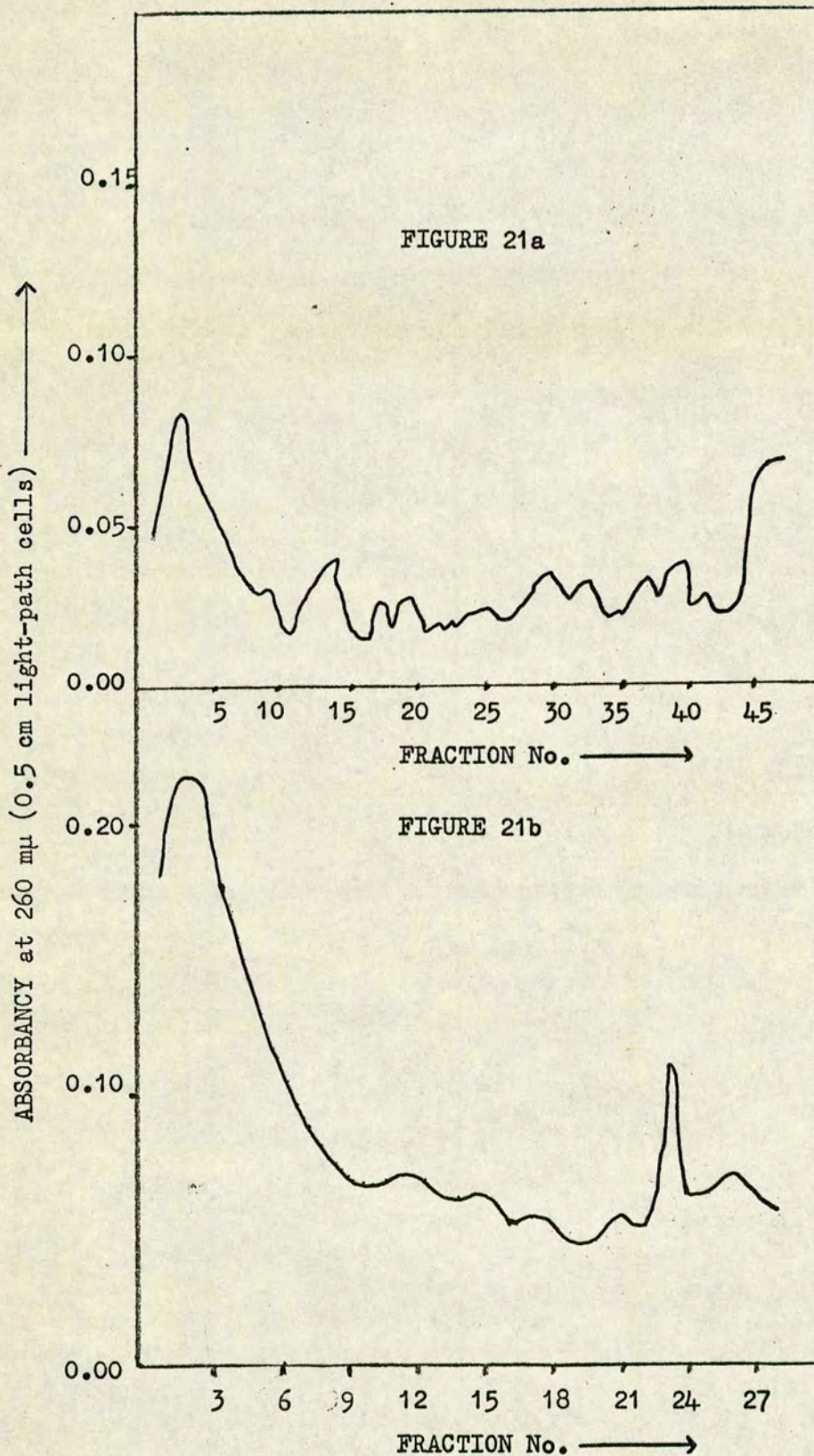


FIGURE 21

CsCl density gradient centrifugation of control preparation 2.5% SLS lysates. Figure 21a. Control preparation from stock 540 sensitive, using identical preparation method to that for μ particles. No marker DNA was added. There were 22 μg of diphenylamine reacting material in the lysate. 6 drop fractions, diluted with 1 ml SSC. Figure 21b. Lysate of eluate from the cellulose column step of the preparative procedure, using stock 540 sensitive. No marker DNA added; there were 45 μg of diphenylamine reacting material in the lysate. 8 drop fractions, diluted with 1 ml SSC. Both gradients spun 69 hours at 32000 rpm at 19°

in the solvent used here (SSC), then the guanine + cytosine content is 30.6 mole %. Thus the values obtained from buoyant density centrifugation and determination of T_m are substantially in agreement, and the result obtained is in agreement with those of Smith-Sonneborn, Green and Marmur (1963), Gibson (1964) and Suyana and Preer (1965).

As mentioned earlier, it was not found possible to prepare DNA from μ particles by the pronase method or by the method of Marmur (1961). Instead, μ particles were lysed with 2.5% SLS and the lysate subjected to CsCl density gradient centrifugation. Generally, the results were comparable to gradient runs using purified DNA. However, there was also a peak of RNA, which has a greater density than DNA at the bottom of the tube, and a zone of protein at the top, usually all floating on the surface. The presence of these materials did not affect the results.

Figure 20 shows the result of density gradient centrifugation of a μ particle lysate. It is evident that the lysate contains DNA of sufficiently high molecular weight to form a peak in the gradient, but the buoyant density of this DNA is estimated to be 1.694 gm/cm^3 . This is very similar to the density of Paramecium macronuclear DNA. Control preparations from animals not bearing μ particles, did not contain DNA which could form a peak in a CsCl gradient (figure 21a); this is in agreement with a low DNA content in such preparations. If the crude eluate from the cellulose column step is taken and lysed (again using sensitive cells) DNA of density 1.693 gm/cm^3 is observed, but the amount is very small compared with the amount found in μ particles at a much later stage of extraction. This DNA is almost certainly P.aurelia DNA (figure 21b).

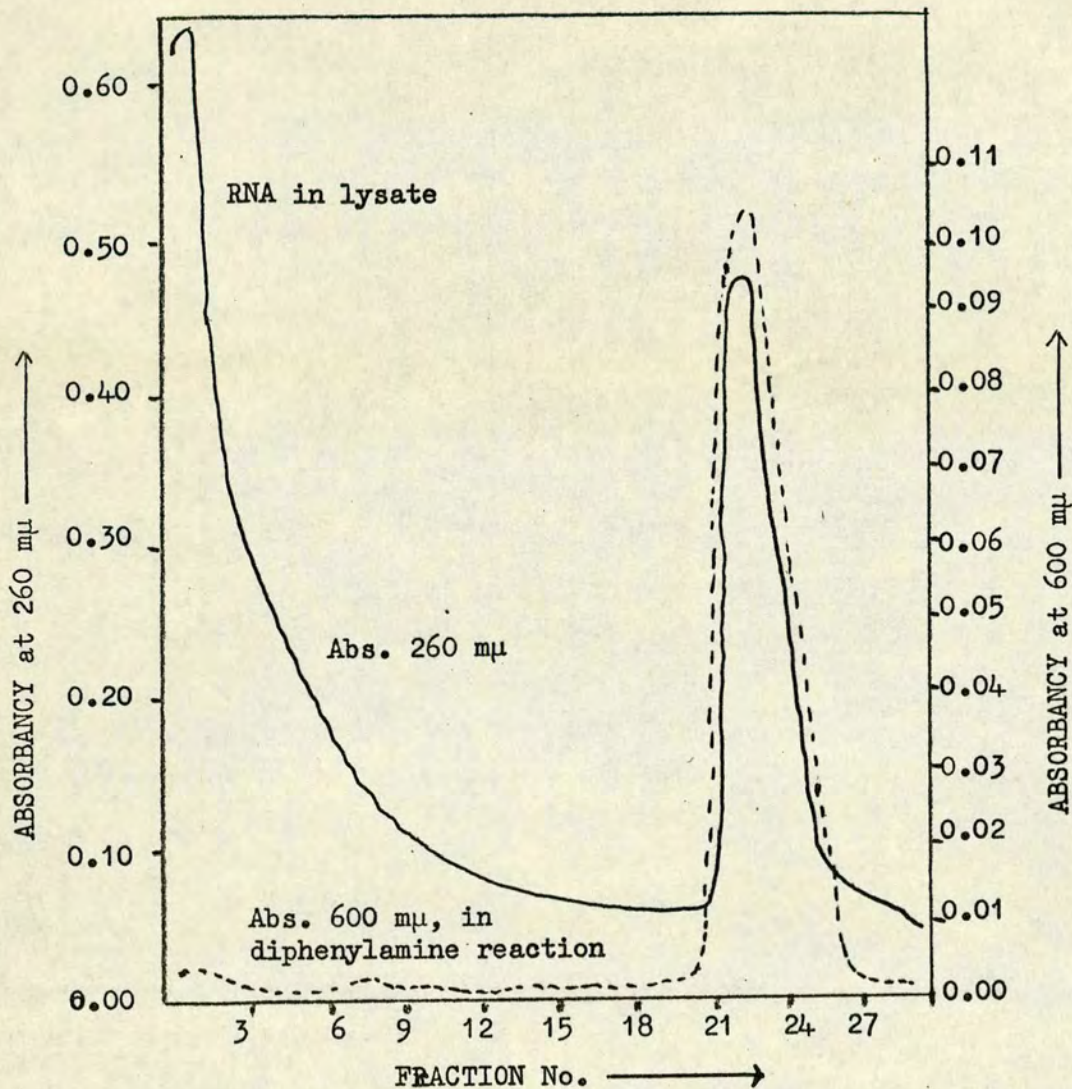


FIGURE 22

CsCl density gradient centrifugation of mu particle 2.5% SLS lysate + *P. aurelia* macronuclear DNA. The lysate contained 102 μg of DNA, and there were 79 μg of *P. aurelia* DNA. Recovery of DNA in the peak was about 135 μg ; i.e. more than either of the component DNA's. This, and the shape of the peak, indicate that the DNA's have mixed, which suggests that the two DNA's, from mu particles and macronuclei, are of very similar or identical density.

70 hours at 32000 rpm at 19°. 8 drop fractions, diluted with 1.5 ml of SSC, and read at 260 mμ in 0.5 cm light-path cells.

For the diphenylamine reaction, 0.2 ml of the diluted fraction was taken and assayed as described by Burton (1956).

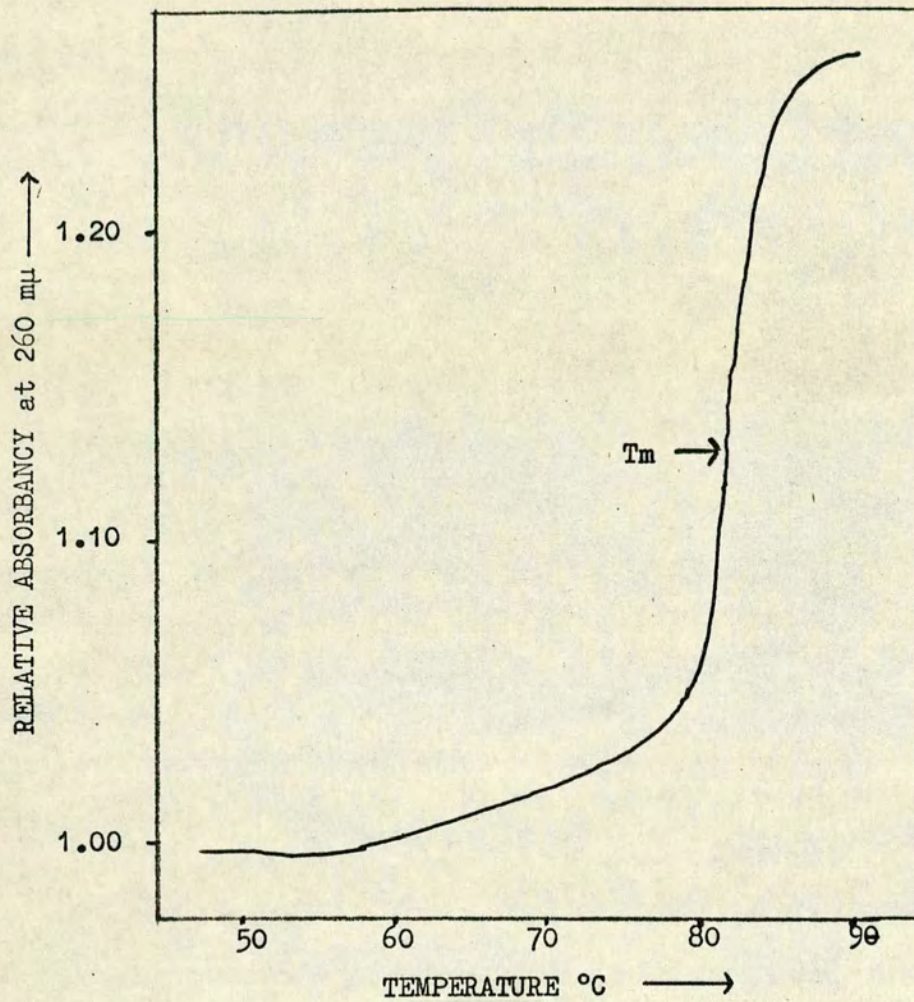


FIGURE 23

Melting curve of DNA from isolated mu particles. Solvent:- SSC.
Mid-point of absorbancy increase = 82° = Tm.

In order to check whether the use of lysates was affecting the position of a peak in the gradient, and also to test if P.aurelia DNA and the DNA in the mu particle preparations were in fact of very similar density a mixing experiment was carried out in which purified macronuclear DNA was added to a mu lysate and the mixture was then subjected to density gradient centrifugation. The macronuclear DNA used was the same preparation as that in figure 16a; indeed the experiment shown in figure 22 was carried out during the same centrifugation as that in figure 16a. It is evident from figure 22 that the use of a lysate does not affect the validity of the results, and that the DNA present in mu preparations is indeed of similar density to macronuclear DNA. Figure 22 also shows the results of assaying fractions by the diphenylamine method, and shows that this gives similar results to the measurement of absorbancy at 260 m μ .

The T_m of the DNA present in the preparations of mu particles was also determined. The preparation used for the experiment shown in figure 23 was in fact the pooled and dialysed peak fractions from the experiment shown in figure 20. Although the absorbancy rise is lower (about 25%) than macronuclear DNA it is evident that the DNA behaves typically for a double-stranded DNA. T_m was found to be 82.0° (mean of experiments with 3 different preparations), suggesting a guanine + cytosine content of 31.0 mole %, substantially in agreement with the result of CsCl density gradient centrifugation, which gives 32.3 mole % guanine + cytosine.

Thus the DNA present in preparations of mu particles is similar to or identical with the macronuclear DNA of P.aurelia in composition.

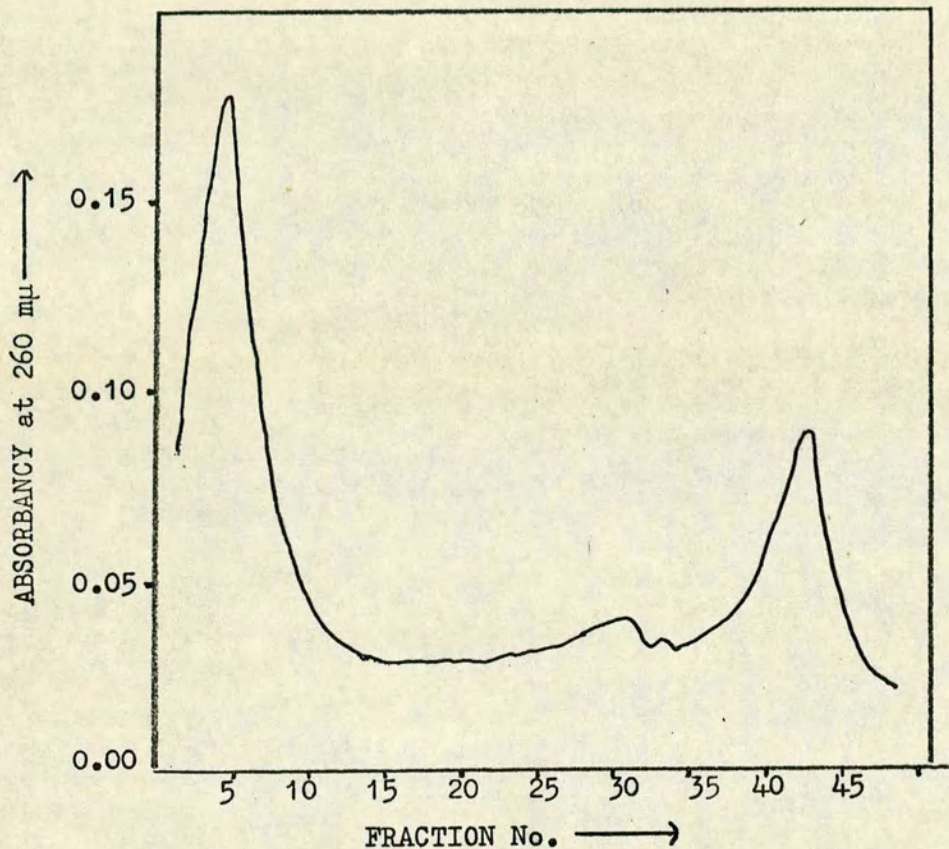


FIGURE 24

CsCl density gradient centrifugation of 2.5% SLS lysate of mu particles which were treated with 10 $\mu\text{g}/\text{ml}$ DNase for 45 mins at 37° before lysis. No marker DNA was added after lysis. The lysate contained 38 μg of DNA.

68 hours at 32000 rpm at 18°.

6 drop fractions, diluted with 0.8 ml of SS0, and read at 260 mμ in 0.5cm light-path cells.

It was possible that this DNA was macronuclear DNA of the Paramecium, derived from broken nuclei, and possibly absorbed to the outside of the particle. If this were the case, the absence of DNA in control preparations would not be a satisfactory control, for since there are no particles present in such preparations, there would be nothing for such DNA to absorb to. Mu particles were therefore treated with deoxyribonuclease before lysis; this would determine whether the DNA observed was outside the mu particle, or inside, protected by the membrane from nuclease action. After isolation, mu particles were resuspended in 0.05M phosphate, pH 6.5 + 5 mM MgCl₂. DNase (Seravac Ltd., Maidenhead) was added to 10 µg/ml, and incubated at 37° for 45 minutes. The mu particles were then spun down at 1000 g. for 10 minutes, washed with SSC, then lysed as before, and the lysate subjected to CsCl density gradient centrifugation. The result is shown in figure 24, and it is evident that mu particle DNA is almost unchanged. Very slight degradation has taken place, but, under these conditions, macronuclear DNA was completely degraded. Hence it would seem that the DNA found in preparations of mu particles does indeed come from inside the particle itself, being protected from nuclease action by the membrane surrounding the particle.

The material banding in CsCl gradients has been established to be DNA on the basis of the following criteria:- firstly, peak material was always checked for a typical nucleic acid absorption spectrum in the U.V. region, which it was always found to possess; secondly, both macronuclear and mu particle DNA preparations gave temperature-absorbancy curves typical of DNA (figures 19 and 23); thirdly, peak material always gave

a positive reaction with diphenylamine; and fourthly, peak material was DNase-sensitive. This is a very germane check, as electron micrographs show that P.aurelia contains many glycogen granules, while in Tetrahymena pyriformis glycogen has been confused with DNA (Suyama, Preer and Bonner, 1965). It has been shown (Padilla and Barber, 1965) that glycogen in T.pyriformis bands at a density of 1.65-1.68 gm/cm³ in CsCl gradients; glycogen also absorbs UV light at 260 mμ. The report of Suyama, Preer and Bonner (1965) is now known to have been in error, but it does show how easily confusion may arise. However, glycogen has not been confused with DNA in this work, as all the properties of the material are compatible with the hypothesis that it is DNA.

In summary, therefore, it seems that the mμ particle possesses DNA, presumably inside the particle, and protected by a membrane. The buoyant density and T_m, and hence base composition of this DNA is similar to, or identical with, the base composition of the macronuclear DNA of P.aurelia. There is of course no intrinsic reason why this should not be so. Many reports of the characterisation of DNA from chloroplasts and mitochondria have laid stress on the observation that the DNA of the organelle has a different buoyant density from the DNA of the nucleus (Sager & Ishida, 1963; Ray & Hanawalt, 1964; Luck & Reich, 1964; Rubinowitz et al. 1965; Suyama & Preer, 1965, and others). But this does not seem to be a general case, for Dawid (1966) has shown that in the frog Rana pipiens egg mitochondrial DNA has an identical density to the whole egg DNA, and also in Xenopus laevis there is only a very small difference. Corneo et al (1966) and Borst and Ruttenberg (1966) have shown a similar relationship between the mitochondrial and nuclear DNA's of

guinea pigs, sheep and chickens. Hence it would seem that it is perfectly possible for two DNA species of identical density to coexist in the same cell. Further, in the case of the mu particle it is not an integrated cell organelle which is under consideration, but a symbiont belonging to a different kingdom of organisms from the host cell. It is quite possible that the DNA of the mu particle and the macronucleus of the Paramecium could be similar in composition purely by chance, and as pointed out, by analogy with the mitochondrion, this need not have any implications for the relationship between the two DNA species.

(6) Ribonucleoside triphosphate incorporation by mu particles

Several workers have shown that the DNA contained in mitochondria and chloroplasts is capable of coding for RNA, the synthesis of which is carried out through the mediation of the enzyme RNA polymerase (Kalf, 1964, Kirk, 1964, Luck and Reich, 1964). As the mu particle contains DNA and may be capable of the synthesis of its own cell wall, it may be that it has the ability to carry out a number of biochemical transformations. If this is so, then proteins and hence RNA would be required. Therefore it seemed possible that the mu particles might possess a mechanism of RNA synthesis, and experiments were carried out to test this. Experiments were also carried out with isolated macronuclei for comparative purposes and also with control material.

The assay mixture of Weiss (1960) was used to test for the ability to incorporate C^{14} -ATP, C^{14} -UTP or H^3 -GTP into acid-insoluble material.

Table 5 gives typical results on the incorporation of C^{14} -ATP and H^3 -GTP into acid-insoluble material by isolated mu particles lysed by 3 cycles of freezing and thawing, and on the incorporation of C^{14} -ATP by

TABLE 5

RIBONUCLEOSIDE TRIPHOSPHATE INCORPORATION BY ISOLATED LYSED MU PARTICLES AND CONTROL PREPARATIONS

All figures are μM of triphosphate incorporated into acid-insoluble material in 15 mins at 30° , per mg protein in the experimental material. Figures for macronuclei are taken from Table A2 in the appendix

Additions/omissions to assay mixture	mu particles				control		nuclei
	C14 ATP μM	% inhibition by treatment	H3 GTP μM	% inhib.	C14 ATP μM	% inhib	C14 ATP μM
None (complete system)	595.7	0.00	367.5	0.00	0	0	56.3
No ATP	---	---	72.9	80.2	---	---	---
No GTP	77.8	86.9	---	---	0	0	14.1
No CTP	230.0	61.4	31.9	91.3	0	0	15.1
No UTP	287.1	51.8	109.6	70.2	0	0	20.9
No triphosphates	180.2	69.7	81.9	77.7	0	0	38.6
+ Actinomycin D*	260.0	56.3	59.0	83.9	0	0	30.7
+ Ribonuclease [†]	42.3	92.9	---	---	---	---	4.7
No Mg ⁺⁺	0.0	100	---	---	---	---	---

*Concentrations of actinomycin D were $12.5\mu\text{g}/\text{ml}$ in the experiment with macronuclei, and $15\mu\text{g}/\text{ml}$ in the experiments with mu particles

[†]Concentrations of ribonuclease were $10\mu\text{g}/\text{ml}$ in the experiment with nuclei, and $25\mu\text{g}/\text{ml}$ in the experiments with mu particles

Control preparations gave negative results in every experiment (7 replications).

similarly treated control preparations. For comparison, some results on C^{14} -ATP incorporation by isolated macronuclei are given; these results are taken from Table A2 in the appendix. All results have been converted to μM of labelled triphosphate incorporated into acid-insoluble material in 15 minutes at 30° . The various additions to and omissions from the assay mixture are given in the legend to Table 5. It will be noted that ATP is incorporated nearly twice as well as GTP by isolated μ particles (595.7 μM ATP against 367.5 μM GTP); this ratio of incorporation of the nucleotides is of the order expected if RNA complementary to the DNA in base ratio is being synthesised. Incorporation of C^{14} -UTP was very similar to that of C^{14} -ATP by μ particles (561.3 μM for the time and conditions given) but the results of experiments with UTP are not given in Table 5, as only one series was run, due to the difficulty of obtaining C^{14} -UTP; in general the results confirmed those obtained with C^{14} -ATP.

From Table 5, therefore, it is evident that the μ particle possesses a very active nucleoside triphosphate incorporating activity. Control preparations always gave negative results; this further shows the insignificance of contamination with residual A.aerogenes. For optimal activity, the system present ⁱⁿ μ particles required all four nucleoside triphosphates and the presence of Mg^{++} ions. The concentration of Mg^{++} ions that was optimal was 5 mM. The system was susceptible to Actinomycin D, known to inhibit DNA-dependent RNA synthesis, and synthesised an RNase-sensitive product. Also, as mentioned above, the ratio of incorporation of ATP or UTP to the incorporation of GTP was compatible with the synthesis of RNA complementary in base ratio to the

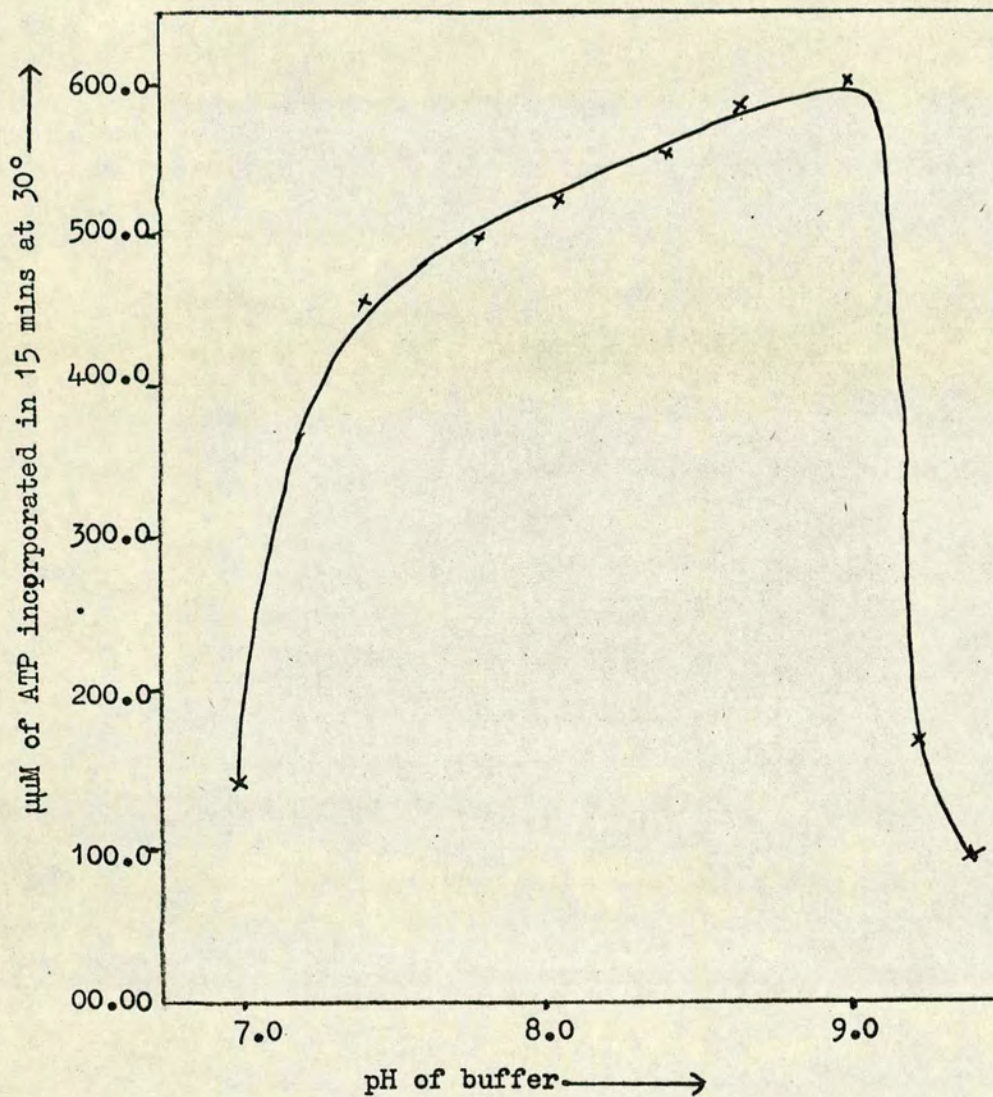


FIGURE 25

pH optimum of nucleotide triphosphate incorporation by isolated μ particles. 8-C¹⁴-ATP was used as the labelled triphosphate, and the reaction mixtures were buffered with 0.05M tris-HCl, at pH's from 7.0 to 9.4. Conditions otherwise were as described in the text.

DNA present in the mu particle. Optimum pH for the reaction was fairly broad, but was in the range pH 8.1-pH 9 (figure 25). This is the range of pH found to be optimal for DNA-dependent RNA synthesis (Weiss 1960; 1962). These findings all agree in suggesting that RNA is being synthesised in a DNA-dependent reaction by the mu particle.

It is very unlikely that the activity observed in the mu particle is due to enzyme from macronuclei that has become adsorbed to the outside of the particle. Mu particles were prepared from animals that were in the stationary or decline phase of growth. Frequently these animals were entering autogamy, up to 10% being in autogamy in some experiments. Results obtained with macronuclei isolated from animals that are entering autogamy suggest that there is a reduction of RNA synthesis before autogamy (Stevenson, 1967), as incorporation is much lower in such macronuclei than in macronuclei isolated from animals in the logarithmic phase of growth. The macronuclei used in the experiment referred to in Table 5 were isolated from late log phase cells. Thus the incorporating activity found in mu particles is about 10 times greater than that found in macronuclei from log phase cells; there must be a correspondingly greater discrepancy in the case of macronuclei from stationary phase cells. Apart from the activities found in cytoplasmic organelles such as chloroplasts, mitochondria and mu particles, RNA polymerase is an exclusively nuclear enzyme (Weiss, 1962). It is of interest that Kirk (1964) found that there was much greater RNA polymerase activity in broad-bean chloroplasts than in nuclei, parallel to the relationships found between mu particles and macronuclei.

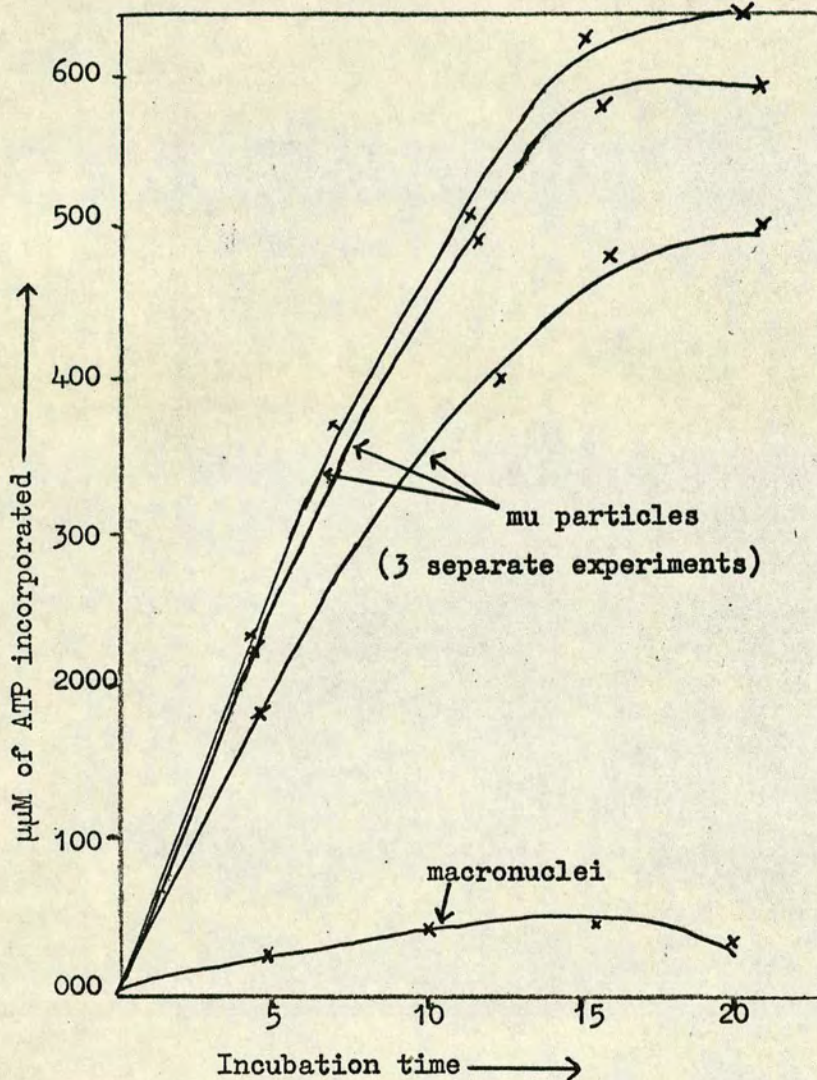


FIGURE 26

Incorporation of nucleotide triphosphate by isolated mu particles and by isolated macronuclei. 8-C-14-ATP was used as the labelled triphosphate. Conditions were as described in the text.

There is variation from experiment to experiment in the amount of inhibition observed when one or more of the nucleoside triphosphates is absent from the assay mixture; this was observed throughout the experiments and may reflect variations in the amounts of available nucleoside triphosphates or precursors in the mu particles or macronuclei. There was also variation in total incorporating activity from one preparation to another, but almost always not more than $\pm 15\%$ of the results in Table 5. Storage for long periods (>1 month) in the frozen state greatly reduced the incorporating activity of preparations, and it was also found that reagents had to be fresh for optimal incorporation to be observed. New reagents were made up at 2-3 week intervals. Also, the more contaminating material there was present in a particle preparation, the lower was the incorporating activity.

The time-course of incorporation of C^{14} -ATP by isolated mu particles is shown in figure 26, which shows the result of 2 separate experiments to demonstrate the degree of reproducibility normally observed. Incorporation almost always ceased after 15-20 minutes, but occasionally continued for 25-30 minutes. Results with C^{14} -UTP and H^3 -GTP gave similar shaped time-courses. For comparison, a result with isolated macronuclei is also given; this is taken from figure A4 in the appendix. Loss of TCA-insoluble label continued up to 60 minutes; after this time mu particles had lost about half the label incorporated after 15 minutes, but macronuclei had lost nearly all the TCA-insoluble label after 60 minutes. In neither case was there any further increase of TCA-insoluble label upon prolonged incubation after incorporation had ceased; this

indicates that there is no contamination due to bacteria, but there are degradative enzymes present in both systems.

In conclusion, it seems that the mu particle possesses a mechanism of DNA-dependent RNA synthesis that is independent of a system found in macronuclei. The system shows all the properties of DNA-dependent RNA synthesis and is much more active than the system found in isolated macronuclei; this might however, be due to better preservation of the mu particle system during isolation. However, possession of RNA synthesis implies the presence of proteins and metabolism.

DISCUSSION

Before commencing a discussion of the significance of the results of these studies it may be helpful to briefly recapitulate them. A reliable and reproducible method for the isolation of the mu particle of P.aurelia stock 540 has been developed. Such isolated mu particles closely resemble the mu particle as observed in the Paramecium cytoplasm in fine structure. The findings of Beale and Jurand (1960) on fine structure were in general confirmed. Contamination of preparations with residual food A.aerogenes was negligible and the amounts of other contaminating cell particulates etc. were low (less than 5%). Isolated mu particles contained DNA, RNA, protein and probably carbohydrate and lipid. Some staining reactions were tried - mu particles were Gram negative, not acid-fast and did not possess flagella. Analysis of hydrolysates of lyophilised mu particles by paper chromatography and in an aminoacid analyser showed the presence of α -diaminopimelic acid (DAP), and possibly of muramic acid, components typically found in the bacterial cell-wall. DNA could be extracted from isolated mu particles; its base composition appears to be very similar to that of DNA of isolated macronuclei of P.aurelia. The DNA of the mu particle was double-stranded and coded for RNA, which was synthesised in a DNA-dependent reaction.

The immediate conclusion from these results is that the mu particle is indeed a bacterium, as suggested by Sonneborn (1959) and Beale and Jurand (1960). The presence of DAP is the most convincing evidence for this. Salton (1964) states that the presence of DAP is a distinguishing feature of the bacteria and blue-green algae, being found in the cell-wall glycosaminopeptide but in no other cell components. Earlier studies by Work and Dewey (1953) had also shown this; in an extensive survey of

118 micro-organisms they found DAP in almost all bacteria, but not in yeasts or other fungi, protozoa or plant viruses. DAP was absent from Tetrahymena pyriformis, a close relative of P. aurelia. The data in Table 4 (facing page 33) also show that γ -aminobutyric acid was present in isolated mu particles. Work and Dewey found that this aminoacid was found in many bacteria but rare in other organisms.

Mu particles also resemble bacteria in size, shape and in staining reactions. These are of course, very unspecific criteria. The complex chemical make-up, and especially the presence of DNA and RNA, precludes the possibility of a viral nature, a possibility that is also very unlikely on other grounds, such as the appearance of dividing mu particles, and the structure of thin sections in the electron microscope. Mu particles are surrounded by a double membrane, but the internal contents are undifferentiated (figure 10, facing page 25). Nuclear bodies, as have been observed in many bacteria (Kellenberger, 1960) do not appear to be present, as they are not evident even after the fixation procedure designed to show them (Kellenberger, Ryter and Séchaud, 1958). Beale and Jurand (1960) concluded that DNA is spread throughout the interior of the particle, and have also claimed (Beale and Jurand, 1966) that fine filaments resembling DNA may be observed throughout the interior of the particles. In view of this, it is of interest that the DNA content of the mu particle is quite high (about 5% of dry weight, Table 3, facing page 28). This could be because there is a large amount of DNA occupying much of the interior of the particle, but present in a dispersed state that does not show it up as a region of increased electron density. Possibly this might be some adaptation to the intracellular environment.

Sonneborn (1959) suggested that a rather similar condition might hold in the kappa particle.

Mu particles appear to divide by constriction and not by formation of a transverse septum. In this they appear to differ from other bacteria, which show a cross-wall which can be demonstrated in the electron microscope (see, for example Bradfield, 1956). The fact that the process of cell division appears to differ from that of other bacteria is not a critical drawback to the acceptance of mu as a bacterium; it may be that other bacteria divide in a similar way to the mu particle, and, furthermore, it is difficult to obtain an accurate idea of dynamic processes such as cell division from static electron micrographs.

Hence, on several grounds, the hypothesis that the mu particle is a bacterium appears to be justified. There are inconsistencies, but these are not critical. All other theories of the nature of the mu particle have a greater number of inconsistencies with observation than the hypothesis of a bacterial nature, so this may be taken as established.

Proving a bacterial nature for the mu particle is, however, only a start to the problem. Questions are immediately raised concerning mode of entry to the cytoplasm, possible function in the host animal, comparable particles in other organisms, and the affinities that the mu particle may possess with other particles or organelles in the cell cytoplasm. This last is of particular interest at the present time in view of the revival of the theories of Altmann (1890), Mereschowski (1905), and Famintzin (1907) by Ris (1961) and others that mitochondria and chloroplasts are descendents of ancient cytoplasmic symbionts. If this is so, then the

mu particle would be a stage in a series which would range from intracellular symbionts of all types to cell organelles such as ^{the} chloroplast and mitochondrion. This hypothesis is in most favour at the present time to account for the origin of some cell organelles. Recent indicative work of interest will be discussed later, but this discussion will first concern itself more with the mu particle before going on to discuss its possible relationships to other particles found inside the cell.

The mu particle is large for an intracellular particle, being bigger than a mitochondrion, a lysosome or a kinetosome. As a bacterium living endosymbiotically in the cytoplasm, it must have an external origin from the Paramecium, and have invaded the cytoplasm by some route. Almost the only possible route is via the buccal cavity. There is no evidence that the comparatively rigid and highly complex cell wall of Paramecium is capable of undergoing any process comparable to pinocytosis, while electron microscopy shows that although there is some tearing of cell membranes and a slight outward flow of cytoplasm upon discharge of food vacuoles through the cytopyge, the opening is small and short-lived (Jurand, 1961). The cytopyge is therefore unlikely as a route of infection for the mu particle. However, a difficulty involved in suggesting infection via the buccal cavity is that it appears to be very rare or non-existent for food vacuoles to lyse as a result of breakage of the vacuolar membrane; and it appears that everything taken up by the buccal cavity is placed into a food vacuole. Thus, infection via this route implies the breakage of a food vacuole and escape of the invading mu particle from it. Jurand (1961) only observed breakage of the vacuolar membrane on excretion of the contents through the cytopyge. Of course,

breakage of the vacuolar membrane is not excluded, and, in the circumstances, this would seem the best explanation of the means by which the mu particle invaded the Paramecium cytoplasm. Having escaped from the vacuole, the particle retained its viability and was not excreted. Beale and Jurand (1966) point out that many wild stocks of P.aurelia have been found to contain endosymbiontes, but that these frequently disappear upon cultivation of the animals. This would suggest that endosymbiosis is a flexible condition, which may be established and lost with variation in environment. It is well known that the kappa and lambda particles may be infected into non-bearer animals that are genotypically competent to maintain them; though this has not been achieved with the mu particle, it may be quite possible; the correct conditions may not have been found.

Of interest in relation to this is the situation that is found in the case of the symbiotic algae of P.bursaria. The algae carried by different stocks have been found to vary (Bomford, 1965) but stocks do not appear to differ genetically in ability to maintain symbionts. Several workers have shown that it is possible to remove algae from a stock and later to re-establish the symbiosis (Oehler, 1922; Siegel & Karakashian, 1959; Bomford, 1965). But the algae in P.bursaria are known to be ingested via the mouth, and even inside the Paramecium are still surrounded by a vacuolar membrane, which is very clear in electron micrographs. But the symbiosis in P.bursaria is clearly not so highly specified as that in P.aurelia, specific nuclear genes not being necessary, and the symbionts still being surrounded by a membrane.

Coming now to the function of the mu particle in the host animal, the most immediate question that arises is that of the

mechanism and function of mate-killing. Little work has been done for the mu particle. Siegel (1954) showed that in the syngen 8 mate-killers physical contact of the mate-killer and sensitive animal is necessary, and that the effect of the mu particle was on the nuclei of the sensitive animal. He postulated that the most intensive action must be on the pre-zygotic macronucleus, as death occurred before the new post-zygotic nuclei came into action. But mu also acted on the micronuclei; these were either lost or became unable to give rise to macronuclei. This may be due to interference with nucleic acid metabolism. The course of events with syngen 1 mu particles appears very similar. In the light of this, the best hypothesis would seem to be that the mu particle produces a substance or substances (a "toxin") which interferes with some vital process in the re-organising cell. However, it is difficult to devise an approach to this problem which would provide some insight of the mechanism.

It is difficult to form an idea of the function of mate-killing; indeed it seems genetically pointless as it results in the death of half of the exconjugant animals. It may be that mate-killing is an incidental issue, a side effect caused by the metabolism of the particles themselves in a cytoplasm lacking the products of the M genes necessary for particle maintenance. This is supported by the fact that the different mate-killer stocks of syngen 1 differ in mate-killing ability - for example stock 551 is considerably weaker than stock 540 or stock 548, and quite frequently fails to kill the exconjugant deriving its cytoplasm from the sensitive stock. In other syngens, non-killer particle bearing stocks are known, for example the pi particles (Hanson, 1954) and the nu particles (Sonneborn, Mueller and Schneller, 1959).



Turning from the mate-killing question to the biochemical relationship of the symbiosis between mu particles and Paramecium, the results in this thesis give some insight into this. They suggest that the mu particle must be capable of carrying out some complex syntheses. The incorporation of ribonucleoside triphosphates suggests the presence of DNA-dependent RNA synthesis; in many ways this is a basic synthesis, as metabolism via enzymes would be impossible if the coded information in DNA could not be transferred to make RNA capable of directing the synthesis of protein. Indeed, the presence of an RNA-synthesising mechanism implies a protein-synthesising mechanism, and some further experiments, not included here, suggest that the mu particle has ribosomes distinct from those of the Paramecium. Ribosomes from the mu particle appear to have a monomer unit of sedimentation value 73S; Paramecium has an 80S monosome (Reisner and Macindoe, 1967; Sommerville, 1967). If this is so, then the mu particle will probably possess the complete information transfer mechanism (Experiments attempting to show aminoacid activation by isolated mu particles failed for technical reasons). Mu particles do, however, incorporate C^{14} aminoacids into acid-insoluble material, suggesting protein synthesis.

The results on aminoacid composition imply that the mu particle may be capable of directing the synthesis of its own cell-wall, since this has a unique method of synthesis in bacteria (Salton, 1964). It may be able to synthesise components such as DAP, though it could obtain compounds like this ready-made, digested from the food bacteria by the Paramecium, and then absorbed from the cytoplasm by the mu particle. But even if DAP and other components are obtained ready-made, the mu particle must still build a cell-wall out of them, and such a synthesis requires considerable metabolic ability.

Other Paramecium particles are also able to perform complex syntheses. The lambda particle of stock 299 can synthesise the B vitamin folic acid. Soldo (1963) showed that stock 299 bearing lambda did not require folic acid when grown in axenic medium, while stock 299 not bearing lambda did require folic acid, suggesting that the lambda particle can synthesise this vitamin and supply it's host's needs. This was confirmed when van Wagtendonk, Clark and Godoy (1963) showed that the lambda particle did not require folic acid when grown outside the host-cell on a complex artificial medium. Thus the lambda particle clearly has an active pathway for the synthesis of this vitamin. Kung (1966) has shown that the kappa particle can respire and utilize glucose in vitro. This implies likewise a high degree of metabolic ability; indeed it suggests the presence of the entire respiratory mechanism.

In the case of the mu particle, further experiments have shown that it can convert L -malate to succinate, via citrate. This implies the presence of the tricarboxylic acid cycle in the mu particle; an energy-yielding metabolism must be present if there is a tricarboxylic acid cycle. Taken along with the other experiments just discussed, this means that the mu particle must be biochemically quite able; indeed the particles generally cannot be as poor in enzymes as Sonneborn (1959) suggested.

Therefore, it seems that kappa, lambda and mu all possess complex biosynthetic mechanisms separate from those of the Paramecium. But they are quite likely to be obligate endosymbiotes, for although the lambda particle has been grown outside the host cell, the medium used was highly complex (van Wagtendonk et al., 1963); so complex a medium would

probably only occur in nature as an intracellular environment. From the viewpoint of the degenerate symbiont theory of the origin of mitochondria and chloroplasts, kappa, lambda and mu would represent a more recent symbiosis in evolutionary terms, in which integration is beyond the stage of the free-living organism. Paramecium has become the essential habitat for the particles, but can still live without them. However, the occurrence of genes for particle maintenance suggests that factors necessary for an inseparable relationship are in the process of evolution.

The endosymbiotes of P.aurelia are not unique. The symbiotic algae of P.bursaria have been mentioned earlier; many protozoa of classes other than ciliates carry these, for example, Foraminifera, Radiolaria, Heliozoa and Flagellata. The algae of P.bursaria have probably been extensively studied simply on account of the convenience of Paramecium as an experimental organism. However, of more interest and relevance to the present discussion are bodies similar to kappa, lambda and mu found in other organisms. Stocks bearing bacterium-like particles have been found in Euplotes patella and E.eurystomus by Faure-Fremiet (1952), while Katashima (1965) found mate-killer stocks of E.patella. Siegel and Heckmann (1965) found a killer stock of E.minuta, and Heckmann, Preer and Straetling (1967) showed that bacterium-like particles were present in this stock and some others that were also killers. Many years ago, Kirby (1941) gave an extensive review of many cases of intracellular symbiosis by micro-organisms, but pointed out that in many cases there was no critical evidence that the bodies observed were indeed bacteria or

anything else. Sonneborn (1959) also makes this point in his review of intra cellular symbiosis, in which he gives many examples, too numerous to quote fully. He also discusses the occurrence of particles in the cells of insects which appear to be similar to the particles of Paramecium; again he stresses the lack of critical evidence. Koch (1956) has specifically reviewed the situation in insects. It is of interest to note, however, that examples of similar particles appear to be very rare in other invertebrates, and in vertebrates. Confining consideration to the protozoa, however, it is clear that there are intra-cellular symbionts in many species; but the apparent preponderance of particles in ciliates may only reflect the more intensive study of this class.

The remainder of the discussion will consist of a brief review of evidence for the idea that mitochondria and chloroplasts are self-determining, self-reproducing structures. The kinetoplast of trypanosomes and the kinetosomes (basal bodies) of ciliates will also be considered. The discussion will conclude with an assessment of the relevance of these findings to those with the mu particle and other cytoplasmic symbionts of P.aurelia.

Mitochondria have been thought to be self-duplicating for many years, mainly on the basis of cytological evidence (Lewis and Lewis, 1914; Frederic, 1958), though the idea of genetic continuity was enhanced by the petite mutants of yeast (Ephrussi, 1953, 1956) and the poky mutants of Neurospora crassa, which show maternally inherited respiratory deficiencies (Mitchell & Mitchell, 1952). These examples have been reviewed by Wilkie (1964). More recently, evidence

has been adduced that the mitochondrion contains DNA; this has been demonstrated by electron microscopy (Nass & Nass, 1963; Nass, Nass and Afzelius, 1965; Swift, 1965), autoradiography (Swift, Kislev and Bogorad, 1964; Stone & Miller, 1964) and by biochemical methods (Luck & Reich, 1964; Rabinowitz et al, 1965; Suyama & Preer, 1965; Corneo et al, 1966; Dawid, 1966; Borst & Ruttenberg, 1966 and others). Frequently this DNA has been recognised by its buoyant density in CsCl gradients, this having been found to differ from the density of the DNA of the nucleus. However, Corneo et al (1966) and Borst & Ruttenberg (1966) point out that this is not necessarily the case, and that in some organisms the mitochondrial DNA has the same buoyant density as the nuclear DNA. Generally, the mitochondrial DNA has been found to be double-stranded and of high molecular weight. Cummins, Rusch and Evans (1967) have shown that mitochondrial DNA from the slime-mould Physarum polycephalum differs in nearest-neighbour frequency of nucleotides from the nuclear DNA of this organism; they point out that the frequency, particularly of G, is closer to bacterial DNA's than DNA's from other organisms. Electron microscopic studies have also emphasised this point, showing that the appearance of the DNA in the EM is more like the appearance of bacterial DNA.

The presence of DNA in mitochondria suggests, but does not demonstrate, that the mitochondria are self-reproducing and self-determining. However, further work has made this conclusion very likely. The experiments of Luck (1963), who used radioactive (C^{14}) choline to label the mitochondria of N. crassa show that after a pulse of label followed by shifting the growing mycelium to non-labelled medium,

C^{14} -choline is distributed equally to all the mitochondria in the mycelium. This experiment clearly suggests that mitochondria are self-duplicating, for, if they were produced de novo, then unlabelled mitochondria would be observed, these being the ones produced after the shift to unlabelled medium. As labelling is uniform, the result argues against de novo origin. The experiments of Parsons (1964^a, 1964^b) with T.pyriformis were rather similar, except that tritiated thymidine was used to label growing cells. Labelling occurred at any stage of the cell cycle, and label was distributed uniformly amongst all the mitochondria during 4 generations in unlabelled medium. Parsons' experiments agree with those of Luck in suggesting self-duplication of mitochondria, and also suggest that the DNA of the mitochondrion may be replicated there. Parsons and Simpson (1966) have shown that isolated rat liver-mitochondria incorporate tritiated thymidine triphosphate and other deoxyribonucleoside triphosphates into their DNA; this could be a mechanism for DNA replication, while Reich and Luck (1966) have shown that N.crassa mitochondrial DNA has physical continuity through several cycles of replication, and that this replication is at least partially independent of the replication of nuclear DNA. Reich and Luck also showed that the pattern of inheritance of mitochondrial DNA was the same as that associated with uniparentally inherited abnormal mitochondrial phenotypes, for example poky. Woodward and Munkres (1966) found that the mitochondrial structural protein (MSP) in maternally-inherited respiratory-deficient mutants differs from the MSP of wild-type N.crassa and this appeared to

result from alterations to the mitochondrial DNA; the aminoacid replacements were consistent with the genetic code. This suggests that the mitochondrial DNA determines the mitochondrial structural protein.

The work discussed above shows that the mitochondrial DNA is capable of producing more DNA and seems to have a hand in determining at least the structure of the mitochondrion. It has been shown that a DNA-dependent RNA synthesis occurs in mitochondria; Luck (1965) showed that radioactive GTP could be incorporated into an acid-insoluble product by a reaction which had the characteristics of RNA polymerase activity. Kalf (1964) and Wintersberger (1964) had made similar, but not so detailed findings. This suggests that mitochondrial DNA is functional in that it can code for RNA.

It has been known for some years that mitochondria are capable of incorporating aminoacids into acid-insoluble material by a process which appears to be protein synthesis via ribosomes (Rendi, 1959; Kalf & Simpson, 1959; Roodyn, Reis & Work, 1961; Kalf, 1964, and others). This aminoacid incorporation is inhibited by chloramphenicol, as is bacterial protein synthesis (Kroon, 1965). It has been pointed out that chloramphenicol only inhibits protein synthesis by the bacterial 70S type of ribosome, not by the 80S type of ribosome; this suggests that mitochondria may have 70S ribosomes (Vazquez, 1966). Swift (1965) has shown by electron microscopy that ribosome-like particles are present in mitochondria. Rosenbaum and Holz (1966) found that isolated mitochondria of T.pyriformis were capable of the aminoacid activation step of protein synthesis. There can be little doubt that mitochondria possess a mechanism of protein synthesis via ribosomes which is

independent of the cytoplasmic protein synthesis.

The kinetoplast of trypanosomes is known on the basis of electron microscope studies to be a specialised mitochondrion (Steinert, 1960; Rudzinska, D'Alesandro and Trager, 1964). It changes characteristically in structure and function with stage of life-cycle and host (review by Trager, 1965) and is known to be self-duplicating. The kinetoplast is known to contain DNA (Trager, 1965), and DuBuy, Mattern & Riley (1965) showed that Leishmania enrietti DNA from the kinetoplast had a distinct density in CsCl from that of the nucleus. Simpson (1965) made a similar finding for L.tarentolae. Dyskinetoplastic forms may be induced with acridine dyes and other DNA antagonists (Inoki, 1956). These forms are often non-viable, greatly altered in their ability to infect various hosts, and the DNA of the kinetoplast appears to be lacking (Trager and Rudzinska, 1964).

To turn now to the chloroplast. There is far more information available, bearing on the concept of the chloroplast as a self-determining, self-duplicating organelle, and anything approaching a complete review is impossible in the space available. The very extensive work with higher plants cannot be included on account of its sheer bulk, and for the purposes of this discussion work with micro-organisms, particularly the phytoflagellate Euglena gracilis and the alga Chlamydomonas reinhardi gives a fair picture of the current state of ideas and research.

The chloroplast has been thought to be self-reproducing for many years, and many investigators provided microscopic evidence of this. More recently, Manton (1959) showed this clearly in the phytoflagellate Chromulina pusilla and Green (1964) has given an elegant cinematic

demonstration of the division of chloroplasts in the alga Nitella. In passing, it is of interest to note that there are apparently no reports of this type with higher plant chloroplasts.

The study of chloroplast development in E. gracilis has provided much information about the inheritance and capabilities of chloroplasts. When grown in the light, E. gracilis normally has 8-12 plastids (Lyman et al, 1961; Gibor and Granick, 1962b). On culturing wild-type cells in the dark, the chloroplasts degenerate into small colourless proplastids (cells with these degenerate chloroplasts are said to be bleached) which will differentiate again to plastids on exposure to light. This bleaching is thus impermanent and reversible. / Inducers of permanent hereditary bleaching include high temperature U.V. light, antihistamines, streptomycin, erythromycin, kanamycin, nitrofurans and nitrosoguanidine amongst others. Treatment with these agents under appropriate conditions results in degeneration of chloroplasts to proplastids. Such bleached strains do not revert to chloroplast carriers. Gibor and Granick (1962a) report that the proplastids appear normal, but are incapable of differentiating into chloroplasts. These authors also reported the results of experiments with microbeams of U.V. light. Irradiation of the cytoplasm alone, shielding the nucleus, caused bleaching; irradiation of the nucleus alone did not cause bleaching. A healthy, unirradiated nucleus could not cure a bleached cytoplasm, and an irradiated nucleus did not cause bleaching of a healthy cytoplasm. The conclusion is that the determining factors do not reside in the nucleus. Lyman, Epstein and Schiff (1961) showed that the U.V. action spectrum for bleaching had a maximum at

260 μ i, suggesting that nucleic acid was the target, a conclusion reinforced by the known mode of action of some other bleaching agents.

The work of Sager and collaborators with streptomycin induced non-chromosomal mutants of C.reinhardi is also relevant here. Sager (1964, 1966) has given reviews of this. Growth of cells on agar containing sublethal concentrations of streptomycin produces cells which are mutant in any of several ways - streptomycin resistant or dependent, acetate-requirers unable to grow photosynthetically and other auxotrophic and slow growing types. The mechanism of the mutagenic process is not understood. The first non-chromosomal (NC) mutant was a streptomycin resistant one (Sager, 1954) which had the following properties:- transmission from one parent to all progeny (that is 4:0 segregation in tetrads), maintained through four backcrosses, stability in clonal growth in the absence of streptomycin, and stability to a very wide range of environmental variation. Such mutants did not arise in the absence of streptomycin (Sager, 1962a); and a large number have now been characterised as for the first streptomycin resistant mutant. Further work has established that NC genes show post meiotic segregation (i.e. in divisions after meiosis in the zoospore) but do not segregate at any particular division and also show independent assortment.

Sager considers that the NC genes are particulate carriers of primary genetic information and that in some cases linkage of NC genes is involved. However, the chemical basis and cellular localisation of NC genes is still unknown. It seems most likely that these genes may be located in the chloroplast or mitochondrion - many of them affect chloroplast function, and they have also been found to have a

intragenic mutation rate, comparable to that found in bacteria and bacteriophage. This would suggest an organisation that is different from a chromosomal one, as understood in higher organisms. The DNA of mitochondria has been shown to resemble that of bacteria in ultra-structural organisation (Swift, 1965). Sager and Ishida (1963) have shown the presence of DNA in the chloroplast of C.reinhardi, but its function remains unknown.

Many workers obtained evidence that chloroplasts contained DNA, but some of this work was open to criticism. Ris and Plaut (1962) have reviewed this earlier work. More recent work has left no doubt that the chloroplast does indeed possess DNA. Gibor and Izawa (1963) overcame the objection that DNA found in isolated chloroplasts might be contaminating nuclear DNA by isolating chloroplasts from enucleated Acetabularia. Their results clearly indicated the presence of DNA in the chloroplast. Other workers have used the CsCl density gradient method to detect differences in buoyant density of nuclear and chloroplast DNA. Besides Sager and Ishida (1963), Ray and Hanawalt (1964; 1965) Leff et al (1963) and Edelman, Schiff and Epstein (1965) demonstrated the presence of DNA in the chloroplast. Several other groups made similar findings with higher plants. On the basis of the above studies there can be no doubt that DNA of a distinct type occurs in the chloroplast.

Chloroplasts have also been reported to be able to synthesise RNA. Kirk (1964) reported that labelled ATP was incorporated into isolated bean chloroplasts, and that the reaction had all the characteristics of DNA-dependent RNA synthesis via RNA polymerase. He also showed that the chloroplast reaction was activated by different divalent metal ions to the nuclear RNA polymerase reaction. Schweiger and Berger (1964) made rather

similar findings with chloroplasts from enucleated Acetabularia. The results of Brawerman (1962) and Brawerman and Eisenstadt (1964) also suggest that RNA may be made in the E.gracilis chloroplast.

Brawerman (1962) found that the plastid RNA of E.gracilis was associated with ribosome-like particles, while Ishida and Sager (1963) found 70S ribosomes associated with the chloroplast of C.reinhardi. Lyttleton (1962) showed the presence of 66 S ribosomes in the chloroplasts of spinach leaves, while Clark, Matthews and Ralph (1964) and Clark (1964) reported the presence of polysomes in Chinese cabbage leaf chloroplasts. Sager (1964) and Swift (1965) give electron microscopic evidence for the presence of ribosomes in chloroplasts. Earlier, the presence of aminoacid activating enzymes in chloroplasts was reported (Bové and Raacke, 1959). It seems then, that chloroplasts are capable of protein synthesis via their own ribosomes, for as in the case of mitochondria, the organelle ribosomes are of the 70S type, compared to the 80S cytoplasmic ribosomes.

Finally, brief consideration will be given to centrioles and basal bodies (kinetosomes). The kinetosomes are recognised by Lwoff (1950) as specialised forms of centrioles. The centrioles of animal cells have not been observed to arise de novo but always arise from pre-existing centrioles. Mazia (1961) and collaborators have shown that a new complement of centrioles is formed during one division in readiness for the next; since chromosome replication does not appear to take place during division this implies that centrioles duplicate independently of the chromosomes. Lwoff (1950) reached the same conclusion for the kinetosome; indeed he maintains that this is the model self-reproducing particle. In the case of centrioles

reproduction is by outgrowth on a molecular scale at right angles to the original structure - that is, the parent is used as a pattern for the assembly of a daughter particle, but the evidence is not so clear in the case of kinetosomes.

The idea that DNA contained in the centriole or basal body might be responsible for its precise duplication has been current for some years. Seaman (1959) isolated kinetosomes in bulk from T.pyriformis and claimed to find DNA, but his preparations were very poor. A later report (Seaman, 1962) is subject to the same criticism. Other experiments using cytochemical methods (Randall and Jackson, 1958; Randall, 1959) also gave ambiguous results. More recently Randall and Disbrey (1965) using fluorescence microscopy and autoradiography have obtained results suggesting that DNA may be present in small quantity, but results are still uncertain. Work in this field has been hampered by the lack of good isolation methods for kinetosomes; several workers have made some progress (Argetsinger, 1964; Hoffman, 1965; Satir and Rosenbaum, 1965) but preparations are still poor; in particular there is morphological alteration from the in vivo state. Accurate analysis is thus not yet available.

Little or nothing is known of any genetic continuity of other cell organelles such as Golgi bodies and lysosomes.

From this review of the genetic potentialities of mitochondria, chloroplasts and kinetosome, several conclusions may be drawn. The first is that these organelles are self-reproducing and do not arise de novo; daughter organelles are produced from pre-existing ones with great frequency and accuracy. If de novo origin does occur, it must be on a

small scale or be from structures below the resolution of the electron microscope. Many claims of the observation of de novo origin of mitochondria and chloroplasts have been made; but some are contradictory. For mitochondria, Novikoff (1961) has critically evaluated some claims, stressing the uncertainty of determining movements of organelles from static pictures such as electron micrographs. The evidence in the case of kinetosomes is much poorer, and de novo origin is still a possibility.

Secondly, the occurrence of DNA in these cell organelles constitutes a powerful argument that they are at least partially autonomous. Of particular relevance here are the findings that the DNA is able to replicate itself (Chiang, Kates and Sueoka (1965) have shown semi-conservative replication of the chloroplast DNA of C.reinhardi) and appears to show no homology to the nuclear DNA. Shipp, Kieras and Haselkorn (1965) have shown that the chloroplast DNA of tobacco leaves has no homology to the nuclear DNA, and Dawid (1965; 1966) has made a similar finding with frog-egg mitochondrial DNA. Thus it seems that these organelles possess DNA which is not just an on-site controller for the nuclear DNA but appears to be an active determining agent for the organelle. The work of Woodward and Munkres (1966) with N.crassa MSP and the work of Sager's group on C.reinhardi are particularly relevant here as is the work on E.gracilis. Swift (1965) has also stressed the results of autoradiography.

Thirdly, the presence of an RNA synthesising system and a protein synthesising ^{system} implies further autonomy, though these systems would be expected to be present if the DNA has the functions that are indicated. What is significant is the finding that organelle ribosomes are smaller than those found in the cytoplasm. This is certainly true in

the case of the chloroplast, and very likely in the case of the mitochondrion. It is well-known that bacterial and blue-green algal ribosomes are smaller than those of other organisms, having a sedimentation coefficient of about 70S, while those of other organisms are about 80S. Organelle ribosomes appear to be of this 70S class. This enhances the idea of degenerate symbiosis for it does not seem likely that the cell would make 70S ribosomes solely for use in the organelles. It seems a better argument that a symbiont possessing this type of ribosome should keep it, even though its functions were becoming more specialised and circumscribed in the course of evolution.

In summary, it may be said that chloroplasts and mitochondria possess the essential mechanisms for the autonomous continuation of existence. They contain DNA which appears able to replicate, can direct the synthesis of RNA and specify protein, which can be synthesised by an independent mechanism. They can carry out the basic functions of life. It could be permissible to regard such an organelle as an organism - after all a virus cannot carry out the functions that a chloroplast or mitochondrion can - and obviously does, with great frequency and accuracy.

How the mu particle may fit into this picture is clearly indicated. It too possesses DNA, can synthesise RNA, and may be able to synthesise protein. In addition, it is clear that it has affinities with free-living bacteria. But its only known habitat is the Paramecium cytoplasm, just as a chloroplast or mitochondrion only has the cytoplasm of the cell in which they are contained as a habitat. Where the mu particle does differ from these organelles is that it lacks their specialization of function. Enzymes specified by nuclear genes have become localised on them with the

result that these organelles may be regarded almost as frameworks that hold enzymes, substrates and cofactors in the correct places for efficient functioning. No such localisation of function has taken place on the mu particle, for as Paramecium can survive and reproduce without them they cannot perform any essential function, though they may be able to carry out some synthesis the host cell cannot, as the lambda particle can. But integration may be proceeding; perhaps the mu particle cannot revert to a free-living state, while factors may be evolving in the Paramecium to result in an inseparable relationship.

There are many problems remaining. Much remains to be done on the biochemical abilities of the mu particle and how these fit in with the activities of the host cell. It would be of great interest to find out if mu particles could carry out some metabolic functions that the host cell cannot, as in the case of the lambda particle, likewise to know whether there are deficiencies in the metabolism of the mu particle that are remedied by activities of the Paramecium. Unfortunately the greatest aid to such studies, an axenic culture medium for syngen 1 of P.aurelia, is at present lacking. It would also be of interest to elucidate the mechanism of the process of mate-killing, but a fruitful approach is difficult to devise. Despite the technical drawbacks, however, such studies would be of value in providing an approach to an understanding of the way in which the complex and well-regulated cell machinery has evolved.

APPENDIXCell-fractionation of Paramecium aurelia.INTRODUCTION

In the course of the studies described in the present thesis numerous cell-fractionation experiments were carried out and several cell organelles were isolated from homogenates. The organelles which have been isolated are macronuclei, mitochondria, ribosomal monomers and, of course, mu particles. It was felt that it would be worthwhile to assemble the details of the methods used and the results achieved so that they might serve as a basis for further work which might involve the use of isolated cell organelles.

Cell-fractionation in Paramecium presents some difficulties, principally due to the large number of types of particulates in the homogenates. Examination of a homogenate under the phase-contrast microscope shows the presence of free cilia, discharged trichocysts, large portions of body wall (sometimes including whole gullets), kinetosomes, mitochondria, lysosomes, fragments of cell-membranes, macronuclei and micronucleic and cytoplasmic crystals. Also present are ribosomal monomers and polysomes (Sommerville, 1967) and residual food bacteria if monoxenic culture has been used.

Faced with this diversity of particulates, simple centrifugal separation is unsatisfactory, as Preer and Preer (1959) found. For example, many cell-fractionation schemes involve centrifugation at 500-1,000 g., followed by centrifugation at 5,000-15,000 g., yielding pellets

which have been designated 'nuclear' and 'mitochondrial'. Applied to Paramecium, it is found that the 'nuclear' pellet contains macronuclei, (but apparently no micronuclei), body wall and gullets, cytoplasmic crystals and very small amounts of smaller cell organelles, presumably carried down by the larger matter, while the 'mitochondrial' pellet contains mitochondria, cilia, trichocysts, kinetosomes, lysosomes, some cell membrane and the residual food bacteria. Such fractions are clearly of no value for definitive biochemical studies, being too crude and heterogeneous, and it is therefore necessary to apply further methods of cell-fractionation. The density gradient has proved to be a versatile method, while separation of nuclei using sedimentation in concentrated sucrose solutions has also proved satisfactory. In this appendix, methods for the isolation of macronuclei, mitochondria and ribosomal monomers are detailed.

EXTRA STRONG

MATERIALS AND METHODS(1) Isolation of macronuclei

Animals were grown, harvested and washed using the methods described in the main body of this thesis, with the exception that the growth temperature was 31°.

Washed cells (4-20 gms. wet weight) were suspended in 50 volumes of 0.1% V/V Tween 80-0.001 M CaCl₂ and stirred using either an MSE homogeniser operating at half-speed or an MSE "Ato-mix" blender (MSE Ltd., London) operating on reduced voltage (65-80 V). After 4 to 7 minutes stirring, 70-90% of the macronuclei were liberated. Nuclear release was checked, and purification monitored, by staining with acetocamine-fast green, and examining at 80 x magnification.

The homogenate was centrifuged at 600 g. for 6 minutes (MSE MS 18 centrifuge), and the supernatant discarded. The pellet was washed twice with 0.25 M sucrose-0.001 CaCl₂, and recentrifuged as above. The nuclei were freed of contaminating unbroken cells, cytoplasmic debris and enucleate remains ("pellicles") by resuspension in 2.4 M sucrose-0.001 M CaCl₂ and centrifugation at 40000 g. average in the 3 x 40 ml swing out rotor of the MSE "Superspeed 50" ultracentrifuge. Macronuclei formed the pellet, while contaminating material floated. The pellet was washed twice with 0.25 M sucrose-0.001 M CaCl₂, or, if ribonucleoside triphosphate incorporation was to be assayed, with 0.25 M sucrose. All the preparative steps were carried out at 0-4°. The method is based on those of Fisher & Harris (1962) and Widnell and Tata (1964).

(1) Other methods with macronuclei

DNA was estimated by the method of Burton (1956). RNA by the orcinol reaction (Ogur & Rosen, 1950) and protein by the method of Lowry et al (1951). Assay for ribonucleoside triphosphate incorporation was carried out by the method of Weiss (1960) with modifications as described in the main part of this thesis.

Electron microscopy of isolated macronuclei was carried out by Dr. A. Jurand. The nuclear pellets were fixed in sodium barbital-sodium acetate buffered 1% OsO₄ + sucrose to make isotonic; 0.3 mg/ml. CaCl₂ was added to the fixative to improve preservation of membranes. Fixation was at room temperature for 30 minutes.

Small clumps of fixed nuclei were taken, dehydrated through ethanol, and then embedded at the top of an araldite capsule. The embedded nuclei were sectioned, stained with 1% KMnO₄ + 2.5% uranyl acetate, and the sections were rinsed with 0.25% citric acid. They were examined in the Philips EM 75 electron microscope.

(2) Isolation of mitochondria

Animals were grown, harvested and washed as before. The packed animals (3-8 ml.) were resuspended in 4 volumes of 0.44 M sucrose - 0.001 M EDTA and homogenised using a Tri-R stirring motor at about 3500 rpm for 3 minutes. The homogenate was centrifuged at 400 g. for 5 mins., and then the supernatant was centrifuged at 1000 g. for 15 minutes. The pellet was resuspended in 12 ml. of 0.44 M sucrose - 0.001 M EDTA and 4 ml. was layered on to each of 3 sucrose gradients. The gradients were 27-60%

sucrose (in 0.001 M EDTA) and the volume of each gradient was 34 ml. Gradients were prepared using a mixing device of the type described by Britten & Roberts (1960). Centrifugation was for 4 hours at 1° at 2000⁰ rpm (45000 g. average) in the 3 x 40 ml. swingout rotor of the MSE "Superspeed 50" ultracentrifuge. After coasting to a stop, tubes were removed and the gradients were fractionated using the MSE tube piercer and pumping the contents out. 11 fractions were collected using a drop-counting fraction collector. There was generally a small pellet; this was resuspended in 3.0 ml. 0.44 M sucrose - 0.001 M EDTA. Mitochondria formed a distinct band at a sucrose concentration estimated to be 53%.

Succinic dehydrogenase (SDH) was assayed on each fraction as described by Bonner (1955); this method estimates the reduction of potassium ferricyanide, measured as decrease of absorbance at 400 m μ in the Beckman DB spectrophotometer. Protein in fractions was measured by the method of Lowry et al (1951) and DNA by the method of Burton (1956).

(3) Isolation of ribosomal monomers

This work was done as a control for some work on ribosomes in the mu particle. Washed packed cells were resuspended in 5 volumes of 0.1 M tris-HCl pH 7.4, 0.025 M KCl, 0.005 M MgCl₂ (TKM) and homogenised with a "Tri-K" stirring motor for 1 minute. The homogenate was centrifuged at 3000 g. for 10 minutes, the pellet was discarded and 0.5 ml. of the supernatant was loaded onto a 32 ml. 5-20% sucrose gradient in TKM. Gradients were prepared as described before. They were centrifuged at 15000 rpm (24000 g. average) for 16 hours at 3° in the 3 x 40 ml. swingout rotor of the MSE "Superspeed

50". After coasting to a stop, gradients were fractionated in a similar manner to those used for preparing mitochondria except that 16 fractions were normally taken. Absorption at 253 m μ was estimated using the LKB "Uvicord" flow-cell (LKB Produkter, Sweden) and recorded using a Beckman potentiometer recorder.

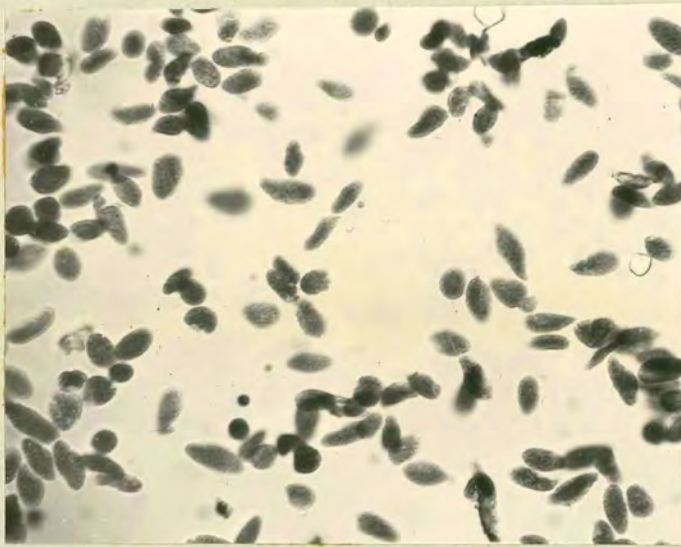


FIGURE A1

Photomicrograph of isolated macronuclei of *P. aurelia*, stained with acetocarmine-fast green. X160

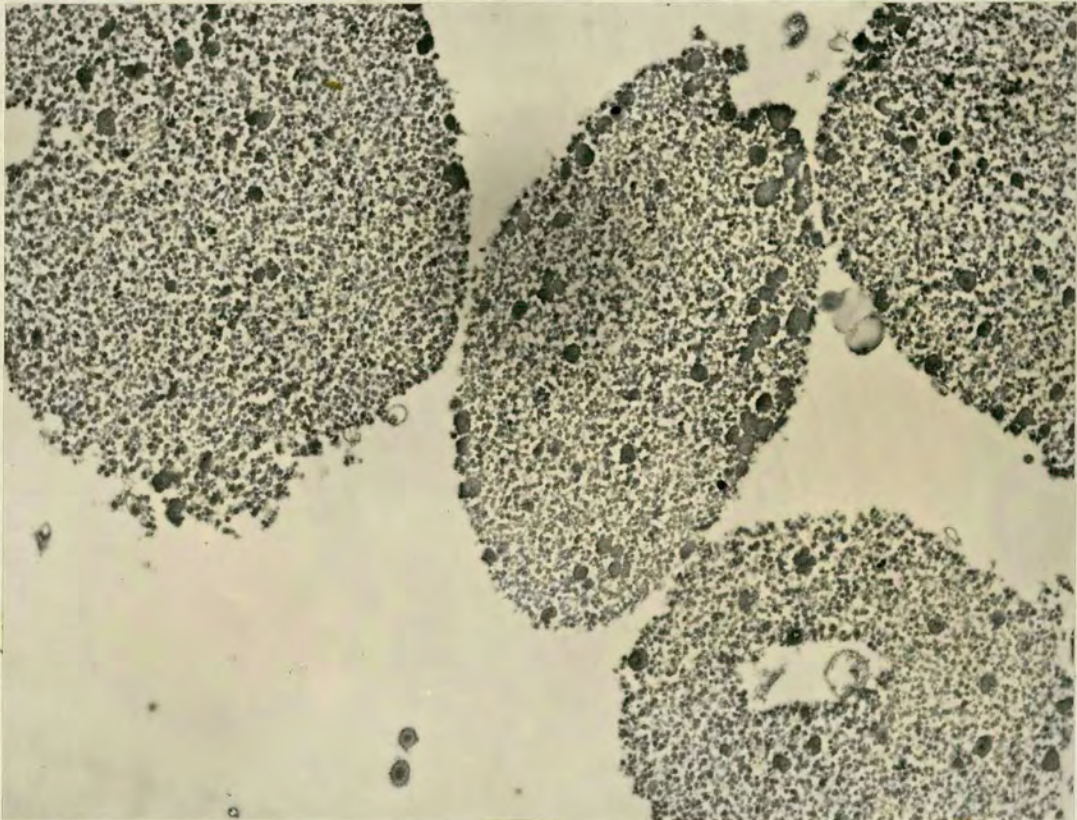


FIGURE A2

Electron micrograph of isolated macronuclei of *P. aurelia*, prepared as described in the text. The macronuclei have lost their membranes, and some breakage has occurred. The "matrix" is less electron dense, but the large and small bodies are well-preserved. Philips EM 75, X5400.

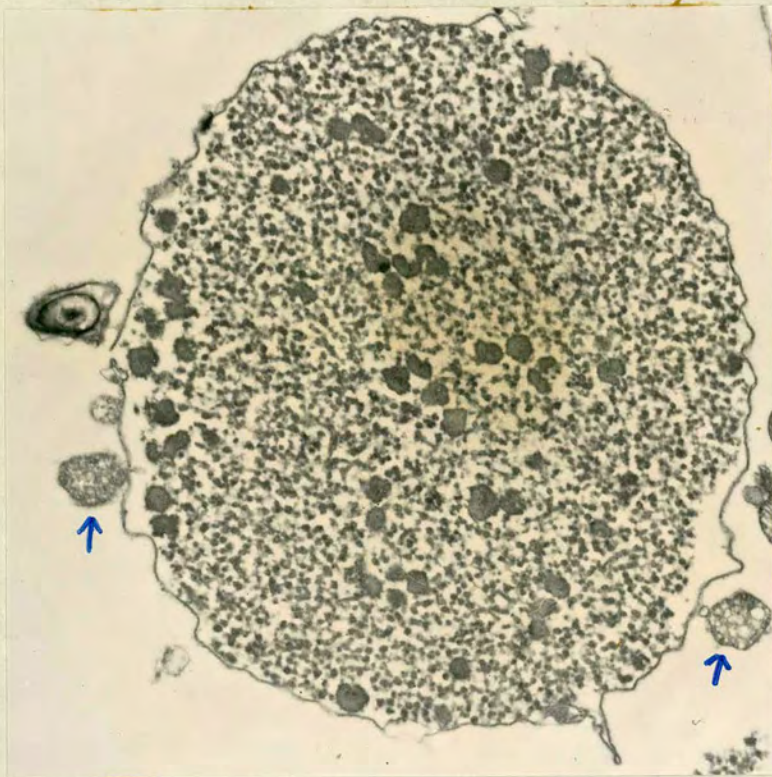


FIGURE A3

Electron micrograph of isolated macronucleus of P. aurelia, similar to figure A2, except that the nuclear membrane is better preserved, and the internal structure is also better. Contaminating abnormal mitochondria are attached to the membrane (arrows). Philips EM 75, X9000.

RESULTS(1) Isolation of macronuclei

Figure A1 is a photomicrograph of the final preparation of macronuclei, stained with aceto-carmin-fast-green. The macronuclei have retained the variation in shape typical in vivo, and seem to have undergone little fragmentation. A small amount of cytoplasmic debris is present but intact cells are not found.

Figures A2 and A3 are electron micrographs of the isolated macronuclei. Figure A2 shows that while the macronuclei have lost the majority of the outer membrane, and the matrix has become less electron dense, the fine structure is well-preserved, the large and small bodies described by Jurand, Beale and Young (1962) being very evident. The loss of the membrane is common in aqueous methods of extraction. Figure A2 also shows that the principal contaminating material consists of the cytoplasmic crystals that are commonly found in Paramecium. Although these are very evident in the electron micrographs they are only rarely visible in the preparations under the light microscope. Figure A3 shows a macronucleus in a better state of preservation, having retained the nuclear membrane almost intact. Also visible is another occasional contaminant - grossly abnormal mitochondria. A further contaminant of the preparations of macronuclei was whole gullets of Paramecium, though there were never more than 1-2% of these. However, it is evident that this preparation method produces macronuclei in a fairly good state of preservation and quite high purity. The yield, estimated by the recovery of DNA, was 30 to 50%.

TABLE A1

COMPOSITION OF P. AURELIA MACRONUCLEI AND WHOLE CELLS IN TERMS OF DNA,
RNA AND PROTEIN

	DNA (%)	RNA (%)	PROTEIN (%)
Macronuclei of stock 540 sensitive animals	4.4	16.9	78.6
Stock 540 sensitive whole cells	1.6	10.7	87.7

DNA, RNA and protein were estimated by the methods described, and the amounts of each, in $\mu\text{g/ml}$ of suspension, were added together, and the total set to 100%. Results were expressed as percentages of this total.

TABLE A2

INCORPORATION OF C^{14} ATP AND C^{14} UTP INTO ACID-INSOLUBLE MATERIAL BY
ISOLATED MACRONUCLEI

Additions/omissions to assay system	μM ATP incorporated	% inhibition by treatment	μM UTP incorp.	% inhib
None (complete system)	56.3	0	50.1	0
No CTP	15.1	73.2	--	--
No GTP	14.1	75.0	--	--
No UTP	20.1	62.9	--	--
No triphosphates	38.6	31.4	--	--
+ deoxyribonuclease*	33.7	40.1	33.1	34.0
+ ribonuclease ⁺	4.7	91.6	--	--
+ actinomycin D ⁺	30.7	45.5	0	100
No ATP	---	---	13.4	73.2
None, then post-incubation with 50 $\mu\text{g/ml}$ DNase.	50.0	11.1	--	--
None, then post-incubation with 10 $\mu\text{g/ml}$ RNase.	0.0	100	0.0	100

*DNase, 50 $\mu\text{g/ml}$. ⁺RNase, 10 $\mu\text{g/ml}$. ⁺Actinomycin, 12.5 $\mu\text{g/ml}$.

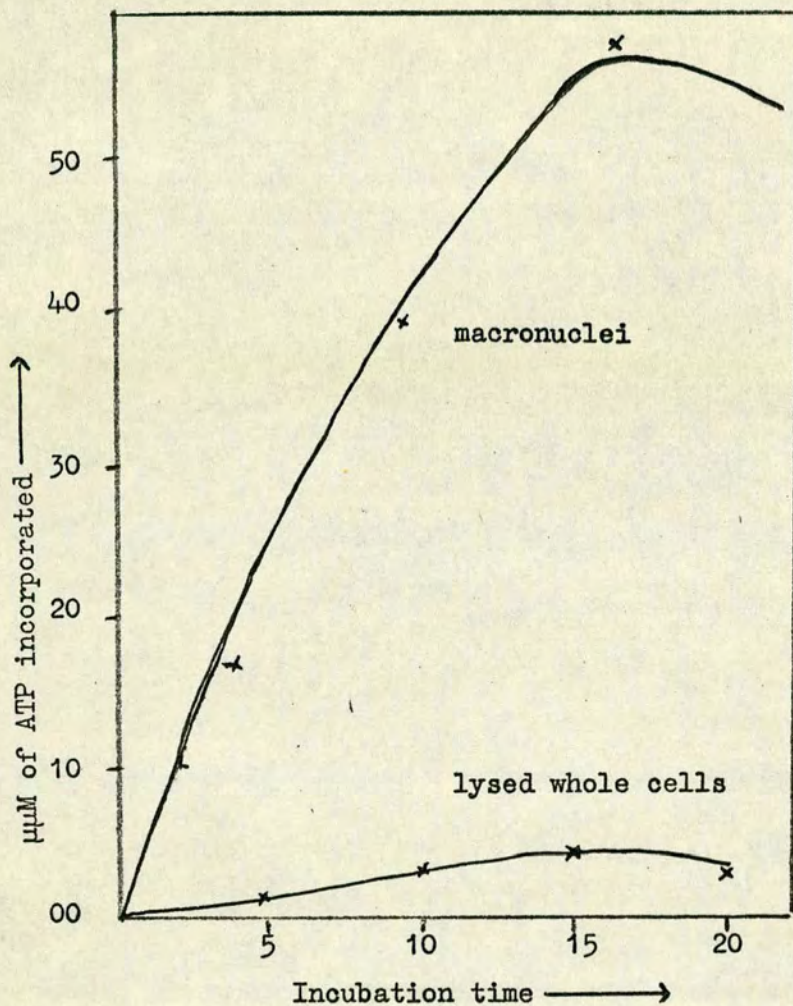


FIGURE A4

Time-course of the incorporation of ribonucleoside triphosphate by isolated macronuclei and freeze-thaw lysed whole cells. 8-C¹⁴-ATP was used as the labelled triphosphate. Conditions otherwise were as described in the text.

(2) Composition of macronuclei

Table A1 gives data on the composition of macronuclei in terms of DNA, RNA and protein. The figures may not be representative of the true composition of macronuclei, due to losses and exchanges with the cytoplasm, which always occur with aqueous purification methods (Allfrey et al, 1952).

(3) Ribonucleoside triphosphate incorporation by macronuclei

Data are presented in Table A2 on the incorporation of (8-C¹⁴) ATP and (4-C¹⁴) UTP into acid-insoluble material, using the assay mixture described. The amount of incorporation (in terms of $\mu\mu\text{M}/\text{mg}$. protein) was variable from experiment to experiment but it was consistently observed that if macronuclei were prepared from cells that were entering autogamy, incorporation was lower. The cells in experiment II (Table A2) were 10-15% in autogamy while those in experiments I and III were less than 5% in autogamy. In experiments I and II, (8-C¹⁴) ATP was used and in experiment III (4-C¹⁴) VTP.

Figure A4 shows a time-course of incorporation and shows that the macronuclei had a much higher rate of incorporation than a whole-cell preparation (about 25 fold more). There may also have been some loss of activity from the nuclei, as the supernatant of the 600 g. centrifugation, used to pellet the nuclei, also had some incorporating activity, but whether this was due to loss from the nuclei or contamination with residual food-bacteria is not known.

However, it is evident that the macronuclei prepared by this method are structurally and functionally well preserved.

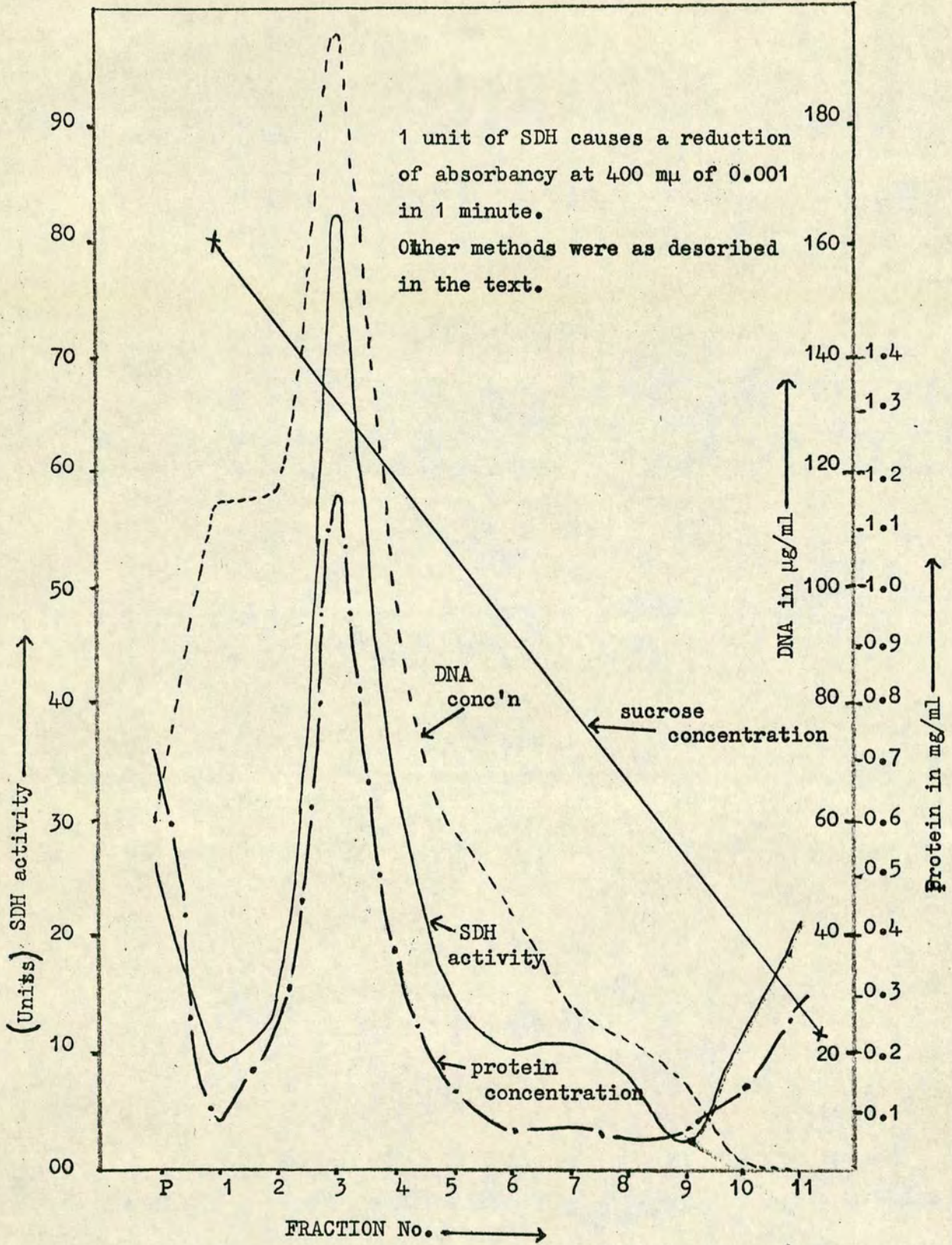


FIGURE A5

Sucrose gradient preparation of mitochondria. 27 to 60% gradient in 0.001M EDTA. Centrifuged for 4 hrs at 20000 rpm at 1°. 11 fractions + pellet (P).

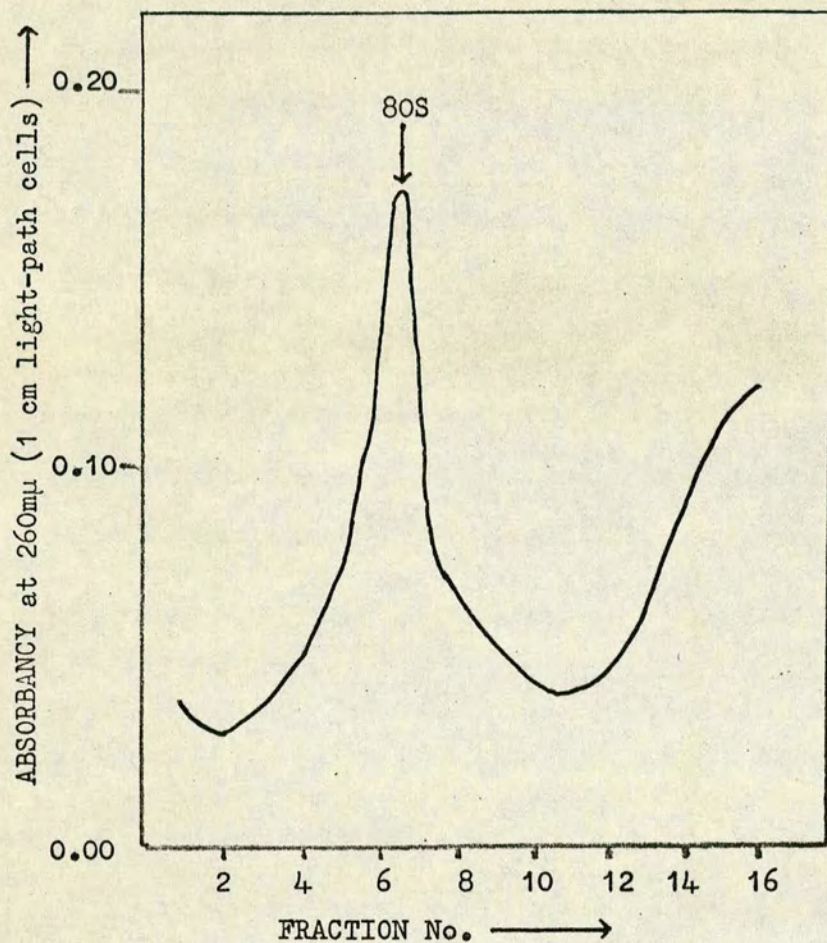


FIGURE A6

Sucrose gradient centrifugation of a 30000g supernatant of a Paramecium homogenate, prepared as described in the text. 5 to 20% sucrose gradient (32ml), load 0.5ml of supernatant. Centrifuged 16 hrs at 15000 rpm at 3°. 16 2ml fractions, the absorbancy at 260mμ being read in 1 cm light-path cells.

(4) Isolation of mitochondria

Figure A5 shows the result of a sucrose gradient preparation of mitochondria, using succinic dehydrogenase, known to be associated with the Paramecium mitochondria (Watanabe et al 1954), as marker. It is evident that there are coincident peaks of SDH activity, total protein and DNA in tube 3. This result also suggests that DNA is present in, or associated with, isolated mitochondria, but attempts to isolate and characterise this DNA resulted in failure.

Examination of the peak fractions from a sucrose gradient under the phase-contrast microscope showed that the preparation consisted very largely (>90%) of mitochondria, either in the native rod-shaped state (Preer & Preer, 1959) or somewhat swollen. Principal contamination appeared to be broken cilia and a few basal bodies.

(5) Isolation of ribosomal monomers

Figure A6 shows the result of a sucrose gradient centrifugation of 30000 g. supernatant of a Paramecium homogenate. The main peak has a sedimentation coefficient of 80S and would seem to consist almost entirely of ribosomal monomers, as no shoulders or separate peaks are evident. Sommerville (1967) and Reisner and Macindoe (1967) have shown that polysomes are present in P.aurelia and have identified them on sucrose gradients. These workers have also shown that the ribosomal monomer of P.aurelia has a sedimentation coefficient of 80S.

DISCUSSION

The methods described here for the preparation of organelles have been found necessary in order to obtain preparations in which contamination with other organelles is reduced to a low level.

The method of preparation of macronuclei is less laborious than that of Skoczylas et al (1963), and probably gives a better yield, but comparison is difficult in view of the different methods of estimation used. It appears also to be the first published method for the isolation of macronuclei that yields a biosynthetically active preparation, for although Rosenbaum & Holz (1966) tested isolated macronuclei of Tetrahymena pyriformis for aminoacid activation ability their results were negative; however, this may have been due to leaching out of components for their preparations were active before washing.

The DNA content of isolated macronuclei is low, compared to values reported for some mammalian nuclei (Allfrey et al, 1952; ~~Lovtrup-Rein~~ & MacEwen, 1966), but it is higher than the content reported for yeast nuclear bodies (Rozijn & Tonino, 1964). The low level of DNA may be due to losses from the DNA-containing region near the outer boundary of the macronucleus, described by Jurand, Beale and Young (1962). It is also evident that, from the electron micrographs, ~~the~~ the "matrix" as described by these authors surrounding the large and small bodies of the macronucleus is low in electron density; they found it to be considerably higher, indicating loss of DNA. If loss of DNA is occurring to a significant degree, then the overall yield will be better than the 30 to 50% estimated by recovery of DNA in the macronuclei. The RNA content is high; this may

be due to absorption from the cytoplasm, or may indeed reflect the true composition, as Gorovsky (1965), using electron microscope cytochemistry, found that the macronucleus of T.pyriformis was very RNA-rich. However, to gain unambiguous data, macronuclei will have to be prepared by non-aqueous methods, as only these methods preclude exchanges of material between the nucleus and the cytoplasm (Allfrey et al. 1952).

The isolated macronuclei contain an RNA-synthesising system which seems to be similar to those described in other organisms, for example by Weiss (1960, 1962) and Semal, Spencer, Kim and Wildman (1964) in its DNA dependence, requirement for all four ribonucleoside triphosphates, and inhibition by actinomycin D. An interesting feature of this system was the lessened inhibition of incorporation observed when three triphosphates were absent, compared to that observed when only one triphosphate was absent (Table A2). This suggests synthesis of the homopolymer of the supplied labelled triphosphate; a similar effect was observed by Moyer, Smith, Semal and Kim (1964) with tobacco leaves. The variability of inhibition by actinomycin D is difficult to explain: all concentrations of actinomycin between 10 and 50 $\mu\text{g/ml}$ gave a similar amount of inhibition in any one preparation. It may be that ability to bind actinomycin varies from preparation to preparation, possibly due to slight differences of treatment during isolation.

The isolation of macronuclei in a metabolically active condition is a useful step, permitting study of synthesis and metabolism in the macronucleus. The isolation method could be improved, as it does yield

a preparation which is quite significantly contaminated and is not very well preserved; although sufficient of the fine structure would seem to remain for autoradiographic studies of synthesis and metabolism; however the preparations are adequate for biochemical studies also. The defects of the method are those of all aqueous methods - namely leaching out of components, and loss of membrane.

Of the isolation method for mitochondria, there is little to be said. In general, the method is similar to that used by Luck (1963, 1965) to isolate mitochondria from Neurospora crassa, and a similar method has been employed by other workers, for example Rabinowitz et al. (1965) and Dawid (1966). Results similar to those obtained in studies such as these have been obtained. Microscopically they appeared normal, and they did possess SDH activity, as found by Watanabe et al. (1954). They appeared to possess a rather high amount of DNA, the ratio of protein to DNA being about 10 to 1, but this could be an artificial result due to the inaccuracy of the method of Lowry et al (1951) for the estimation of protein. As this method effectively measures the tyrosine and tryptophan content of the protein, it may be that the tyrosine and tryptophan contents of the standard protein, bovine serum albumin, and the Paramecium mitochondrion, are not comparable. However, the coincidence of peaks of SDH activity, protein and DNA content, and observations with the phase-contrast microscope, all indicate that this is a useful preparation method for mitochondria. Indeed, it is necessary to isolate mitochondria by some method such as this to obtain a homogeneous preparation.

The isolation of ribosomal monomers was principally designed as a control experiment for some other work with ribosomes from mu particles. The sucrose gradient is a widely used method for the fractionation of ribosomes and their sub-units, and the results here merely demonstrate its general applicability. The work of Sommerville (1967) has more extensive use of this method, and includes separation of various ribosomal classes.

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