ELECTRON MICROSCOPY OF THE CILIATE PROTOZOA

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SUMMARY

1. The ultrastructural localization and synthesis of a group of proteins, the immobilization antigens, has been investigated in Paramecium aurelia.

2. A technique for the preparation of tissues, suitable for the ultrastructural localization of cytoplasmic antigens, has been developed. Paramecia fixed in osmium tetroxide at 37°C are equilibrated with 12.5% glycerol, and frozen sections of this aqueous suspension of cells are cut at -30°C. This technique results in minimal cell destruction, yet provides access for antibodies to the interior of the cell.

3. Three antibody markers have been used in this investigation, fluorescein (Coons et al. 1941), ferritin (Singer 1959), and iodine (Johnson et al. 1960). The limitations of each marker have been investigated. Iodine^{125} conjugated antibodies, at an iodine:globulin ratio of 1:1, have been found to be the most useful. Unlike either fluorescein or ferritin, there was no detectable loss in antibody activity or specificity, no change in the charge of the globulin, and finally no appreciable change in the size of the globulin due to conjugation. A quantitative analysis of autoradiographs has been described which allows a comparison of the reactions of different iodinated globulins with tissues, irrespective of the absolute retention of radioactivity.

4. Extensive non-immune labelling has been encountered. This is believed to be a structural adsorption (Klotz 1953) rather than an electrostatic adsorption (Mayersbach 1959, Mayersbach and Schubert 1959). A second non-immune reaction has been described, namely globulin-globulin adsorption. The latter reaction imposes severe restrictions on the use of any two-stage

immunological technique.

5. Using conjugated globulins, from antibodies prepared against purified immobilization antigens, the only conclusively demonstrable immobilization antigen *in situ*, is found on the outer surface of the pellicle and cilia. Only one immobilization antigen has been detected in cells from stable cultures: no other "secondary immobilization antigens" have been found with a similar or differing ultrastructural distribution.

6. The presence of immunologically active immobilization antigen in the cytoplasm has been demonstrated by the selective precipitation of ribosomal material on sucrose gradients, using iodine^{125} conjugated antibody prepared against purified immobilization antigen.

7. The incorporation of Sulphur^{35}, from radioactively labelled bacteria into *Paramecium aurelia*, has been followed by electron microscope autoradiography. The incorporation of isotope into the pellicle and cilia is compatible with a flow of precursor molecules from the food vacuole to the cytoplasm, and from the cytoplasm to the pellicle. Under normal culture conditions the isotope is incorporated into the pellicle, and also into the immobilization antigen, in less than four minutes. The density of the isotope in the pellicle has been shown to be related to the incorporation of isotope into the immobilization antigen, as shown by scintillation counting of specific antibody-antigen precipitates in immunoelectrophoresis gels.

8. During serotype transformation in stock 168, from serotype 168G to 168D, the simultaneous demonstration of the incorporation of 168D antigen

and newly synthesised radioactive protein in the pellicle and cilia (using ferritin conjugated antibody and electron microscope autoradiography, respectively), indicated that all the 168D antigen was synthesised after the stimulus to transform was given. It is concluded that immobilization antigen expression is controlled during transcription or translation, or both, and not by selective protein distribution.

9. The synthesis and incorporation of 168D antigen into the pellicle was first detected thirty minutes after the stimulus to transform was given, indicating a slowing down in the general metabolism of the cell during serotype transformation. The synthesis of antigen, characteristic of the original conditions of culture (168G antigen), continued for five to twenty-eight hours after the synthesis of the new antigen had begun.

10. The ultrastructural localization and synthesis of immobilization antigen has been considered in relation to other systems similarly analysed, and a possible ultrastructural pathway of immobilization antigen synthesis has been proposed. The control of immobilization antigen synthesis during serotype transformation has been discussed.

INTRODUCTION

Two species of ciliate, Paramecium aurelia and Tetrahymena pyriformis have contributed, perhaps more than any other species, to an understanding of the biochemical and structural organisation of this class of protozoa. One of these, Paramecium aurelia has been subjected to extensive examination in the electron microscope, and has been shown to possess an exceedingly complex ultrastructural anatomy (see Jurand and Selman 1969).

In recent years there have been dramatic advances in the field of molecular biology, and many biological processes, notably deoxyribonucleic acid (DNA) synthesis and protein synthesis, have been described in molecular terms. It has become common practice to discuss these events in terms of the ultrastructural organisation of the cells as shown by electron microscopy. However, until very recently there have been relatively few attempts to demonstrate biochemical processes using the electron microscope. Three basic techniques, all developed from light microscopy, have been exploited. These are; the localization of enzymes (see review of Holt and Hicks 1962), the localization of antigenic substances using various electron-dense antibody markers, such as ferritin (Singer 1959) mercury (Pepe 1961) or haemocyanin (Eskland 1967), and finally the investigation of the biosynthetic incorporation of radioisotopes by autoradiography (Caro and van Tubergen 1962).

Paramecium aurelia has been used exclusively as the subject of this investigation. In particular an attempt has been made to localize the sites of synthesis and structural integration of a group of immunologically identifiable substances - the immobilization antigens, within the ultrastructure of this organism. These immobilization antigens have been studied in great detail (Sonneborn 1950, Beale 1957). It has been shown that they vary between different stocks, and also within a stock depending on the conditions of culture. Any one stock is capable of synthesising as many as twelve different immobilization antigens, but usually only one is synthesised at any one time. For example stock 168 of syngen 1, expresses serotype 168S below 18°C, 168G between 18°C and 25°C, and 168D above 25°C.

The immobilization antigens are known to be proteins (Preer 1959b), with a molecular weight of 310,000. They are believed to contain three identical trimers with a molecular weight of approximately 106,000, each of these subunits consisting of three different polypeptide chains of molecular weight 35,000 (Steers 1964). The observed immunological variation between different immobilization antigens has been correlated with differences in the electrophoretic mobility (Bishop and Beale 1960), and the peptide composition of the isolated antigen molecules (Jones and Beale 1963, Steers, 1962).

The immobilization antigens have been shown to be localized in the pellicle and cilia by cell fractionation techniques (Preer & Preer 1959), and associated with the outer surface of the pellicle and cilia membranes by fluorescence microscopy (Beale & Kacser 1957, Beale & Mott 1962) and immune electron microscopy (Mott 1963). Subsequently Sommerville (1967), using cell fractionation techniques has found evidence for the presence of newly synthesised antigen on membrane bound ribosomes.

The control of immobilization antigen expression has been thoroughly investigated (see reviews of Beale 1954, 1957, 1964). Antigenic specificity

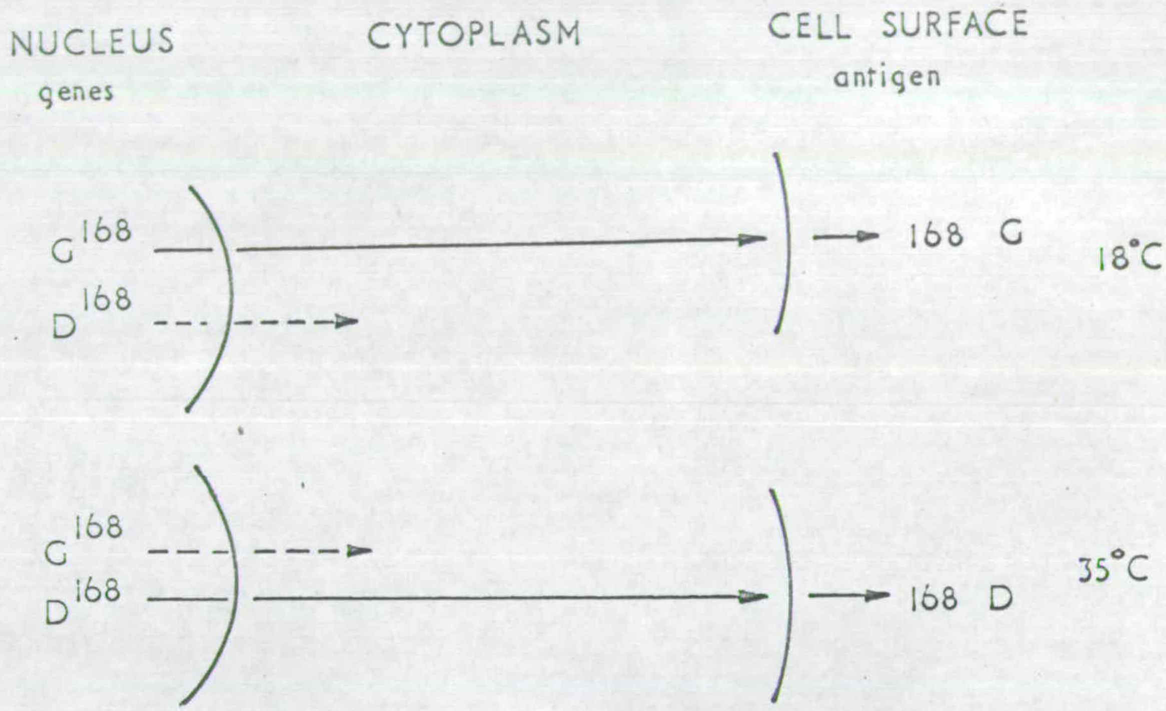


FIG.1 Diagram to show the effect of temperature on the expression of two of the immobilization antigens that may be synthesised by *Paramecium aurelia* of stock 168.

is determined by a series of unlinked genes, whose expression is controlled by both the cytoplasm and the environment. Under certain conditions, such as a change in temperature, or the presence of chemical agents e.g. patulin (Austin et al. 1957), transformation from one serotype to another takes place. This has been shown to be caused by a process involving the "switching-off" of one antigen determining gene and the simultaneous "switching-on" of another, although the exact mechanism of this switch, in molecular terms, is still not understood (see fig. 1).

The successful application of the ferritin conjugated antibody technique to the localization of the immobilization antigen on the outer surface of the pellicle (Mott 1964), contrasts with the unsuccessful attempt to detect immobilization antigens in the cytoplasm of frozen sections using the relatively insensitive fluorescent antibody technique (Beale and Mott 1962). However, in view of the recent work of Macindoe and Reisner (1967) and Sommerville (1967), who have found evidence for the association of immobilization antigens with ribosomal material, the greater part of the work to be described here has been the attempt to demonstrate the ultrastructural localization of the immobilization antigens throughout the cell, using various conjugated antibody techniques.

The results of such exclusively immunological techniques have two inevitable limitations. The first is that only substances possessing the same immunological characteristics as the complete immobilization antigen can be detected by the conjugated antibody - this may exclude the localisation of cytoplasmic immobilization antigen precursors. The second is that the information obtained does not give any indication of the movement of the antigens, during synthesis, from one structure to the next. Both these limitations have been avoided by a series of investigations in which the incorporation of various radioactive precursors, from the food bacteria (Aerobacter aerogenes) into paramecium, has been followed by electron microscope autoradiography. This technique has been used successfully for similar investigations in other tissues e.g. collagen synthesis in fibroblasts (Ross and Benditt 1965), protein renewal in mitochondria (Bergen and Droz 1969), protein renewal in retinal rods (Young and Droz 1968), and lipid synthesis (Stein and Stein 1968). In the experiments to be described here the passage of radioactive isotope has been followed through the various organelles of paramecium, and the movement of the isotope related to the synthesis of the immobilization antigen.

The possibility of making direct measurements on the reactions of both ferritin and iodine^{125} conjugated antibodies, (the latter developed during this investigation), with the different organelles of paramecium, notably the pellicle, has allowed certain investigations to be made into the fundamental problem of the control of immobilization antigen expression. The control of immobilization antigen expression has been studied not only in cultures of stable serotype, but also during serotype transformation where particular attention has been paid to the very early synthesis and incorporation of the immobilization antigens into the pellicle.

It is hoped that these investigations may contribute to a greater understanding of the synthesis of these antigenic proteins, and that they may provide information that will lead to a more meaningful interpretation of the control of immobilization antigen expression.

MATERIALS AND METHODS

1) Culture techniques

Cultures of *Paramecium aurelia* of Syngen 1, stocks 90D and 168D were grown at 31°C on baked lettuce medium, innoculated with *Aerobacter aerogenes*, stocks 168G and 504G (Syngen 9) were grown on the same medium at 18°C. A brief explanation of some terminology is perhaps necessary. A SYNGEN consists of a group of stocks that are reproductively isolated from all other stocks, i.e. conjugation will only occur between stocks within a syngen. A STOCK is a culture of paramecia derived from a single animal isolated from the wild. A culture synthesising only one immobilization antigen is said to be of a particular SEROTYPE (Sonneborn 1947, 1950).

All experiments used actively growing cultures. For the preparation of purified immobilization antigen, 500 litres of each serotype were grown on grass medium buffered with Na_2HPO_4 to pH 6.8. The mass cultures were then harvested by centrifugation at 1000 g. for 4 minutes in an oil testing centrifuge. All cultures were tested for purity of serotype by the immobilization test immediately before harvesting.

2) Preparation of Purified Immobilization Antigens (after Preer & Preer 1959, Preer 1959a Jones 1965)

It is essential that the preparations of purified antigens, used to induce antibodies against 168D and 168G antigens, do not produce antibodies against the heterologous antigen due to the contamination of the original cultures with spontaneously transformed cells, e.g. from serotype 168D to 168G. It has been shown that antigens 90D and 168D are identical (Beale 1954),

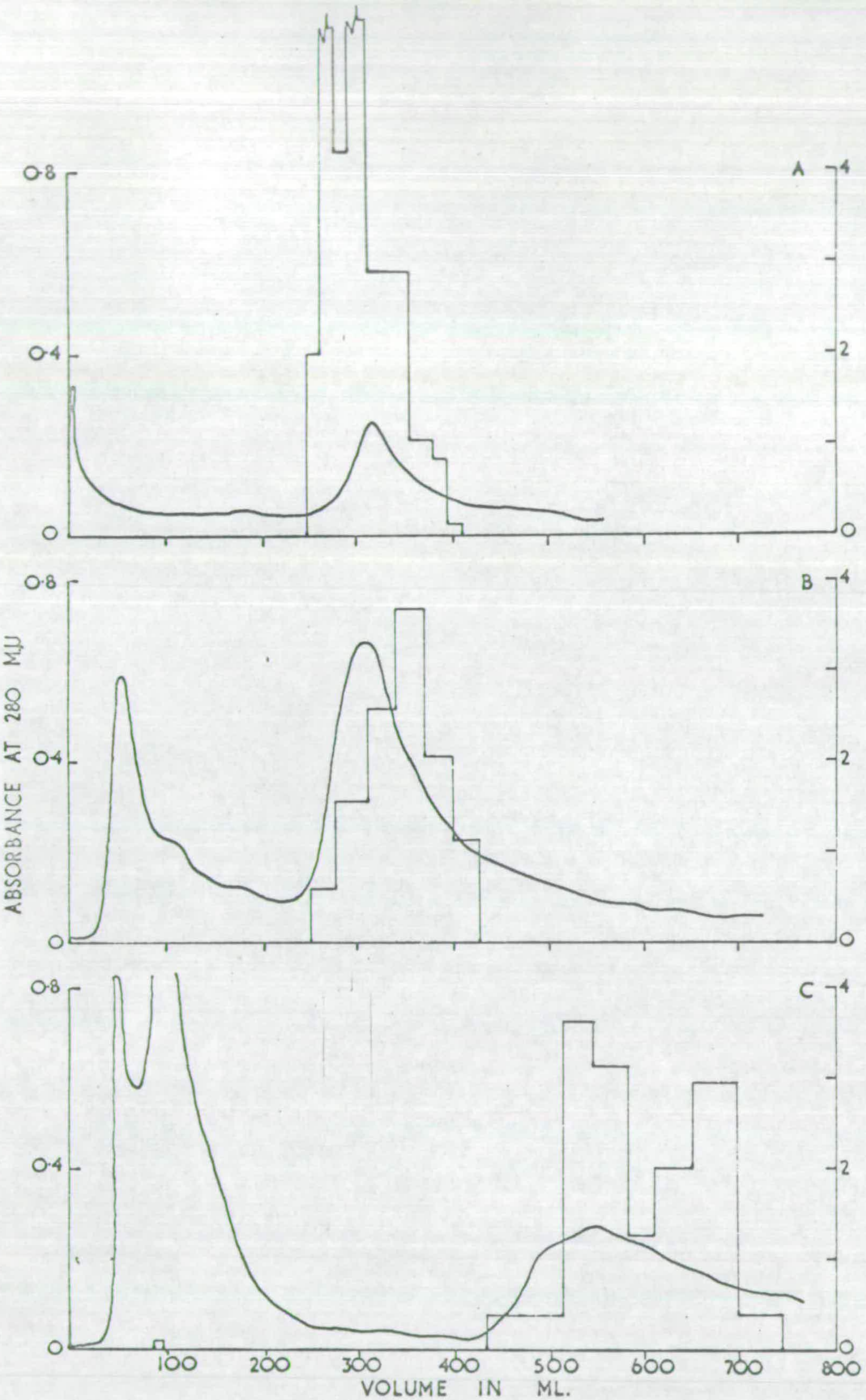


FIG. 2 Elution profiles of the purification of the immobilization antigens by ion-exchange chromatography on S.E.Sephadex, using an 800 ml. gradient of 0.05M sodium acetate/acetic acid buffer between pH. 4.2 and 5.2. Concentration of the immobilization antigen was estimated by the immobilization inhibition test (page 11)

Figs. 2a,b 168G Antigen

2c 90D Antigen

however if serotype 90D were to transform spontaneously serotype 90G would be formed, this antigen (90G) has been shown to be immunologically completely unrelated to antigen 168G (Beale 1954). Therefore stocks 90D and 168G have been used to induce antibodies against 168D and 168G antigens respectively, thus ensuring that the antibodies produced will be serotype specific.

Four volumes of salt alcohol solution (0.0025 M Na_2HPO_4, 0.0025 M NaH_2PO_4, 0.0225% NaCl, 15% C_2H_5OH), were added to the harvested paramecia, left at $4^{\circ}C$ for one hour, then centrifuged at 40,000 rcf for five minutes. The supernatant was decanted and stored at $-20^{\circ}C$. Solid ammonium sulphate was added to give 75% saturation and the mixture stood at $4^{\circ}C$ for 3 hours, after centrifugation at 11,700 r.c.f. for 15 minutes the pellet was dissolved and dialyzed against distilled water. The solution was centrifuged at 40,000 g for 15 minutes and freeze dried. A 2-3% solution of the freeze dried protein in sodium acetate buffer pH 4.2 0.05M, was passed through a column of S.E. Sephadex C50 (Pharmacia), a continuous 800 ml gradient of sodium acetate buffer from pH 4.2 to 5.2 was applied (see fig. 2). The E_{280} was monitored by hand on a Beckman D.B. Spectrophotometer. Each antigen showed a characteristic and repeatable elution pattern (168G between pH 4.67 and 4.72, antigen 90D between pH 4.75 and 5.13). After dialysis against distilled water the peak fractions were assayed by the immobilization inhibition test (Bishop 1963). Those fractions with high antigenic activity were pooled, freeze dried and stored at $-20^{\circ}C$.

3) Preparation of Antibodies (after Herbert 1967).

Either 4 mgm of purified antigen in 0.5 ml of 0.9% NaCl, or 0.5 ml of packed homogenised cells was prepared and injected into an equal volume of a mixture containing 9 parts Drakeol 6VR (Pennsylvania Oil Refining Co.) and 1 part Arlacel A (Kodak). The antigen and oils were recycled through the syringe until an even white emulsion was formed. This was similarly injected into a solution of 2% Tween 80 (Koch Light) in 0.9% NaCl, then checked by phase microscopy to ensure a double emulsion had been produced. This preparation was then injected subcutaneously into the back of a rabbit. After bleeding, the separated serum was heated to 57°C to inactivate complement, because complement has been shown to be very toxic to paramecium (Sinclair 1958). The serum was then dialysed against Maintenance Solution (M.S.), (0.013M NaCl, 0.0003 M $CaCl_2$, 0.0003M KCl, 0.004 M Na-K phosphate buffer pH 7.0), and stored at -20°C.

4) Antibody assay techniques

a) Immobilization Test (Sonneborn 1950)

Aliquots of serum were thawed at 37°C and a double dilution series of tubes from $^1/_5$ to $^1/_{10\,240}$ was made using M.S as diluent. 0.4 ml of each dilution was pipetted into a depression slide and 0.1 ml of culture containing approximately 100 animals added. These slides were kept in a moist box at 18°C for two hours after which they were examined. The highest dilution to immobilize the animals completely was taken as the titre of the serum. Each serum was tested against all stocks to give the specific and cross reaction titres.

The immobilization inhibition test was identical to that described by Bishop (1963), and is analysed as follows:- A dilution of antigen (x) delays the normal time of immobilization (t_o) to a time t_1 such that $t_1 < 2t_o$, where t_o is between 3 and 10 minutes. The antigenic activity of the original preparation is then given as:

$$3(1/t_o - 1/t_1)\ x \text{ units}$$

b) Immunodiffusion

Antigens were used either as whole cell homogenates or purified immobilization antigen solutions (2-5 mgm/ml). The suspending medium was 1% Difco Noble agar in veronal acetate buffer pH 8.6, μ 0.1 + 0.01% sodium azide. 2mm diameter wells were cut 3mm apart (Finger 1964) in the usual hexagonal pattern, 10μl of antibody and antigen were added to the appropriate wells, then left in a moist box for 24 hours, after extensive washing in 0.9% NaCl the gels were stained in 1% Naphthalene Black in methanol: distilled water: acetic acid (5:5:1). Reactions of identity were visible as complete fusion of lines, partial identity as a partial fusion with a 'spur' from the homologous precipitate, and non-identity as a complete crossing of the precipitin bands. (Ouchterlony 1962)

c) Immunophoresis

The standard technique of Graber & Williams (1955) was used. The antibody, antigen, and gel are similar to those used for immunodiffusion. The antigen was electrophoresed at 6.67 volts/cm at 40 m amps. for 3 hours

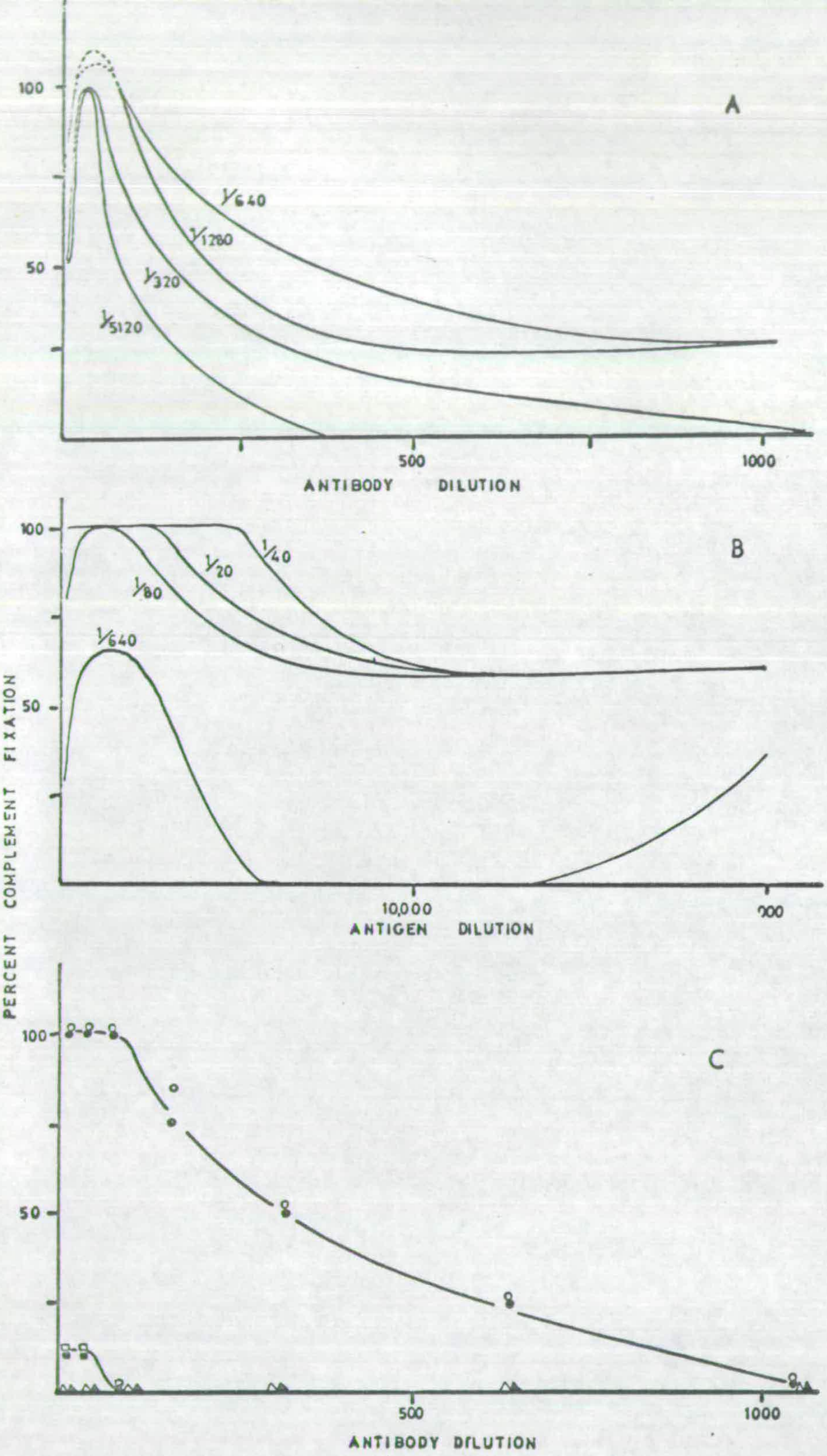


FIG.3 Complement fixing activity of antisera prepared against purified immobilization antigens , using whole cell homogenates as antigens . Techniques described on page 13.

A. The effects of varying the antibody concentration on the fixation of complement using constant antigen dilutions.

B. The effects of varying the antigen concentration on the fixation of complement by constant antibody dilutions .

C. The complement fixing activity of three antisera with both homologous and heterologous antigens (at a dilution of 1/1000).

■-■ anti-168D antibody/168G antigen ○—○ anti-168D antibody/168D antigen

●-● anti-168G antibody/168G antigen □-□ anti-168G antibody/168D antigen

▲-▲normal rabbit serum/168G antigen △-△normal rabbit serum/168D antigen at room temperature for most analyses, or for 18 hours at 4°C for scintillation counting of the radioactive precipitin arcs in the in vivo labelling experiments. The electrode compartments contained the same buffer as the gel but with an ionic strength of 0.5μ. Precipitin band formation and staining were similar to that for immunodiffusion.

d) Complement fixation test

The technique used was that described by Kabat and Mayer (1961), each reaction contained only 1.2 $C'H_{50}$ units. The buffer used was 0.145M NaCl, 0.002M sodium diethylbarbituric acid, 0.003M diethylbarbituric acid, 0.00005M $CaCl_2$ and 0.00015M $MgCl_2$. Red blood cells in Alsever's solution, horse haemolytic serum, and complement (Preserved Guinea Pig Serum) were all obtained from Burroughs Wellcome Ltd. Whole cell homogenates were used as antigens. All reagents were titrated in the usual manner, both antibody and antigen showed optimal reaction concentrations (see fig. 3ab). The final antibody titrations were read by eye and scored in the usual way, i.e. from 0 (no haemolysis) to 4 (complete haemolysis). All reactants were tested singly and in all combinations for 'anticomplementarity' activity, all were negative. The results are shown in fig. 3c.

5) The Preparation and Conjugation of globulins

a) Preparation of globulins

The globulins were precipitated by 50% saturation with ammonium sulphate at 4°C, and after equilibration for one hour the precipitate was removed by centrifugation at 11,700 r.c.f. for 30 minutes, then redissolved in distilled water. Reprecipitation was continued until a white pellet and colourless supernatant remained. The pellet was then dissolved in minimal distilled water and dialysed against running tap water, then either MS or borate buffered saline pH 8.0, or phosphate buffered saline pH 7.5 0.05M, depending on which marker was to be conjugated. The final preparation contained globulins at a concentration of 20-25 mgm/ml as estimated by the Lowry technique (Lowry et al. 1954). This was stored at 4°C.

b) Fluorescein conjugation

The method used was the standard development of the original technique of Coons (1941) using fluorescein isothiocyanate (B.D.H.)

One volume of globulin (dialysed against MS) was mixed with two volumes of 0.5M carbonate bicarbonate buffer pH 9.0, and stirred in an ice bath, powdered fluorescein isothiocyanate (F.I.T.C.) (3mgm for each ml of globulin solution) was dissolved in 'Analar' acetone to give a solution of 3-5 mgm/ml. This solution was added to the globulin, drop by drop, over a period of one hour, with continuous stirring at 4°C. The mixture was then stirred for an additional 24 hours at 4°C. This was then passed through a column, 20 x 5 cm, of Sephadex G.25 fine (Pharmacia) using MS as buffer, to remove the unconjugated F.I.T.C. (Curtain 1961, Fothergill and Nairn 1961, Wagner 1962). The elution of the mixture was monitored on a Uvicord I (E254) (LKB instruments) or Uvicord II (E280) and also a Beckman DB spectrophotometer (E495) with a continuous flow cell. The purified F.I.T.C. conjugated globulin was then dialysed against polyethylene glycol (Lipp 1961, Kohn 1961) until the volume was equal to that applied to the column, then dialysed against MS for 24 hours, and stored at -20°C.

c) Ferritin Conjugation

This method is based on the original technique of Singer (1959), using xylylene diisocyanate as coupling agent (Borek & Silverstein 1961, Isliker 1964). Xylylene diisocyanate (Kodak Rare chemicals) was divided into 1 ml aliquots and stored at 4°C in sealed ampoules to prevent polymerization. The globulins used were as described above, dialysed against 0.05M phosphate buffer pH 7.5.

Ferritin 6x crystallized (Calbiochem) was dissolved in 2 parts of 0.05M phosphate buffer pH 7.5 and 1 part 0.3M borate buffer pH 9.5 at 0°C. The solution (25 mgm/ml) was stirred just fast enough to avoid foaming, while xylylene diisocyanate was added slowly (0.1 ml/100mgm ferritin), the mixture was stirred for 45 minutes at 0°C, then centrifuged at 4,000 r.c.f. for 30 minutes at 4°C. The supernatant was pipetted off and stood at 0°C for one hour to allow the reaction to go to completion. An equal volume (x ml) of globulin and (x/2ml) of 0.3M borate buffer were added to the reaction mixture and stirred gently for 48 hours. The mixture was then dialysed against 0.1M ammonium carbonate overnight to stop the reaction, then dialysed against 0.05M phosphate buffer pH 7.5. After 24 hours the solution was centrifuged at 4,000 r.c.f. at 4°C for 30 minutes, the supernatant decanted and spun at 100,000g for 4½ hours. The pellet was resuspended overnight in fresh phosphate buffer. The double centrifugation was repeated three times to remove all the unconjugated globulin, the final pellet was dissolved in MS (x ml) and dialysed against MS to remove all the phosphates, then stored at $-20^{o}C$.

d) <u>Iodine Conjugation</u> (Johnson, Day and Pressman 1960)

Iodine^{125} as sodium iodide (Radiochemicals) was diluted with 0.002M potassium iodide to a specific activity of 640 μC/ml. For iodination the following solutions were prepared in an ice bath:-

a) 1.0 ml 1M HCl, 0.25 ml iodide (160 μC iodine^{125})

b) 1.0 ml 1M NaOH, 4 ml Borate saline (0.05% sodium tetraborate
0.9% sodium chloride)

c) 1 ml globulin (20-25 mgm, dialysed against borate saline), 3 ml borate Saline pH 8.0.

0.25 ml of Sodium nitrite 0.02M, was mixed with solution A, this was followed by the addition of solution B. The combined solutions were then added slowly to solution C with constant stirring. This mixture was dialysed against borate saline and MS at $4^{o}C$ for four days to remove all the unconjugated iodine (see table 1), finally dialysed against veronal-acetate buffer pH 8.6 0.3M. The final preparation was centrifuged at 1,500 r.c.f. for 30 minutes and stored at $4^{o}C$.

6) <u>Preparation of Tissues</u> a) Fixation

The first requirement of this investigation was to obtain a satisfactory fixation procedure of whole paramecia for electron microscopy. Two methods were developed. First 0.5% osmium tetroxide in 0.3 M veronal

	Radioactivity (Counts per second)	
	Reaction mixture containing globulin	Reaction mixture without globulin
Initial counts in reaction mixture	110	121
Counts after dialysis against		
1 Borate - saline	26	2
2 Maintainenance solution A	26	1
3 " " B	24	0
4 " " C	24	0

TABLE 1 Removal of unconjugated iodine^{125} from the protein fraction after conjugation of globulins with iodine^{125} - after the method of Johnson et al. (1961). Each period of dialysis was for 24 hours against 10 litres of buffer. The radioactivity was monitored with a Geiger-Müller counter.

Source of globulin	Specific activity (counts/min/mgm)
Anti-168G serum	6.039×10^6
Anti-168D serum	7.753×10^6
Normal rabbit serum	19.217×10^6

TABLE 2 Specific activity of globulins conjugated to iodine^{125}. After dialysis (see table 1), 0.1 ml of each serum was added to 10 ml Brays scintillation fluid and counted. The protein concentration of the same solutions was estimated by the technique of Lowry et al. (1954).

acetate buffer pH 8.6, containing 0.075 M sucrose and 0.005 M calcium chloride. At room temperature this fixative still allowed the trichocysts to discharge, severely distorting the cytoplasmic organisation. (See plate 1a) This was prevented by fixation in the same solution at 37°C (Plate 1c). An alternative fixation was developed from the technique of Pitelka (1968):- 2.5% glutaaldehyde in MS at 4°C for two hours, after which the cells were washed three times in MS, then postfixed in osmium tetroxide at 18°C as described above (Plate 1b).

b) <u>Frozen sections</u>

A technique for cutting sections in an aqueous medium was developed. The problems of freezing injury have been extensively studied (Merryman 1966, Mazur 1966, Luyets 1966) and two major criteria are evident. First a controlled rate of freezing of approximately 1°C per minute is necessary to prevent damage by growing ice crystals. Second a balance of the ionic strength of the embedding solution and the tissues has to be maintained during the removal of liquid water by freezing (Mazur 1966). For the purpose of this investigation a very convenient method of controlling the rate of freezing was obtained using a large Leitz freezing microtome with a kryomat cooling apparatus set at -30°C. The preparation of fixed cells was pipetted directly onto the stage of the microtome at 18°C, the stage was then cooled to -30°C, and the rate of cooling achieved resulted in minimal freezing damage. To maintain the ionic strength of the embedding medium two reagents have been used, 12.5% glycerol and 10% dimethyl sulphoxide (Lovelock and Bishop 1959). Experiments using fluorescein conjugated antibodies indicated that neither reagent affected the reactivity of the


A
B
C
KD
CIL
TRICH
MIT
SV
ER

PLATE 1

A. Electron micrograph of Paramecium fixed with 0.5% osmium tetroxide in 0.3M veronal acetate buffer + 0.075M sucrose + 0.05M calcium chloride at 0°C. Note the discharged trichocysts, also substantial leaching of the cytoplasm. Magnification x 25,000.

B. Section of paramecium fixed with 2.5% glutaraldehyde in maintainence solution, postfixed with 0.5% osmium tetroxide in 0.3M veronal acetate buffer pH 8.0. Trichocysts are not discharged. Mag. x 25,000.

C. Micrograph of paramecium fixed with 0.5% osmium tetroxide in 0.3M veronal acetate buffer pH 8.0 + 0.075M sucrose + 0.05M calcium chloride at 37°C. Trichocysts are not discharged and the complex ultrastructure well preserved. Mag. x 50,000.

KEY

cil.	cilium
e.r.	endoplasmic reticulum
g.	glycogen
k.	kinetosome
kd.	kinetodesmus
mit.	mitochondrion
s.v.	smooth vesicles
trich.	trichocysts.

antigens. However dimethyl sulphoxide has been found to increase the contrast of stained electron microscope sections to such an extent that it was difficult to detect ferritin molecules. Therefore glycerol has been used in all antigen localization experiments both for fluorescence and immune electron microscopy. Frozen sections are now routinely prepared in which the preservation of tissues is almost indistinguishable from unfrozen material (see Plate 2). The routine procedures for the preparation of frozen sections and whole cells are given below.

Cultures of paramecium were filtered through cotton wool, and harvested by centrifugation at 1000g for four minutes in an oil testing centrifuge. The concentrated culture was then centrifuged at 1,500g for two minutes in a conical centrifuge tube, the supernatant was decanted and 5 ml of osmium tetroxide fixative at 37°C was added. This was then allowed to stand at 18°C for thirty minutes with occasional agitation. After fixation the cells were washed three times in 10 ml of 0.3M veronal acetate buffer, each wash being of 5 minutes on a rotary mixer. The cells were then equilibrated in 12.5% glycerol in veronal-acetate for 5 minutes. The suspension of cells was reduced to less than 1 ml and then frozen directly on the microtome stage at -30°C, when frozen, 7.5μ sections were cut using a cooled knife, and the sections were placed directly in 12.5% glycerol at 18°C. After washing three times in veronal-acetate buffer the sections were incubated, if desired, with the blocking globulin (diluted 1 in 5) for two hours at 18°C. Again after washing three times the sections were then incubated with labelled globulin

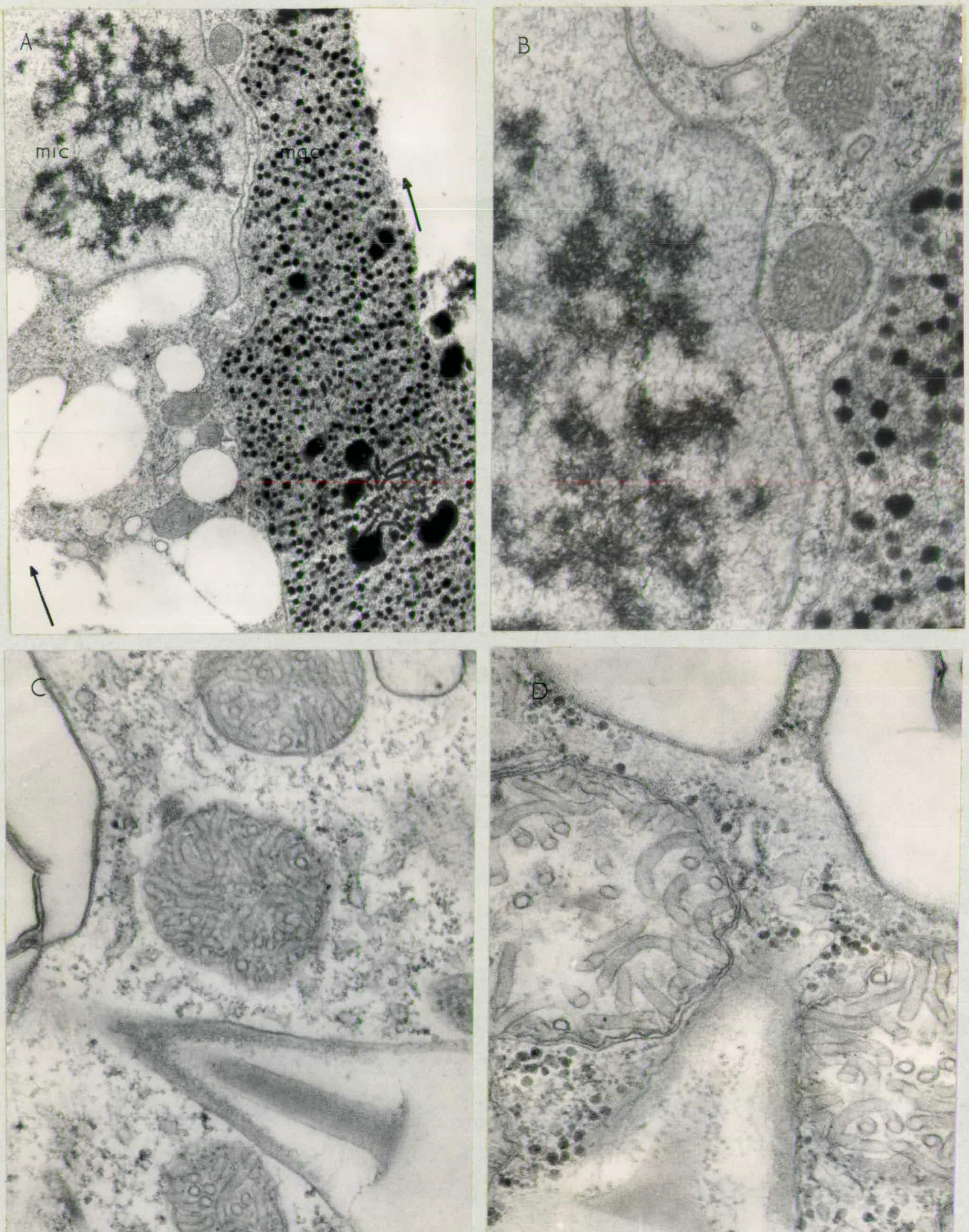

A
mic
mac
B
C
D

PLATE 2

A. Electron micrograph of a frozen section of paramecium prepared as described in the materials and methods page 22. The arrows indicate the plane of sectioning by the freezing microtome. The micronucleus (mic) and macronucleus (mac) are prominent, the latter contains both large and small bodies and an unidentified 'fibrous' configuration. Mag. x 6,250.

B. High power micrograph of the same material showing the preservation of the ultrastructure of the nuclear membranes. Mag. x 32,500.

C. High power micrograph of a frozen section showing the ultrastructural detail of the pellicle, mitochondria and trichocyst. Mag. x 50,000.

D. A section of paramecium fixed in osmium tetroxide but not frozen, showing structures comparable with those in plate 2C. Mag. x 50,000.

for two hours at 18°C. (Fluorescein conjugate diluted $^1/_{20}$; ferritin, $^1/_5$; and iodine^{125} $^1/_1$). After labelling the sections were washed in veronal acetate buffer.

Fluorescein labelled sections were then mounted on microscope slides and viewed under ultraviolet light. Ferritin and iodine labelled sections were subsequently dehydrated in ascending alcohols. Iodine^{125} labelled sections to be assayed by liquid scintillation counting, were sampled from absolute alcohol (see liquid scintillation counting); other samples were air dried for estimation of the dry weight of material. Ferritin labelled sections, and iodine labelled sections for autoradiography were then embedded in araldite as described below (Glauert & Glauert 1958).

Whole cell preparations were similarly treated with the exception of the stages from the equilibration with glycerol to the washing after sectioning. After dehydration in ascending alcohols material was suspended in a 50:50 araldite /alcohol mixture for thirty minutes after which they were resuspended in pure araldite and stood at 18°C overnight. This was followed by 45 minutes at 60°C on a rotary mixer (Jurand and Ireland 1968). The mixture was then pipetted into gelatine capsules and centrifuged at 2,500g for 15 minutes at 60°C. The araldite was then polymerised at 48°C for 72 hours.

c) Homogenates

The paramecia were harvested as described above, the pellet of animals was transferred to a homogeniser tube in four volumes of buffer (either 0.3 M veronal-acetate pH 8.6, or MS pH 7.0 or 0.1 M Tris-HCl pH 7.6 containing 0.025 M sucrose, 0.05 M KCl, 0.01 M $MgCl_2$ and 0.1 M

2 mercaptoethanol). The cells were homogenised with a Tri-R teflon plunger for 15-20 strokes at speed 8. The homogenate was centrifuged at 11,700 r.c.f. for 10 minutes and the pellet was then, either fixed in cold osmium tetroxide for 30 minutes in fixation buffer, or incubated with conjugated antibody for 2 hours at $0^{\circ}C$. After washing, the material previously fixed was incubated with conjugated antibody. This suspension was then washed three times, pelleted firmly to the bottom of the tube, dehydrated and embedded in araldite if ferritin conjugated antibody had been used, or observed directly in wash buffer if fluorescein conjugated antibody had been used.

7) Microscopy

a) Fluorescence

A Zeiss Standard Junior microscope, with dark ground illumination was used. The light source was an HB 200 bulb in a Zeiss high intensity lamp unit with the appropriate exciter and cut out filters. Photography was on Kodak High Speed Ektachrome film with exposures of 45 seconds to 4 minutes.

b) Light microscopy

Light microscope autoradiographs were stained in Methyl green/Pyronin for 30 to 90 minutes, rinsed in 70 and 90% ethanol and air dried. The preparations were viewed under phase optics, and photography was on Panatomic X film with exposures of $^{1}/_{60}$ to $^{1}/_{2}$ second.

c) Electron microscopy

Araldite embedded material (Glauert & Glauert 1958) was sectioned on a Sorval 'Porter Blum' ultramicrotome, for normal investigation pale grey sections (400-600Å) were cut, and for autoradiography gold sections (800-1000Å). Sections were stained in lead citrate (Reynolds 1963) and uranyl acetate (Watson 1958). This procedure stained globulins in the sections as was observed by Mott (1965) using permanganate stain. The sections were stained on 2% uranyl acetate for 15 minutes in the dark, rinsed by dipping into filtered distilled water, then stained on fresh lead citrate for 15 minutes in a carbon dioxide free atmosphere. The grids were then washed by dipping in 0.02M NaOH and distilled water. Ordinary grids were stained any time after preparation, autoradiographs were stained immediately after washing to prevent contamination (Ross and Benditt 1965).

Preparations were examined in an A.E.I. EM6 electron microscope. Estimates of the surface density of ferritin, in grains per unit length, were made by counting the number of ferritin grains on each of ten random micrographs (50,000 times magnification), and measuring the total length of pellicle on each print using a map measurer.

8) Analysis of Radioactivity

a) Autoradiography

Light Microscope:- Cleaned microscope slides were 'subbed' in 0.5% gelatine and 0.05% chromic potassium sulphate, then air dried. Sections 1μ thick, of araldite embedded preparations were expanded by xylol vapour and transferred to the 'subbed' slides. All subsequent procedures were carried out in a dark room using a Kodak Wratten Series 1 Safelight.

Sections were coated with emulsion by the stripping film technique (Pelc 1947). Squares of Kodak AR10 stripping film were inverted onto a water bath at 24°C. After expansion the film was coated onto the preparation and dried. The slides were stored in a light tight box, with calcium chloride and silica gel to prevent fading of the latent image (Lord 1963). The sealed boxes were stored at 4°C. After periods varying between two days and fifteen months the autoradiographs were developed in Kodak D19 developer at 20°C for 4 minutes, washed for 30 seconds in distilled water and fixed in two washes of 5 minutes each in Johnsons Fix-sol solution diluted 1/10, the slides were than washed for one hour in running tap water and stained as described.

Electron microscope autoradiography:- Araldite sections 800-1000Å thick were mounted on carbon coated membranes supported on nickel grids. For each block 50 grids, each containing approximately 20 sections, were prepared. The grids were mounted on microscope slides two or three to a slide, then coated with Ilford L4 emulsion after the method of Caro & van Tubergen (1962), using wire loops 4 cms in diameter. A solution of 10 grammes of emulsion in 20 ml of distilled water was mixed for 15 minutes in a water bath at 46°C in total darkness, then gelled in an ice bath for 2-2½ minutes, and allowed to return to room temperature (20°C) over half an hour. The emulsion, which was judged to give a gold interference colour, using Ilford S902 safelights, was then 'looped' onto the grids. The grids were then sealed in light tight boxes with dessicant. After exposures of between one month and two years at 4°C, autoradiographs were developed as described for light microscope auto-

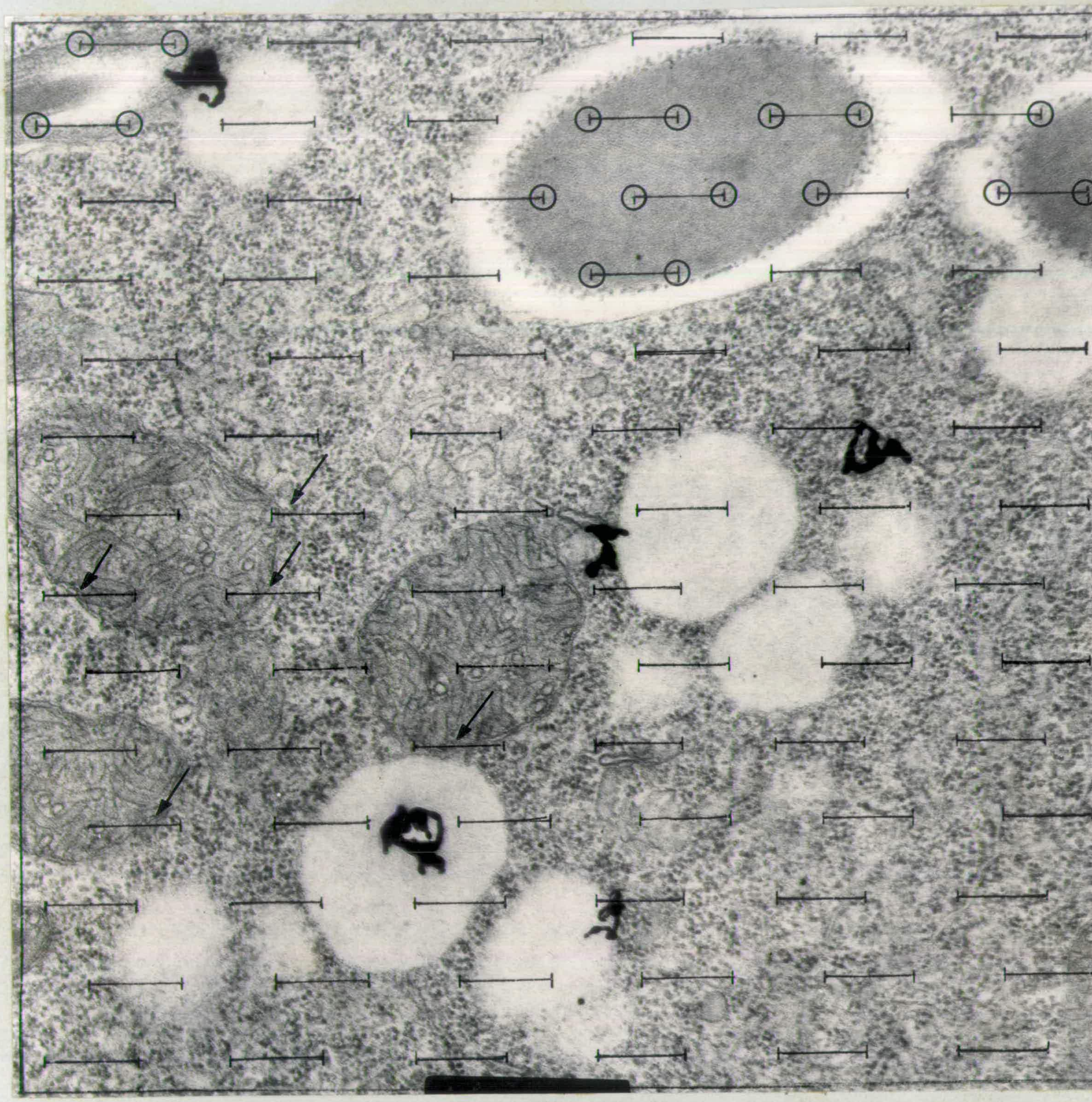


FIG. 4 Technique of analysis of the areas of the different organelles and the length of membranes in electron micrographs of paramecium,(see materials and methods page 23). The ends of each test line ringed are scored as lying on trichocysts. The lines marked by the arrows are scored as hits on the membrane - in this micrograph mitichondrial membrane, pellicle membrane was analysed in the identical manner.

Micrograph x 50,000.

radiography, except that the preparations were washed finally in three changes of distilled water. All the autoradiographs from one experiment were prepared, developed and stained at the same time to ensure homogeneity of the emulsion and development procedure. The grids were removed from the slides and stained individually on uranyl acetate and lead citrate as described above.

The quantitative analysis of the electron microscope autoradiographs is similar to that of Ross and Benditt (1965), (after Chalkley 1943). For each sample ten micrographs were taken at a plate magnification of 8,000 times, not more than four micrographs were taken of any one grid. The only selection of micrographs in experiments using Iodine^{125} conjugated antibodies, was that each plate had to include one cut and one natural surface of the paramecium, this is because even this conjugated antibody did not penetrate more than 2 or 4μ into a section. This selection is certainly the least subjective that could be applied and will ensure a greater, though still representative, sample of autoradiographic grains for a given sample size. For the biosynthetic experiments the sampling technique of Weibel et al. (1966) was used, here a fixed corner of the holes in the electron microscope grid is photographed irrespective of the material present, this ensuring a random sample.

Prints were scored for the localization of all autoradiographic grains. For the analysis of the areas of each organelle, micrographs were placed under the multipurpose test system of Weibel et al. (1966) see fig. 4. Each point was scored as being in one of eight classes of organelle as chosen in table 10, because the surface membrane appears as a linear and not a two dimensioned structure, the number of test lines cutting the membrane were scored as hits. For the localization of immobilization antigen using iodine^{125} conjugated antibody the results are scored as follows:- (see table 10)

a. $$\frac{\text{the percentage of the total grains on the pellicle}}{\text{the percentage of the total area 'hits' on the pellicle}}$$

b. $$\frac{\text{the percentage cytoplasmic grains on an organelle}}{\text{the percentage cytoplasmic area hits on organelle}}$$

These estimates of the relative density of labelling allow a comparison of experiments labelled with different preparations of antibody, exposed for different periods. In experiments, following the incorporation of isotopes from radioactively labelled bacteria, because all the preparations have been treated similarly, and the sections are all of similar thickness as judged by interference colours, the results are scored either as the percentage of the total grains on a given organelle, or the absolute density of grains in each organelle, calculated as:-

$$\frac{\text{Number of autoradiographic grains on an organelle}}{\text{Number of area hits on the same organelle}}$$

Immunoelectrophoresis autoradiography

All samples of homogenised cells from one experiment were run on the same gel, each well containing 10µl of sample. After electrophoresis for 3 hours the gels were washed extensively and air dried. The gel was then fixed to a heavy glass plate, and using a Kodak 6B safelight, a sheet of Kodak Royal Blue x ray film was placed over the gel, and held firmly in place by another sheet of glass. This was stored in a light tight box for 1 to 4 weeks at room temperature. The film was developed in Kodak D X 80 developer for 4 minutes washed and fixed.

b) Liquid scintillation counting

Samples were counted in a Nuclear Chicago Unilux bench top liquid scintillation counter using Brays scintillation fluid (Bray 1960). Since the scintillation mixture is very water tolerant, 0.1 ml of samples of antibody conjugate were pipetted directly into 10 ml of Brays solution for counting. To measure the incorporation of iodine^{125} conjugated antibody onto frozen sections or whole cells, 1 ml samples of each, in absolute ethanol, were collected on membrane filters (Oxoid Ltd.) sucked dry and then put into the scintillation mixture. Counting of the immunoelectrophoresis gels was similar, standard pieces of gel containing the precipitin band were dried onto membrane filters and then placed in the scintillation fluid.

9) In vivo labelling of Paramecia using radioactively labelled bacteria

The incorporation of isotopes into P. aurelia via labelled bacteria has been found much more efficient and more specific than supplying an exogenous source of isotope (Berger & Kimball 1964, Sommerville 1967). Therefore all biosynthetic experiments have required the intial production of labelled bacteria.

a) Labelling the bacteria

Aerobacter aerogenes were cultured on different glucose salts media depending on the isotope being incorporated. The general procedure was as follows; an inoculum of bacteria was grown on non-radioactive medium for 10 to 25 hours at 35°C. The culture was then centrifuged at 12,000 r.c.f. for 10 minutes at 4°C, the pellet was washed twice and resuspended in 2 ml of distilled water. The same medium was then prepared with the required isotope present, 0.25 ml of bacterial suspension was added to 20 or 40 ml of medium. The culture was then grown at 35°C for 2 to 5 hours, the final density of the culture reached 1 mgm/ml. The culture was again centrifuged and washed as before, then resuspended in 2 ml of MS to give a final concentration of approximately 10 mgm/ml, this was stored at 4°C for a maximum of 24 hours before use.

Bacterial labelling media:- all the labelling media are based on those of Roberts et al. (1955)

a) $Carbon^{14}$ acetate:- Medium M 9 was used, containing:-
NH_4Cl 1.0 mgm/ml, Na_2HPO_4 6.0 mgm/ml, KH_2PO_4 3.0 mgm/ml,
NaCl 5.0 mgm/ml, $MgSO_4$ 0.1 mgm/ml, glucose 1.0 mgm/ml.

For labelling, $Carbon^{14}$ sodium acetate 482µC/mg (Radiochemicals) was added at a concentration of 0.5 mgm/ml. To direct the acetate into lipid synthesis the following competitors were added, Aspartic acid 6 mM, glutamic acid 6 mM, and Leucine 6 mM. The glutamic acid was provided to suppress incorporation into the Kreb's cycle, aspartic acid and leucine to suppress incorporation into nucleic acid and protein synthesis. This procedure has been described as giving more than 90% of the C^{14} acetate incorporation into the lipid fraction of E.coli (Roberts et al. 1955). On labelling the paramecia no attempt has been made to direct the incorporation of the C^{14} by the addition of exogenous competitors, consequently the fate of the isotope was probably much wider than in the bacteria, however a substantial proportion of the isotope was expected to be incorporated into paramecium lipid.

b) Sulphur^{35} magnesium sulphate:- Medium M 9 was used as above. All the magnesium sulphate was supplied as $MgS^{35}O_4$ (Radiochemicals) at 810μC/mgm, at a concentration of 0.1 mgm/ml of culture. This labelling procedure was expected to label predominantly methionine and cysteine in the bacteria (Roberts et al. 1955). Since the immobilisation antigen contains 10% of its amino acids in the form of cystine there was expected to be a marked incorporation of this isotope into the immobilization antigen.

b) Labelling Paramecium aurelia with radioactive bacteria

Cultures of paramecium were harvested and concentrated 200 fold into fresh medium without bacteria. Labelled bacteria were added to the culture in the proportions of 1 ml bacteria for each 20 ml of paramecium culture, this was then incubated at the required temperature for 15 minutes. After this time the culture was centrifuged twice through 100 ml of bacterized lettuce medium, then resuspended in 10 times its volume of bacterized medium and replaced in a water bath at the required temperature. Samples containing similar quantities of paramecia were taken at various times after the labelling began.

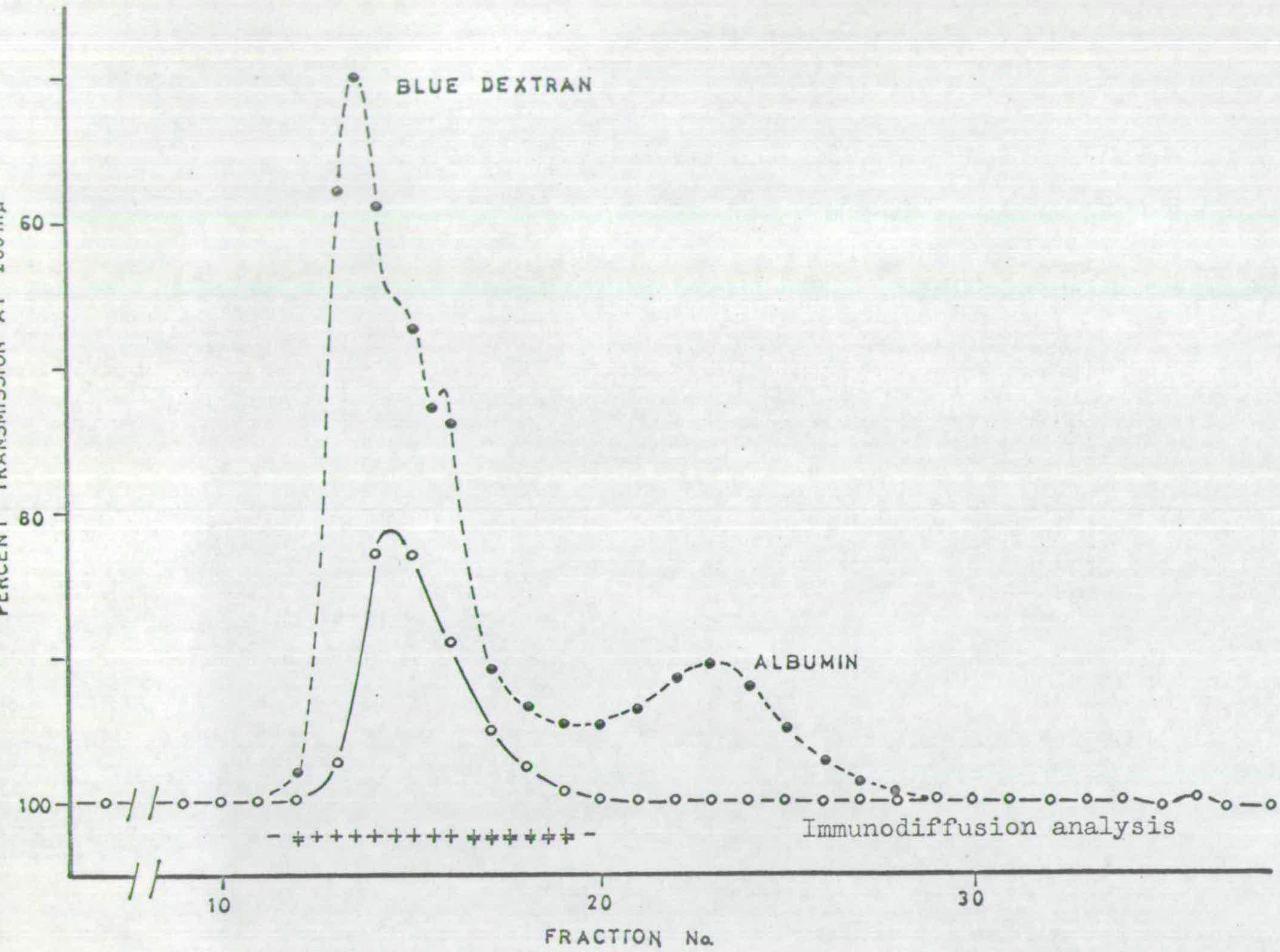


FIG.5a Elution of purified 168G antigen (20mgm.) from a column of Sephadex G.200 in veronal-acetate 0.1μ,pH 8.6. 0—0:also the elution of control preparations of Blue Dextran (M.W.2x10^6) and albumin (M.W. 69,000)●--●. Immunodiffusion analysis of the eluted fractions of 168G antigen , using antisera prepared against whole cell homogenates of 168G cells, were all negative except in the region shown, only one precipitin band was found.

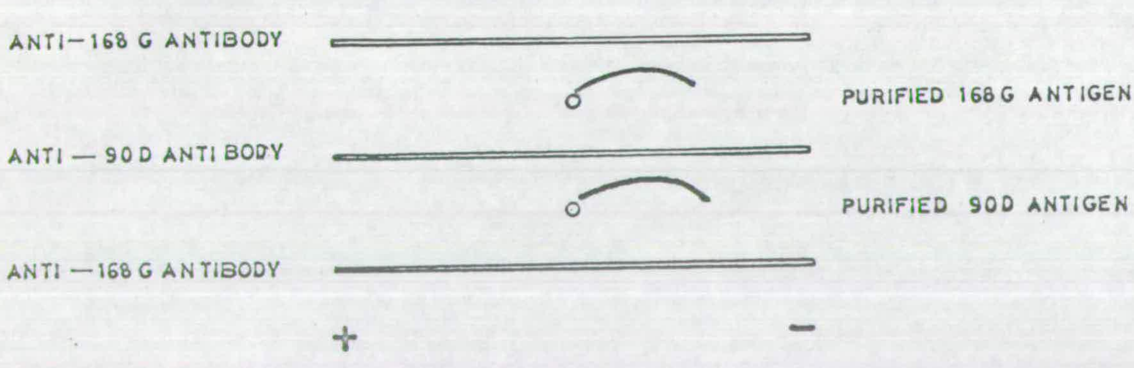


FIG. 5b Immunophoretic analysis of preparations of purified 168G and 90D immobilization antigens. Antisera used were prepared against whole cell homogenates. Electrophoresis and precipitin arc formation are described in the materials and methods section (page 12.)

DISCUSSION OF THE PROPERTIES OF THE CONJUGATED GLOBULINS

The main aim of this investigation has been to reveal the ultrastructural distribution of the immobilization antigens in Paramecium aurelia. The greater part of the work on this problem has relied on the use of antibodies which were conjugated to a variety of marker molecules. It was important therefore to establish that the proteins prepared to induce antibodies were preparations of a single protein - the immobilization antigen. Similarly it was essential to demonstrate that the antibodies produced were specific for the immobilization antigens, both before and after conjugation to the marker molecules. Two further problems influencing the use of the various immunological techniques need to be discussed. These are non-specific labelling of tissues by the globulins, and globulin-globulin interactions. It is convenient to consider these purely immunological aspects of the present work at this stage, and to confine the subsequent sections of this thesis to a discussion of the ultrastructural organisation of Paramecium aurelia and the localization of the immobilization antigens.

1. Demonstration of the purity of the antigen preparations

On elution from S.E. Sephadex each antigen preparation exhibited a single peak of absorption at 280 mμ (Fig. 2). However Jones (1965a) has shown that this criterion alone is not adequate to prove that a single protein is present, since an examination in the analytical ultracentrifuge, of immobilization antigen prepared by the same technique used in the present

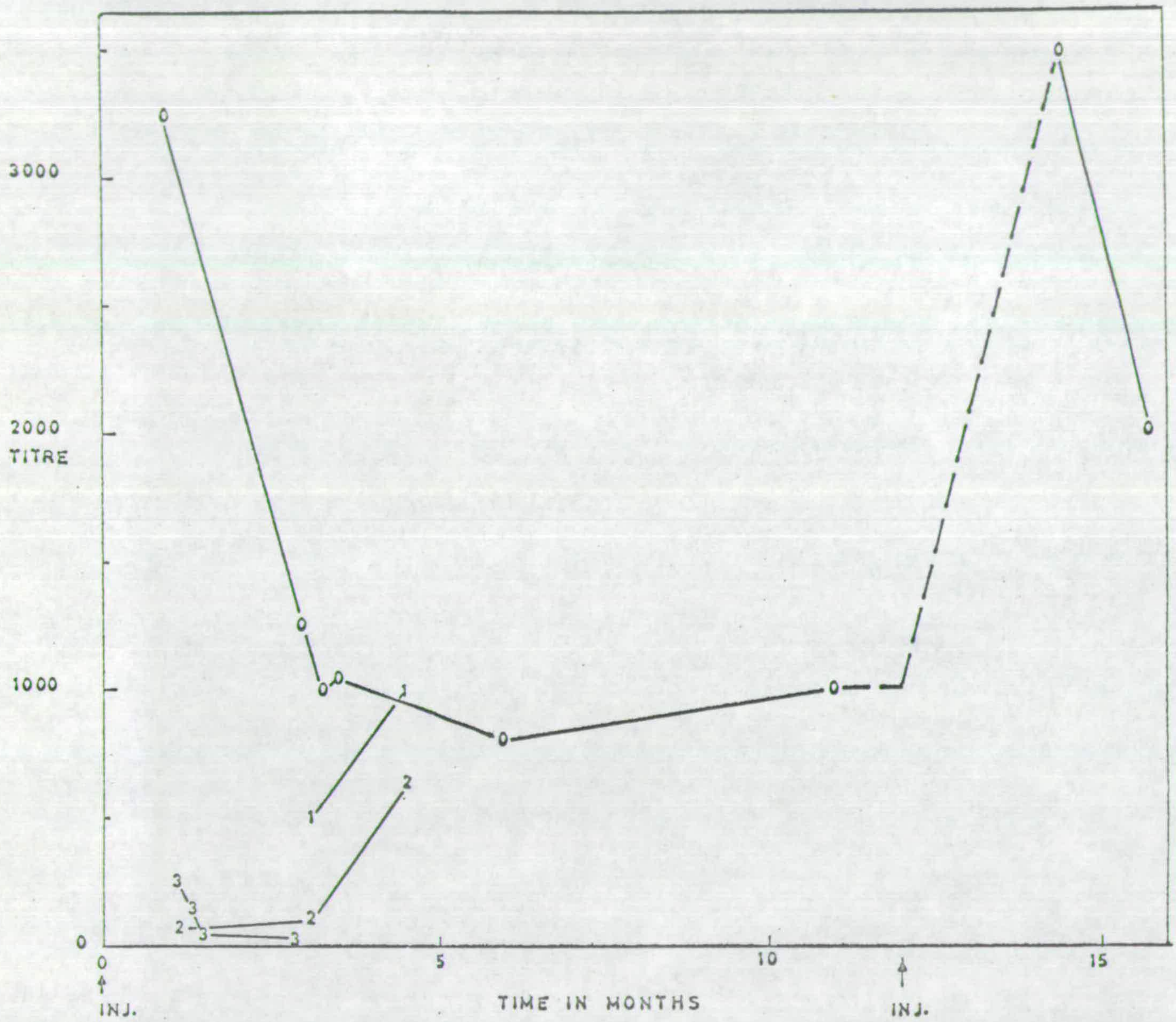


FIG.6a The titre of antibodies stimulated in individual rabbits by the injection of different antigen preparations in 2ml. of emulsion. All rabbits injected subcutaneously at time 0. Techniques are described on page 11. O--O,168G purified antigen; 1--1,2--2,90D purified antigen(4mgm.);3--3, 168G homogenate (0.5 ml. packed cells).

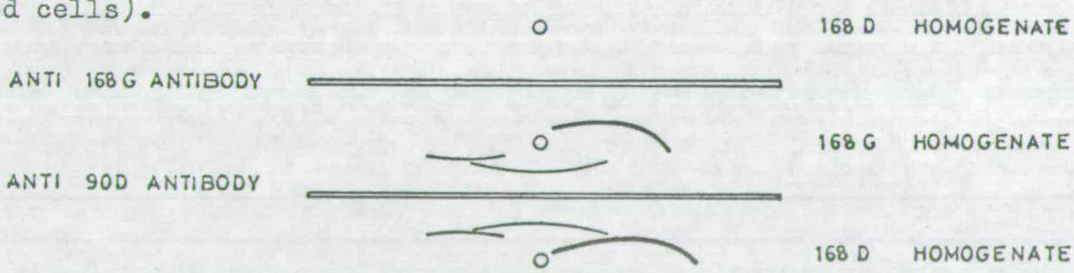


FIG.6b Immunophoresis analysis showing the specificity of two antibody preparations :- a 'monospecific' anti-168G antibody (rabbit 20R), and a 'multispecific' anti-90D antibody preparation (rabbit 41U). Techniques are described in materials and methods page 12.

study, occasionally revealed more than one protein. The composition of each antigen preparation was therefore investigated by gel-filtration on Sephadex G.200 and by immunophoresis. Both techniques indicated that the antigen preparations contained only a single protein (see Fig. 5).

2. Specificity of the antibodies

The purified antigen preparations readily induced the production of antibodies of high titre, which were maintained for long periods in the rabbits (Fig. 6). The specificity of the antibodies was tested by the immobilization reaction to find the antibody titre against immobilization antigens, by immunodiffusion or immunophoresis to demonstrate the number of different antigens against which the antibody reacted, and by the complement fixation test to find the total antibody activity produced against all antigens. The results are given in Table 3, and Figs. 3 and 6. Antibodies prepared against 168G antigen have always been found to be specific for the homologous antigen only, and of high titre, and therefore were used to study the cytoplasmic localization of the immobilization antigen. Antibodies prepared against 168D antigen were sometimes completely specific and sometimes showed precipitating activity against more than one protein (Fig. 6). The specific anti-168D sera were used as the blocking globulin where presaturation was required; and the 'multispecific' anti-168D sera have been used to locate the antigen on the pellicle and cilia of whole cells. Here it has been shown by Mott (1964) that antibodies prepared even against whole cell homogenates are serotype specific. Normal rabbit serum has never been shown to possess any antibody activity against paramecium. Therefore any retention of this globulin by tissues cannot be caused by

SERUM	ANTIGEN	ANTIBODY ACTIVITY		
		Immobilization titre	Precipitin band formation	Complement fixation (%homologous reaction)
Anti-168G purified antigen	168G	1/2,000	one	100
(Rabbit 202)	168D	1/2	-	0 - 12.5
Anti-168D purified antigen	168G	1/5	two	0 - 12.5
(Rabbit 41U)	168D	1/640	three	100
Normal rabbit serum	168G	0	-	0
(Rabbit 61U)	168D	0	-	0

TABLE 3 The immobilizing, precipitating and complement fixing antibody activities of immune and normal rabbit sera. The techniques used are as described in the materials and methods. Whole cell homogenates were used as antigens for the immunodiffusion and complement fixation tests. See also Figs. 3 and 6.

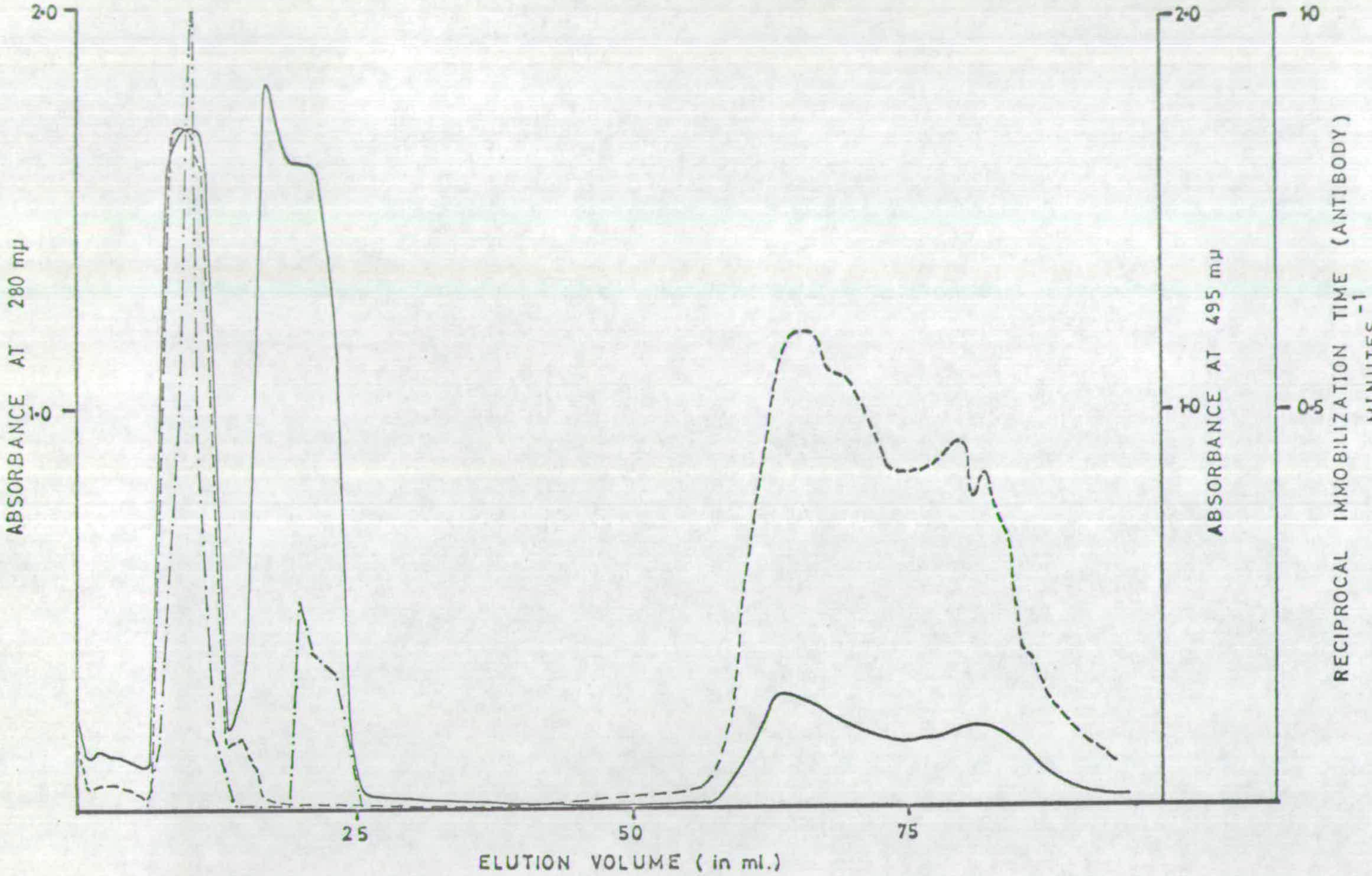


FIG.7 Separation of unconjugated fluorescein and unconjugated globulin from a preparation of fluorescein conjugated globulin (anti-168G). 20ml.of reaction mixture was applied to a column (40 x 2.5cms.) of Sephadex G25 and eluted in maintainance solution,(see page 30).

——— Absorbance at 280mμ.

---- Absorbance at 495mμ.

-·-·- Reciprocal immobilization time (antibody activity).

an immunological reaction and is called non-specific.

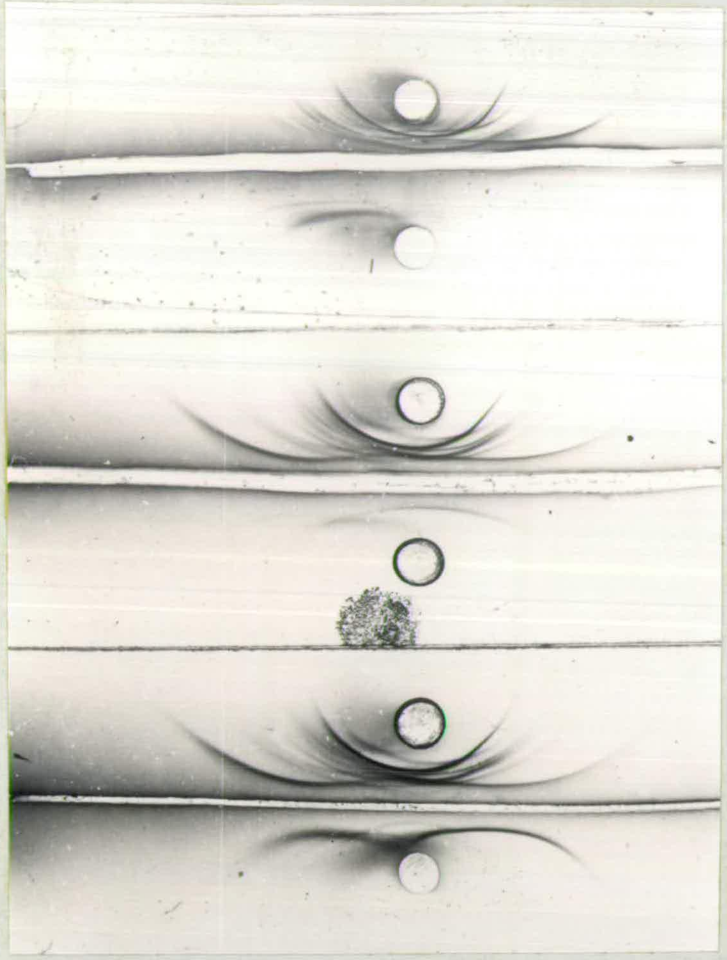
3. The Properties of the conjugated globulins

Three antibody markers were used in this investigation, fluorescein, ferritin, and radioactive iodine^{125}. There have been no previous reports on the attributes of iodine^{125} conjugated globulins as markers for the localization of antigens in situ. A necessary preliminary was therefore to investigate some of the more important properties of the iodine^{125} conjugated globulins and compare them with those of the other two globulin conjugates (section 4c below).

3a. The proportions of conjugated globulin, unconjugated globulin, and free marker in the final preparation.

The preparations of fluorescein-conjugated globulins after fractionation on Sephadex G.25 (Curtain 1961, Fothergill and Nairn 1961, George and Walton 1961) were shown to contain no unconjugated globulin or fluorescein (Fig. 7). This result contrasts with earlier reports in which only the unconjugated fluorescein had been removed by this process (Curtain 1961, Fothergill and Nairn 1961). George and Walton (1961) found that the protein fraction was eluted as two overlapping peaks, the second of which they describe as "less completely labelled with fluorochrome". This differs from the present report where the second protein peak was found to be completely unconjugated.

The final preparation of ferritin conjugated globulin contained some unconjugated ferritin, but the latter has never been seen to label tissues non-specifically in this or other investigations (Andres et al. 1967). Consequently no attempt was made to remove it.



— Whole serum (rabbit 13R).

— Sheep anti-rabbit serum

— Ferritin-conjugated globulin (rabbit 20R).

— Whole serum (rabbit 20R).

— Sheep anti-rabbit serum.

— Fluorescein-conjugated globulin (rabbit 20R).

— Whole serum (rabbit 20R).

— Sheep anti-rabbit serum.

— Iodine^{125}-conjugated globulin (rabbit 20R).

ANODE CATHODE

FIG.8 Immunophoretic analysis of conjugated globulins using sheep anti-rabbit serum as antibody, showing the change in electophoretic mobility of the globulins as a result of conjugation to the marker molecule. Techniques described on pages 12-13.

The preparation containing iodine^{125} conjugated globulins did not contain any unconjugated iodine^{125} (since the latter was removed by dialysis), but was assumed to contain both iodine^{125} conjugated globulin and unconjugated globulin. This assumption was based on the fact that the technique used for labelling the globulin produces a conjugate with an iodine^{125}:globulin ratio of 1:1. Therefore some of the globulin molecules would not be expected to be conjugated.

3b. Charge of the conjugated globulins

When the electrophoretic mobility of three conjugates were compared with that of globulin in whole serum by immunoelectrophoresis (Fig. 8), it was apparent that all the preparations of conjugated globulin contained more than one protein, although the major precipitin arc was due to the gamma-globulin. It was also very apparent that conjugation of the globulin to fluorescein had slightly increased the negative charge of the globulin, and that conjugation to ferritin had increased the negative charge of the globulin most noticeably. These increases in negative charge have been reported by other workers (Curtain 1958, Schiller 1953, Borek and Silverstein 1961, Andres et al. 1967). In contrast it can be seen that the iodine^{125}-conjugated globulin had the same electrophoretic mobility as the unconjugated globulin.

3c. Effect of conjugation on the antigen-binding capacity of the globulin.

The antibody activity of the conjugates was tested by the immobilization test and by immunodiffusion. Fluorescein-conjugated antibodies showed a detectable precipitating activity and an adequate immobilization titre

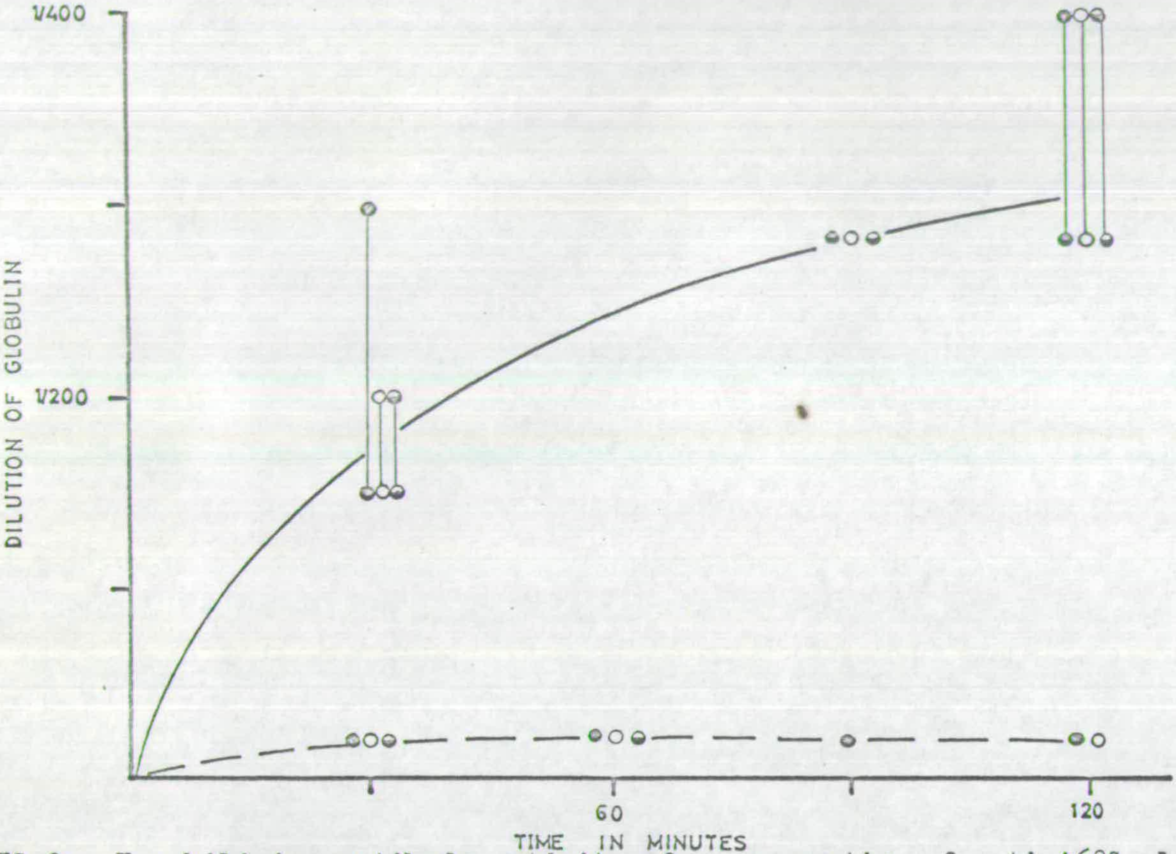


FIG.9a Immobilizing antibody activity of a preparation of anti-168G globulin before and after conjugation to iodine^{125} , all preparations being at the same protein concentration. ●—● unconjugated globulin; O—O iodine^{125} conjugated globulin; ⊖—⊖ globulin "conjugated in the absence of marker molecule".
—— Paramecia serotype 168G (homologous),---- Paramecia serotype 168D (heterologous

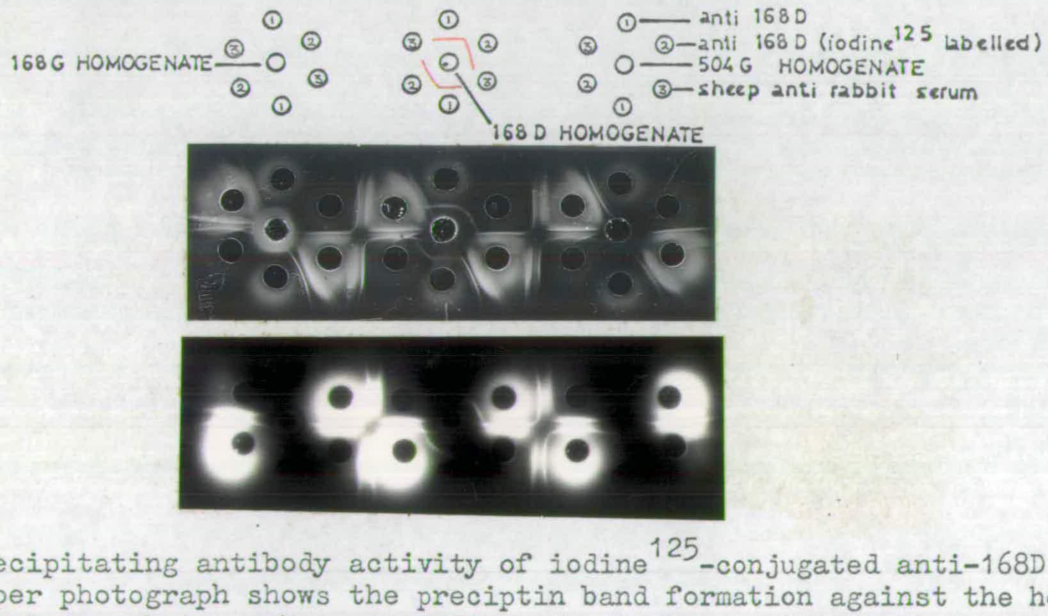


FIG.9b Precipitating antibody activity of iodine^{125}-conjugated anti-168D globulin. The upper photograph shows the preciptin band formation against the homologous antigen The lower photograph anautoradiograph of the precipitin bands produced .Techniques as described in the materials and methods pages 12 and 24.

($^1/_{30}$), although the latter was much lower than unconjugated globulin at the same protein concentration ($^1/_{200}$). Ferritin-conjugated globulins did not possess any precipitating activity, but did show a low specific immobilization of paramecium. Iodine^{125}-conjugated globulins showed a specific precipitating activity identical to that of unconjugated globulin (Fig. 9). The immobilization titre was unaffected by conjugation (Fig. 9). Little change in the antibody activity of the iodine conjugate was to be expected, since Johnson et al. (1960) showed that at least five atoms of iodine^{131} are required to be combined with a single globulin molecule in order to produce a detectable reduction in antibody titre, and in the present work the ratio of iodine^{125}:globulin was 1:1.

4. <u>The Practical Applications of the conjugated globulins</u>

4a. Fluorescein

This marker was used extensively in the investigation of non-specific labelling, due to the ease and rapidity of analysis. As regards the cytoplasmic localization of the immobilization antigens, however, fluorescein was used in preliminary experiments only, because of the relatively poor resolution obtained.

4b. Ferritin

The very high resolution of ferritin as a conjugated antibody marker, makes this conjugate one of the most useful for the localization of surface antigens. Ferritin conjugated antibodies have been used to localize surface antigens on a variety of organisms including: blood group substances on human erythrocytes (Lee 1963, Lee and Feldman 1964), the surface antigens of sea urchin eggs (Baxandall et al. 1962, 1964), influenza virus on chick embryo cells (Morgan et al. 1961a), and the immobilization antigens of Paramecium aurelia (Mott 1964, 1965). In the present work ferritin-conjugated globulin has been found of little use as a cytoplasmic antigen marker, because of its very large size (M.W. 750,000 Hoffman and Harrison 1963). It has never been seen to penetrate more than 1000 Å into the cut surface of a well preserved frozen section. The penetration of this antibody conjugate into the cytoplasm was noticeably dependent upon breaks in the tissue, a finding which could lead to a misinterpretation of the cytoplasmic distribution of the immobilization antigens. In addition to the disadvantage of size, ferritin may be considered the least suitable of the conjugates tested here, because of the gross changes in the charge and antibody activity brought about by conjugation. Other workers have also pointed out the unsuitability of ferritin-conjugated antibodies for the localization of cytoplasmic antigens (Nakane & Pierce 1967, Chapman, personal communication). Despite these objections many workers have continued to make use of the technique, and by this means have successfully localized cytoplasmic viruses (Oshiro et al. 1967, Morgan et al. 1961 b,c, 1962, Kalnins et al. 1966, Rifkind et al. 1960, 1962a, 1964, Lee 1960, Hsu et al. 1963) and cytoplasmic proteins (Andres et al. 1962, Pierce et al. 1963, 1964, Rifkind et al. 1962b). The majority of these investigators used tissues that had been frozen and thawed, and overlooked the resulting ultrastructural damage produced by the technique. A most promising approach to the use of ferritin-conjugated antibody as a label for cytoplasmic antigens, is that of post-embedding staining (Thomson et al. 1967), followed by electrophoretic 'destaining' (Molenaar 1968).

Other molecules have been used to label antibodies, which have then been used to localize cytoplasmic antigens. Many workers have now successfully used peroxidase or acid phosphatase labelled antibody, illustrating the antigenic sites by the histochemical localization of the conjugated enzyme (Nakane & Pierce 1967, Leduc, Scott and Avrameas 1969). Apart from the obvious objection raised by the presence of endogenous enzymes, these marker molecules are still very large (M.W. 40,000). Other techniques using mercury (Pepe et al. 1960, Pepe 1961 a,b,c, 1962), uranium-conjugated antibodies (Sternberger et al. 1963), and haemocyanin (Eskland 1967) all have technical disadvantages. These antibody markers have not been used in the present work.

4c. Iodine

The use of iodine in various isotopic forms, as a protein marker is now well established. Radioactive iodine^{131} has been used to label various proteins including human growth hormone (Greenwood, Hunter and Glover 1963), antibodies (Johnson et al. 1960, Cohen 1951), insulin (Samols and Williams 1961), tissue soluble proteins (Bocci 1964) and others (MacFarlane 1958). Iodine^{131} has also been used as an antibody marker for the autoradiographic localization of antigens (Clayton and Feldman, 1955). Some recent electron microscope observations have localized viral antigens, with apparent success, using antibody molecules made electron dense by conjugation with as many as 40 atoms of non-radioactive iodine, (Mekler et al. 1964, Parfanovich et al. 1965,1966). Iodine^{125} being radioactive and having a lower energy of emission than iodine^{131}, is not only suitable for light microscope autoradiography (Hanna et al. 1966) but also for electron microscope autoradiography (Applegren et al. 1963, Fitch et al. 1962, Kayes et al. 1962, Kuhn and Harford 1963, Stein and Gross 1963). In the present investigation it was felt that the development of a suitable technique for the ultrastructural localization of native antigens using $iodine^{125}$ trace labelled globulins would be an improvement over the majority of previous techniques.

The properties of $iodine^{125}$-labelled globulins prepared after the method of Johnson et al. (1960), at a theoretical globulin:iodine ratio of 1:1, indicate that this conjugate is more suitable as an antibody marker for the detection of cytoplasmic antigens than either fluorescein or ferritin conjugated globulins. The marker molecule is small (MW 125), is substituted into the tyrosine ring on the globulin molecule (Hunter 1967), and therefore causes little change in the size and shape of the globulin molecule. It was also shown that there was no detectable change inthe charge of the globulin, or loss of antibody titre upon conjugation. The relative ease and accuracy with which $iodine^{125}$ can be detected makes it the most useful antibody marker. This isotope can be quantitatively estimated by liquid scintillation counting (Rhodes 1965, Miles and Hales 1968), and localized by light and electron microscope autoradiography. The only disadvantage I have found using this technique is the delay, of between one and twenty months, before electron microscope autoradiographs are ready for analysis.

5. <u>Non-Specific Staining</u>

It is convenient at this point to discuss two non-specific reactions that have been encountered, both of which have caused considerable difficulty in this investigation. These are globulin-tissue adsorption and globulin-globulin adsorption.

5a. Globulin-tissue adsorption

Two methods of fixation for electron microscopy, - osmium tetroxide and glutaraldehyde/osmium tetroxide - were compared for the ability to preserve the antigenicity of the immobilization antigen. Frozen sections of homologous and heterologous cells fixed by each method were treated with fluorescein-conjugated globulin. After osmium tetroxide fixation a bright fluorescence of the homologous pellicle and cilia, and a low fluorescence of the cytoplasm, were seen (see Plate 6A). Heterologous tissue showed the same low intensity of cytoplasmic fluorescence as homologous tissue, but no reaction on the pellicle and cilia. Homologous cells fixed in glutaraldehyde and postfixed in osmium tetroxide did not show any fluorescence on the pellicle, but did show a low cytoplasmic reaction. A similar result was obtained with heterologous cells fixed in glutaraldehyde/osmium tetroxide. This observation is similar to that of Beale and Kacser (1957) who found that formaldehyde fixation destroyed the antigenicity of the immobilization antigen. The cytoplasmic fluorescence observed in sections fixed by glutaraldehyde/osmium tetroxide could not have been due to the presence of active immobilization antigen, since the antigenicity of this protein on the pellicle was destroyed. As the immune sera used for this section of the investigation were shown to possess antibody activity against immobilization antigen only, the observed cytoplasmic staining must be assumed to be non-specific. This was confirmed when a similar fluorescent labelling of the cytoplasm of frozen sections of osmium fixed cells was observed after treating the sections with fluorescein-conjugated normal rabbit globulin.

The cytoplasmic labelling of frozen sections by fluorescein-conjugated globulins for homologous, heterologous and normal rabbit sera, was found to be indistinguishable in intensity in the fluorescence microscope, and also uniform throughout the cytoplasm and nucleus. The uniform distribution of globulin over the cut surface of frozen sections was also noticed in the electron microscope using both ferritin conjugated and unconjugated globulins (Plates 3c and 4).

The retention of the different iodine^{125}-conjugated globulins in frozen sections of paramecium was measured by liquid scintillation counting. It was repeatedly observed that there was a greater retention of globulin by sections which had been treated with normal globulin, than with a heterologous immune globulin or even homologous immune globulin, even when all three globulins were applied at the same protein concentration (Table 4). This difference in reactivity must therefore reflect a difference in the relative protein composition of the immune and non-immune sera, the immune globulin components causing less non-specific globulin-tissue adsorption than other components. It has been shown that the non-specific reaction between normal serum and animal cell nucleoli is caused by a globulin with a slow alpha 1 electrophoretic mobility (Maisel and Lytle 1966).

The non-specific labelling of homogenates of paramecia by fluorescein- and ferritin-conjugated normal globulin was investigated. Osmium fixed or fresh material was treated with the conjugated globulins in a variety of buffers (MS, pH 6.8, Tris-HCl 0.1M, pH 7.6, and veronal acetate 0.3M pH 8.6). In all preparations heavy labelling of all cytoplasmic organelles, notably the kinetosomes, kinetodesmata, and the inner surface of the pellicle, was observed (Plate 3 a,b). The labelling of the inner but not the outer surface of the pellicle by normal globulin, indicates a marked difference in the properties of these two surfaces. It will be seen below that the presence of the immobilization antigens on the outer surface of the pellicle prevents

Serum	Protein concentration of globulin applied to frozen sections (mgm/ml)	Antibody bound radioactivity (counts/min/μg)	Total radioactivity retained by frozen sections (counts/min)	weight of globulin retained by frozen sections (μg)
Normal rabbit serum	1.45	1,856	27,572 ± 1,775	14.8
Homologous antiserum (anti-168D)	1.54	776	7,424 ± 314	9.5
Heterologous antiserum (anti-168G)	1.40	626	2,690 ± 117	4.3

TABLE 4   The retention of iodine^{125} conjugated globulins by frozen sections of paramecia of serotype 168D. Equal aliquots of the same preparation of frozen sections were incubated with 2 ml of each conjugated globulin. The globulin solutions were adjusted to similar protein concentrations as estimated by the Lowry technique. The radioactivity retained in the sections was measured - in 4 samples of each group - by liquid scintillation counting.

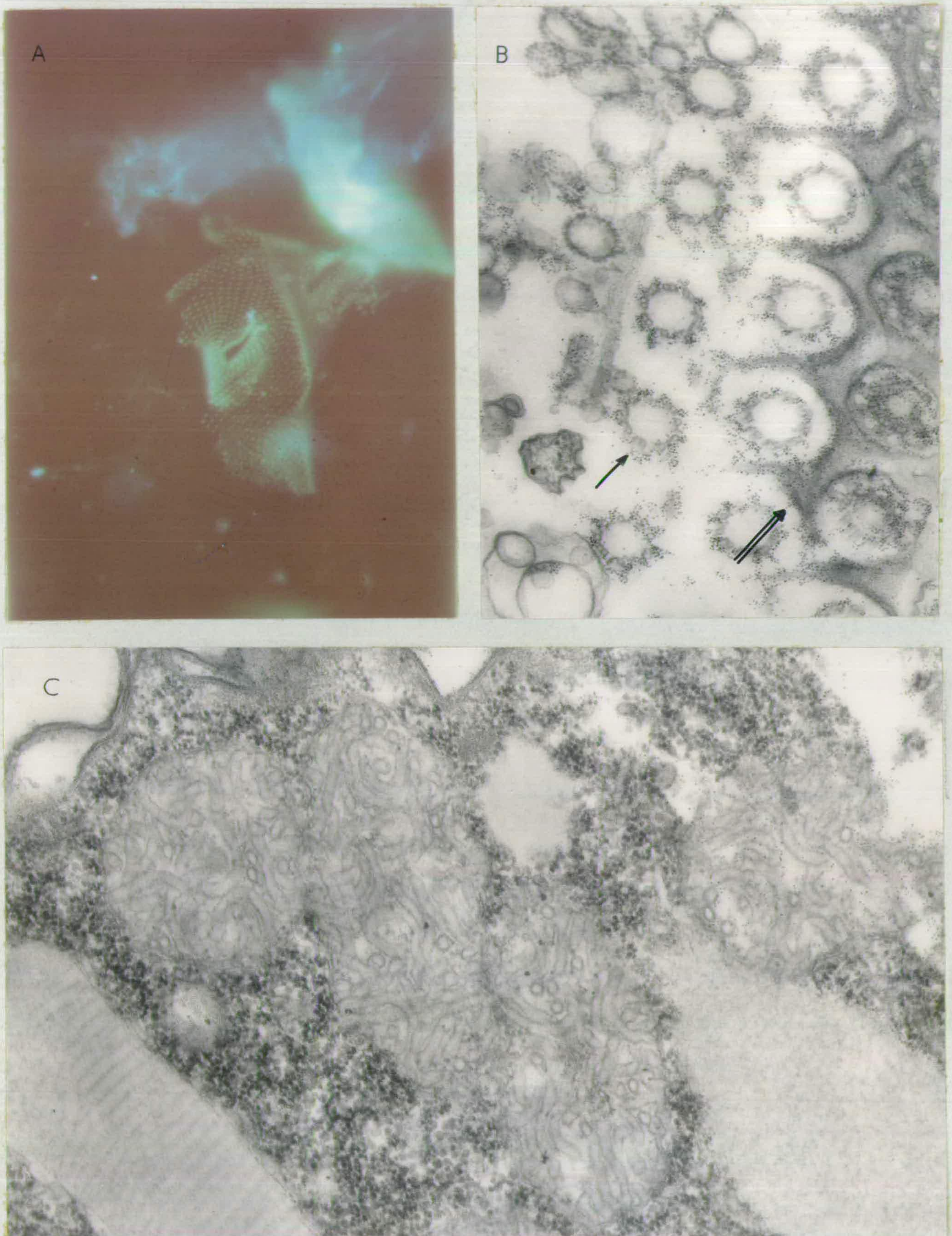

A
B
C

PLATE 3

A. Fluorescence micrograph of a pellicle fragment from an unfixed homogenate of paramecium (serotype 168G) labelled with fluorescein-conjugated normal rabbit globulin. The rows of kinetosomes react non-specifically but not the outer surface of the pellicle and cilia. Mag. x 800.

B. Section of a homogenate of paramecium (serotype 504G) treated with ferritin-conjugated anti-168D globulin, subsequently fixed in osmium tetroxide. Heavy deposits of ferritin conjugated globulin are found on the kinetosomes (arrow), and inner surface of the pellicle (double arrow). Mag x 50,000.

C. Electron micrograph of a frozen section of a cell (serotype 168G) treated with ferritin-conjugated normal rabbit globulin. Non-specific labelling is uniform and heavy over the cut surface, but is not detected on the outer surface of the pellicle. The lack of penetration of the conjugated globulin into the cytoplasm is very apparent. Mag x 50,000.

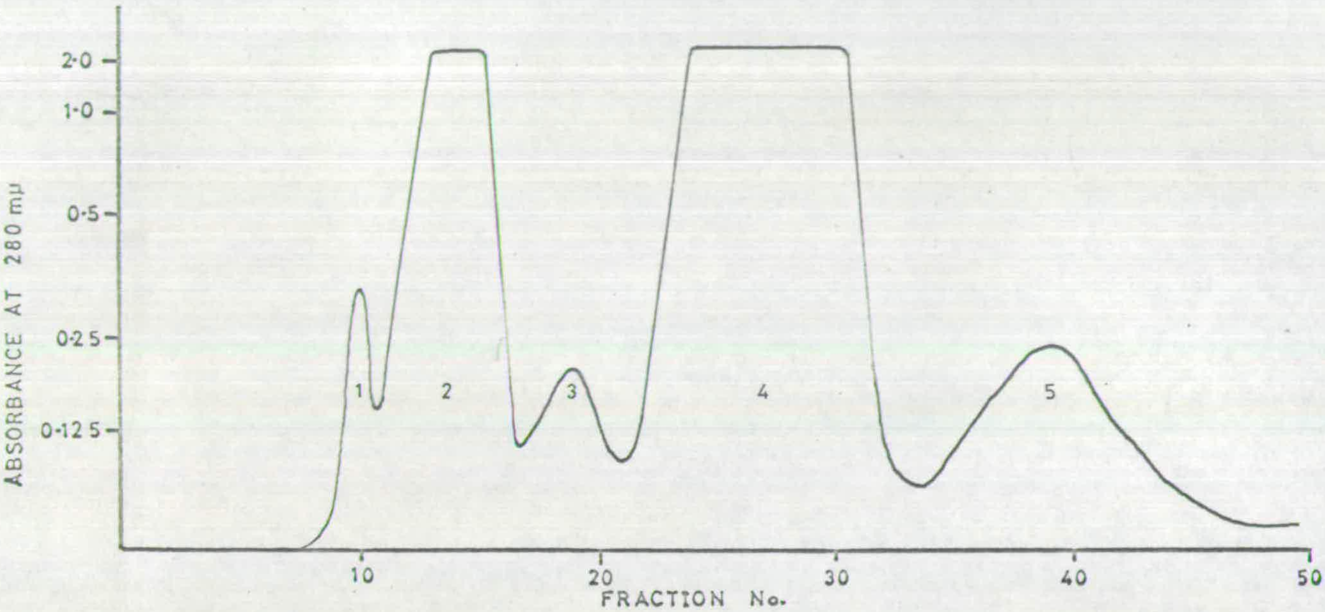


FIG.10 Elution of a 4ml. sample of fluorescein-conjugated anti-168G globulin - previously absorbed with 168D cell homogenate - from a column (25 x 1cm.) of DEAE cellulose . The sample was eluted with 0.01M phosphate buffer pH 7.5 containing a continuous gradient of sodium chloride from 0.00M to 0.20M . Total volume of gradient 500 ml.

PEAK NUMBER		1	2	3	4	5
E_{495}/E_{280}		0.0271	0.0543	0.1250	0.4420	0.1310
IMMUNODIFFUSION ANALYSIS						
Anti-168D antibody		+	-	-	-	-
Anti-rabbit serum antibody		-	-	+	+	+
FLUORESCENT STAINING						
Pellicle	Homologous serotype	-	-	+	+	++
	Heterologous serotype	-	-	-	-	-
Cytoplasm	Homologous serotype	-	-	-	±	+
	Heterologous serotype	-	-	-	-	±

TABLE 5 Analysis of the material eluted from DEAE cellulose after the absorption of anti-168G fluorescein-conjugated globulin with 168D homogenate (Fig. 10). The degree of conjugation of the protein in each peak is given by the E_{495}/E_{280} ratio. The presence of 168D antigen and rabbit globulin in each peak was assayed by immunodiffusion. The specific and non-specific staining properties of the eluted material were shown by treating frozen sections of paramecia of homologous (168G) and heterologous (168D) serotypes with material from each peak, at similar protein concentrations.

the non-specific adsorption of the globulins. The observed non-specific reaction confirms therefore that the immobilization antigens are not present on the inner surface of the pellicle.

Two established methods for the removal of non-specific labelling by fluorescein conjugated globulins - tissue adsorption (Nairn 1962), and ion-exchange chromatography (Goldstein et al. 1961, Riggs et al. 1960), were used simultaneously on the same preparation of fluorescein conjugated (anti-168G) globulin. The globulin was absorbed twice with an equal volume of 168D homogenate, and the supernantant was then eluted from a column of DEAE cellulose in 0.01M phosphate buffer, containing a gradient of sodium chloride from 0.00 to 0.02 M. It can be seen from Fig. 10 and table 5 that with the exception of the last peak eluted from the column there is a correlation between the degree of conjugation - ($E495/E280$), with the retention on the column and the intensity of labelling of the cytoplasm of frozen sections. This indicates that the greater the negative charge of the conjugated globulin, the greater the non-specific labelling. This observation has been reported by other workers (Holbrow and Johnson 1967, Nairn 1962, Goldstein et al. 1961, Riggs et al. 1960, Mayersbach 1959).

However, non-specific labelling by fluorescein and ferritin conjugated globulins has been observed both with homogenates in buffers the pH of which ranged from pH 6.8 to 8.6, and with frozen sections treated with unconjugated globulins. Also there was no apparent difference between conjugated and unconjugated globulins in their efficiency in blocking non-specific labelling (see below). It is concluded therefore that the increased negative charge of a globulin, due to conjugation, is only a contributory and not a major cause of non-specific labelling in this system.

The primary cause of non-specific labelling is believed to be the adsorption of the globulin molecules onto the tissues of the frozen sections or homogenates. This reaction may be due to the complementary structure of some tissue and globulin molecules (Klotz 1953).

5b Globulin-Globulin Adsorption

An alternative method of reducing non-specific staining was attempted by counterstaining or blocking the non-specific staining with rhodamine conjugated normal globulin before reacting the tissue with fluorescein conjugated immune globulin (Smith et al. 1959, Metzger and Smith 1960, Malizia et al. 1961, Brown and Bittner 1961). The rhodamine conjugate was found to be no more efficient than unconjugated globulin in reducing non-specific staining. Therefore in all investigations on the cytoplasmic localization of immobilization antigen using fluorescein conjugated globulins, unconjugated globulin was used for presaturation.

Unconjugated globulins were used to presaturate frozen sections that were subsequently treated with ferritin conjugated globulins prepared from homologous or heterologous sera. It was noticed that the presaturating globulin formed a thick carpet over the surface of the section (Plate 4). Also, the ferritin labelled globulin was present as an even layer over the surface of the presaturating globulin (Plate 4a). This could be due to the exchange of molecules between the unconjugated globulin and the ferritin conjugated globulin (Lee and Feldman 1964), but the intensity of the reaction, and particularly the thickness of the presaturating globulin layer indicates a strong globulin-globulin adsorption.

The existence of this globulin-globulin reaction means that the

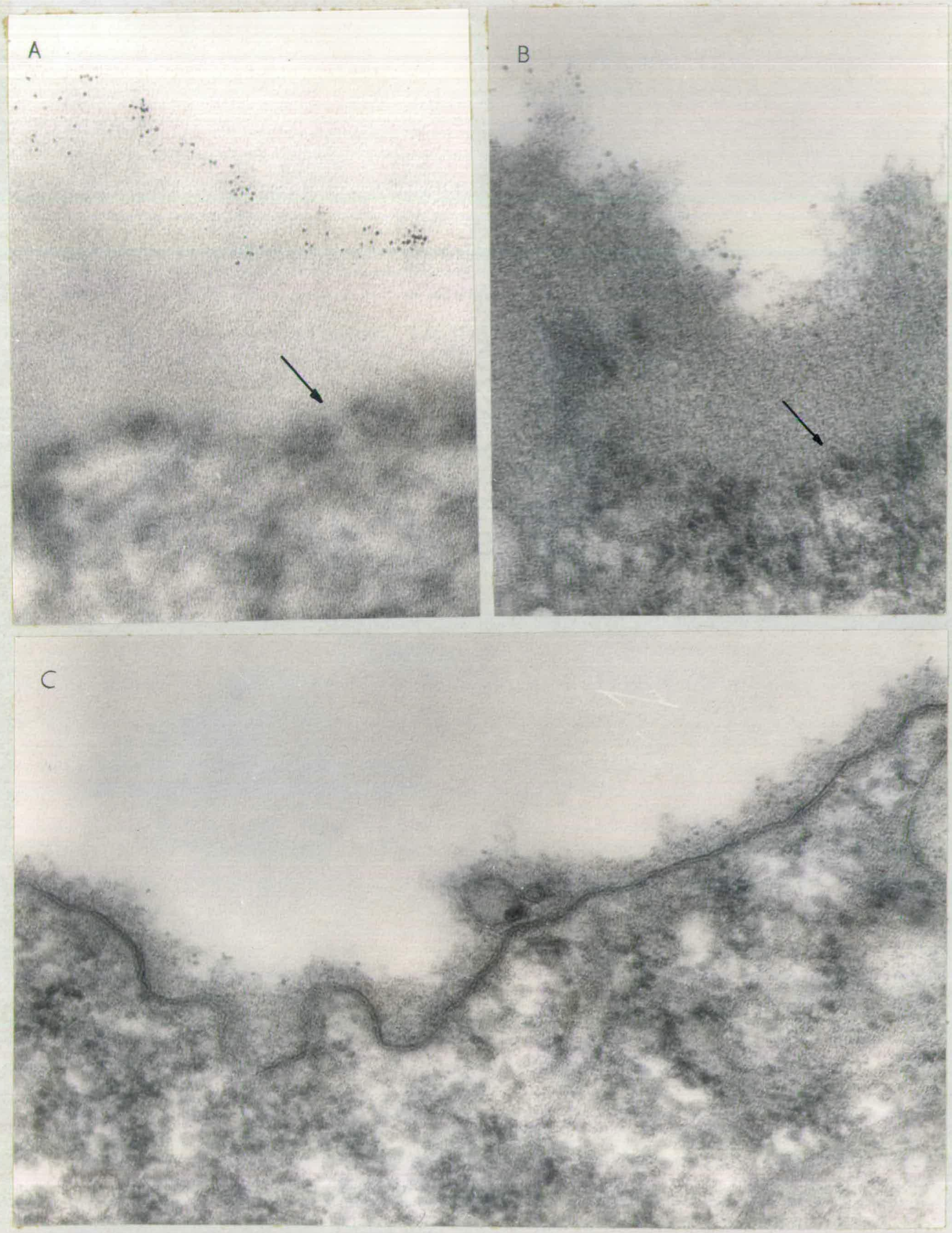

A
B
C

PLATE 4

A-B. Electron micrographs showing the adsorption of ferritin-conjugated anti-168D globulin onto the presaturation layer of unconjugated anti-168G globulin. The presaturation globulin is revealed on the cut surface of the frozen section (arrow) by staining with lead citrate and uranyl acetate (plate 4B). The ferritin conjugated globulin is most apparent in the unstained material (plate 4A). Mag. x 125,000.

C. Section of the membrane surrounding the contractile vacuole from a frozen section of a cell (serotype 168G) treated with unconjugated normal globulin prior to labelling with ferritin conjugated anti-168G globulin. Unconjugated globulin is adsorbed to the membrane, and the ferritin conjugated antibody is seen attached to the presaturating globulin. Mag. x 125,000.

observed labelling by a conjugated specific antiserum after presaturation, depends not only on the specific antigen-antibody reaction, but also on the density of the blocking globulin. A heavy coating of blocking globulin would result in a high labelling with conjugated specific antiserum. This, combined with the finding that blocking may never be complete, makes the interpretation of presaturation experiments less reliable than originally anticipated.

The relative value of the various techniques for the detection of the immobilization antigen may be summarised as follows:-

1) The sera used for the localization of the immobilization antigen <u>in situ</u> are specific for the immobilization antigens.
2) Ferritin conjugated antibodies were of considerable value for the ultrastructural localization of the immobilization antigens on the pellicle and cilia. Here the size of the conjugated globulin would not affect the access of the antibody to the antigenic sites.
3) Fluorescein conjugated antibodies were of value in preliminary experiments only, to detect the immobilization antigens in the cytoplasm of frozen sections. This required the presaturation of the frozen sections by unconjugated globulins in order to reduce the extensive globulin-tissue adsorption between the conjugated globulin and the tissues. The interpretation of these experiments was complicated by the possibility of adsorption between the presaturating and specific globulins.
4) The properties of the iodine^{125} conjugated globulins indicate that this conjugate is superior to either fluorescein or ferritin, as a

marker of cytoplasmic antigens. Iodine^{125} conjugated globulins were used extensively to locate the immobilization antigens *in situ* in frozen sections. The presence of the immobilization antigen in an organelle was detected as a greater proportional labelling of the organelle by homologous immune globulin than by heterologous or normal globulins.

RESULTS

The localization of immobilization antigen using conjugated antibodies.

a) In situ localization 1. The Pellicle.

Live paramecia when placed in homologous fluorescein conjugated globulin were rapidly immobilized and when studied in the fluorescence microscope the cilia and pellicle could be seen labelled with antibody. A few animals were also seen to be lysed thus allowing antibody to enter the cytoplasm. Heterologous animals treated in the same manner were neither immobilized nor lysed, and did not show any fluorescence of the pellicle and cilia, though antibody could be seen ingested into the food vacuoles (Plate 6 a,b).

Similarly, when live paramecia of serotypes 168G and 168D were suspended in ferritin-conjugated globulin against 168G antigen, the homologous cells were rapidly immobilized (within 15 minutes), and after a short while (45 minutes) the swimming of the heterologous paramecia was retarded, although they did not show the characteristic behaviour associated with immobilization. This affect was not associated with an immunological cross reaction, but represents 'toxicity' of the globulin molecules. After fixation and embedding for electron microscopy, both homologous (168G) and heterologous (168D) preparations showed a severe breakdown of the pellicle, and the homologous paramecia in particular revealed a massive leeching of the cytoplasm (see plate 5 a,b). This tissue destruction was not found when paramecia were suspended in a solution of ferritin. The breakdown of the pellicle is therefore presumed to be due to the antibody molecules or possibly other serum components. This breakdown may be responsible, in

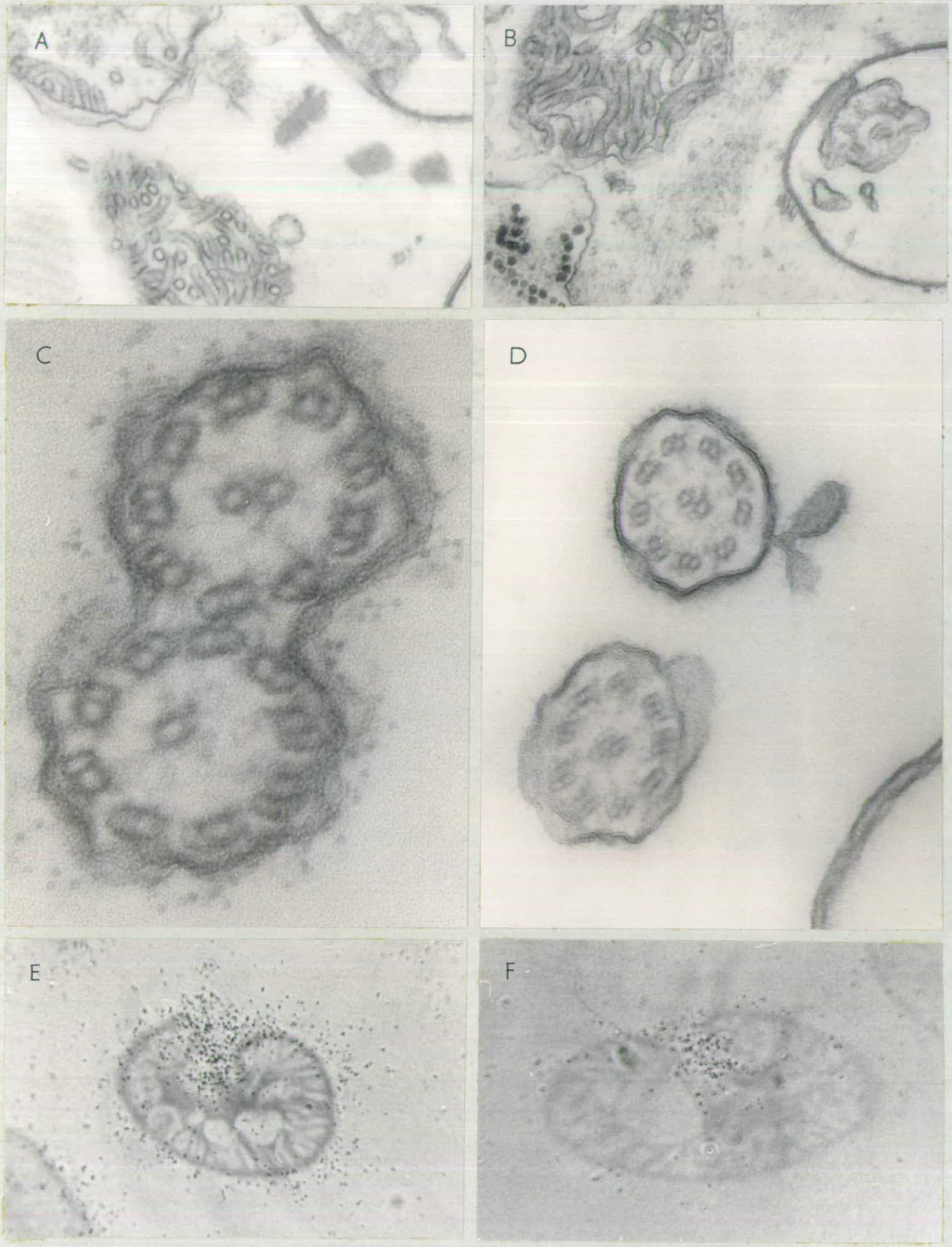

A
B
C
D
E
F

PLATE 5

A. Pellicle of paramecium (serotype 168G) treated live with ferritin-conjugated anti-168G globulin for two hours at 18°C then fixed in osmium tetroxide (Homologous reaction). Mag. x 50,000.

B. Pellicle of paramecium (serotype 168D) treated in a similar manner (Heterologous reaction). Mag x 50,000.

C. T.S. of cilium of osmium fixed paramecia (serotype 168G) incubated with ferritin-conjugated anti-168G globulin. (Homologous reaction). Mag. x 250,000.

D. T.S. of cilium of paramecia (serotype 168D) treated in a similar manner to that above - plate 5c. (Heterologous reaction). Mag. x 125,000.

E. Light microscope autoradiograph of a section of paramecium (serotype 168G) treated with $iodine^{125}$-conjugated anti-168G globulin. (Homologous reaction). Mag. x 320.

F. Light microscope autoradiograph of a section of paramecium (serotype 168D) treated as for plate 5e. Note retention of label in the gullet. Mag. x 320.

part, for the observation that fluorescein conjugated antibody was released from the pellicle of labelled living cells (Beale & Kacser 1957).

If the paramecia were first fixed in osmium tetroxide and then treated with conjugated antibody, both the ferritin and the fluorescein conjugated globulins showed very specific reactions with the pellicle and cilia, and of course no antibody was ingested into the food vacuoles (see plate 5c,d). Since ferritin is particulate when seen in the electron microscope, it was possible to measure the density of antibody on the outer surface of the pellicle and cilia. When the densities of ferritin conjugated globulins from homologous (anti-168D), heterologous (anti-168G) and normal sera on the pellicle of fixed cells of serotype 168G and 168D are compared, (table 6), it is seen that the homologous reaction is much heavier than that produced by either heterologous or normal sera, and also that the reactions of the latter two globulins are not significantly different.

Quantitative estimates have also been made of the reactions of iodine^{125} conjugated globulins with whole paramecia. When samples of fixed whole cells of serotypes 168G and 168D were treated with anti 168G globulin, and the antibody retention measured by scintillation counting, an unexpectedly high heterologous reaction was observed. (table 7) However the same preparations, when examined by light microscope autoradiography, showed that the reaction of the pellicle and cilia (excluding the gullet region) was specific. Calculations of the density of isotope on these structures indicate the same low level of cross reaction shown by both fluorescein and ferritin conjugated globulins (table 8). The high heter-

Serotype of paramecia	Specificity of ferritin-conjugated globulin	Density of ferritin ± standard error (molecules/unit distance)	Percent Homologous reaction	
168D	anti-168D	1.049 ± 0.070	100	
168G	anti-168D	0.041 ± 0.010	3.9	t=1.39 P>0.10
168G	Normal rabbit serum	0.019 ± 0.012	1.8	

TABLE 6 Reaction of ferritin conjugated globulins with the pellicle and cilia of osmium fixed paramecia. Equal volumes of fixed cells of serotypes 168G and 168D were incubated for two hours at 18°C with ferritin conjugated globulins as indicated. The density of the ferritin on the pellicle and cilia was estimated from random electron micrographs as described in the materials and methods page 21.

Cell serotype	Specificity of I^{125} conjugated globulin	Total Counts ± standard error (5 observations)	Counts/mgm dry weight of cells	Percentage homologous reaction
168G	anti-168G	440.0 ± 21.0	146.8	100.0
168D	anti-168G	351.4 ± 20.4	78.9	53.8

TABLE 7 Retention of iodine^{125} conjugated globulin by fixed whole cells of homologous and heterologous paramecia. Techniques as described in materials and methods page 19.

Cell serotype	Specificity of I^{125} conjugated globulin	Density of autoradiographic grains per unit length of pellicle	Percentage homologous reaction
168G	anti-168G	3.40 ± 0.38	100.0
168D	anti-168D	0.25 ± 0.03	7.4

TABLE 8 The density of iodine^{125} conjugated antibody on the pellicle and cilia of fixed paramecia (excluding the gullet region). Ten samples of light microscope autoradiographs were used to calculate each value. The method of analysis was similar to that described for electron microscope autoradiographs (see table 10).

ologous counts were seen to be due to the mechanical retention of isotope in the gullets of paramecia of both serotypes (see Plate 5 e,f). Further quantitative analysis by electron microscope autoradiography of the reactions of $iodine^{125}$ conjugated globulins with the pellicle of frozen sections, have confirmed the results obtained with both the fluorescein and the ferritin conjugated globulins; namely that the pellicle reacts specifically with homologous antibody, and the low reactions of heterologous and normal sera with pellicle material are not significantly different from each other. (See table 11).

It is concluded that the outersurface of the pellicle of a stable culture of Paramecium aurelia of serotype 168G contains only the homologous antigen i.e. it does not contain heterologous antigen and does not react non-specifically.

2. The Cytoplasm

Using fluorescein conjugated globulins, prepared from normal and anti 168G sera, to label frozen sections of cells of serotypes 168G and 168D, no detectable difference in the intensity of labelling of the cytoplasm of any preparation was observed (see Plate 6c).

The possibility remained that the antigen was present in the cytoplasm but could not be detected due to the very heavy non-specific labelling. Therefore attempts were made to block the non-specific labelling, using unconjugated globulins, before reacting the sections with a homologous fluorescein conjugated globulin. (See Plate 6 d,e,). The accumulated results of many experiments are given in table 9. The staining of the pellicle is consistent with the previous results indicating the presence

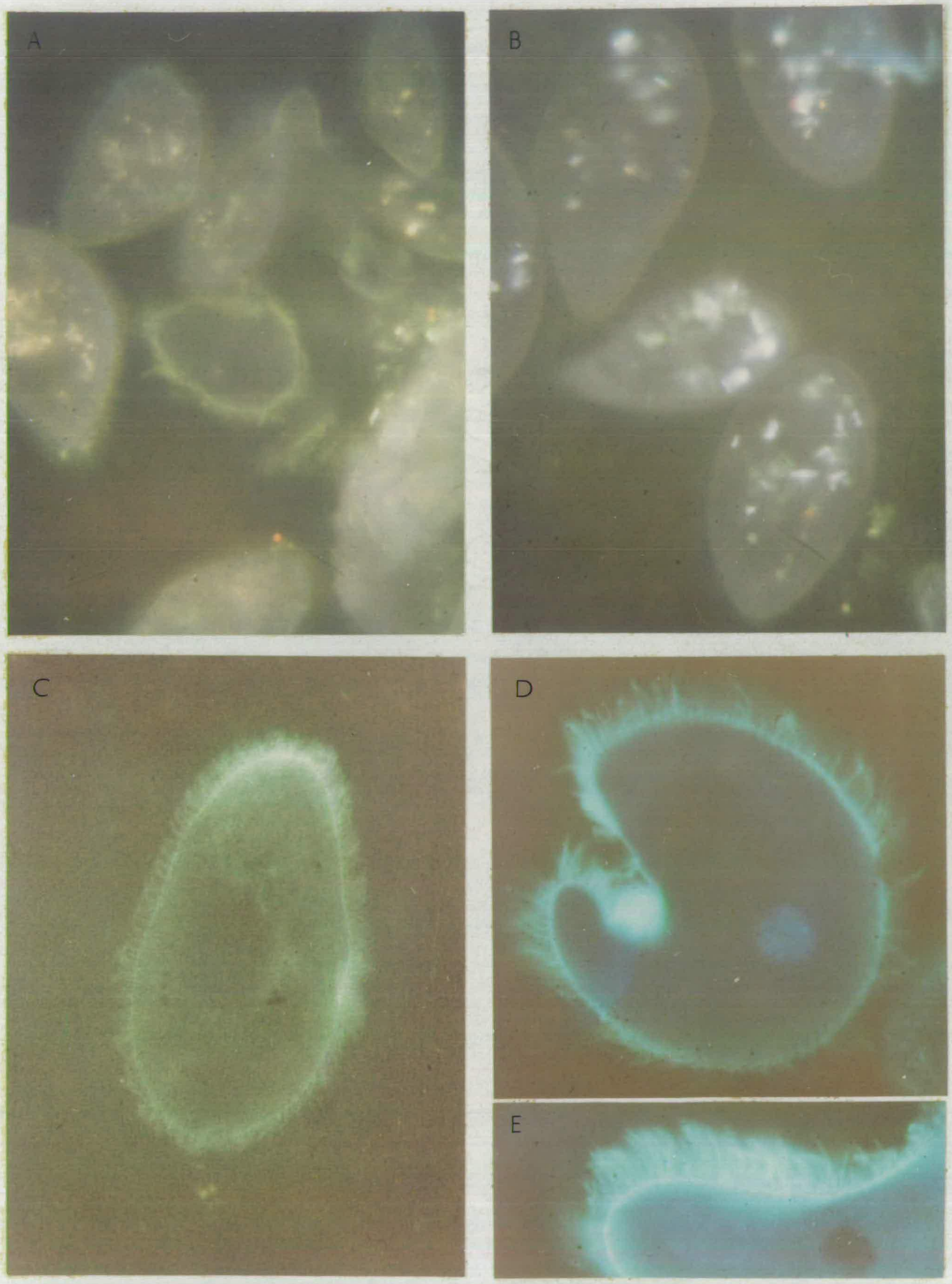

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PLATE 6

A. Fluorescence micrograph of live paramecia (serotype 168G) incubated for two hours at 18°C with fluorescein-conjugated anti-168G globulin. Note fluorescent cilia and pellicle. Mag. x 320.

B. Live paramecia (serotype 168D) treated as for plate 6A. Mag.x320.

C. Frozen section of paramecium (serotype 168G) treated with fluorescein-conjugated anti-168G globulin, the pellicle and cilia react strongly, the cytoplasm is less densely labelled. Mag. x 800.

D. Frozen section (serotype 168G) presaturated with unconjugated heterologous (anti-168D) globulin, subsequently treated with fluorescein-conjugated homologous (anti-168G) globulin. Note the marked reduction in cytoplasmic labelling with little reduction in the labelling of the pellicle and cilia. Mag. x 800.

E. Higher power micrograph of the preparation described above (plate 6D). Mag. x 1,280.

of homologous antigen only. The cytoplasm however, appears to be blocked more successfully by heterologous globulin (anti 168D) than by normal rabbit globulin. This result can be satisfactorily explained in two ways. The first is that the reduction in labelling in both homologous (168G) and heterologous (168D) tissues, after blocking with the heterologous globulin (anti-168D), is due to the reaction of a precursor molecule common to both 168D and 168G antigens, which is blocked by the heterologous globulin but not by normal globulin. The second explanation for the apparent reduction in labelling is that while both blocking globulins are equally effective in saturating non-specific sites, the normal globulin reacts more strongly with cytoplasmic material. This heavier globulin-tissue adsorption combined with the globulin-globulin adsorption described above could result in an artificially greater retention of the fluorescein conjugated globulin by the normal globulin compared to the heterologous globulin. It can also be seen from table 9 that there is a stronger reaction of the fluorescein-conjugated globulin (anti-168G) with homologous cytoplasm (168G) than with the heterologous cytoplasm (168D), except when homologous globulin is used as blocking globulin. This indicates the presence of small quantities of 168G immobilization antigen in the cytoplasm of 168G cells. However, due to the unreliability of this technique these results are not considered conclusive.

The two major difficulties encountered using the presaturation technique with fluorescein conjugated antibodies - namely the lack of resolution and the inability to quantitate the results - do not arise when iodine^{125} conjugated globulins are used. In investigations using this antibody marker

FROZEN SECTION		PRESATURATING GLOBULIN		
Organelle	Serotype	Normal Rabbit Globulin	'Heterologous' (168D)	'Homologous' (168G)
	'Homologous' (168G)	++++++++	‡++++	‡‡++‡‡−
PELLICLE				
	'Heterologous' (168D)	−−−−‡−−	−−−−‡	−‡
	'Homologous' (168G)	‡‡++++‡‡	‡−−‡‡	‡−
CYTOPLASM				
	'Heterologous' (168D)	+−−−‡‡‡	−−−+	‡−

TABLE 9 The fluorescent labelling of frozen sections of paramecia, fixed in osmium tetroxide, after treatment with fluorescein-conjugated anti-168G globulin for two hours at 18°C, this following presaturation - for two hours at 18°C - with unconjugated globulins from normal, anti-168D or anti-168G sera. Each score represents a separate experimental group. Fluorescence was scored as follows:- + bright fluorescence, ‡ pale fluorescence, - no fluorescence.

non-specific labelling has been investigated by directly labelling (without blocking) frozen sections of paramecia of serotypes 168G and 168D with $iodine^{125}$ conjugated normal globulin (see Plate 8b). The reactions of $iodine^{125}$ conjugated anti 168G globulin with frozen sections of paramecia of serotypes 168G and 168D were also studied, and have been called the homologous and heterologous reactions respectively. Organelles that react more heavily with the immune serum than with the normal serum must contain the immobilization antigen. The distribution of the isotope throughout the sections was studied by electron microscope autoradiography, and analysed as described in the materials and methods section. In those organelles where the retention of the globulin is directly related to the volume of the structure, as may be expected with non-specific labelling, the value for the relative distribution of the globulin will be approximately 1. Where there is a specific antibody-antigen reaction in addition to the non-specific labelling a higher value will be expected e.g. 2.4-3.9 for the homologous pellicle (Table 10).

The results of the individual experiments (Table 10) show that those organelles constituting the major part of the cell volume - namely the pellicle and cilia, trichocysts, mitochondria and cytoplasm - the density of isotope is relatively uniform. The other structure e.g. the macronucleus, contractile vacuole and gullet, show a greater variation in the density of isotope since they represent a smaller fraction of the sample which is of a limited size. The results are summarised in table 11.

A comparison of the non-specific and heterologous reactions (Plate 8b and 8a respectively), does not reveal any statistical difference in the density of labelling of any organelle. It is concluded therefore that cells of a stable culture expressing serotype 168D on the pellicle and cilia, do not contain any detectable 168G antigen in any organelle.

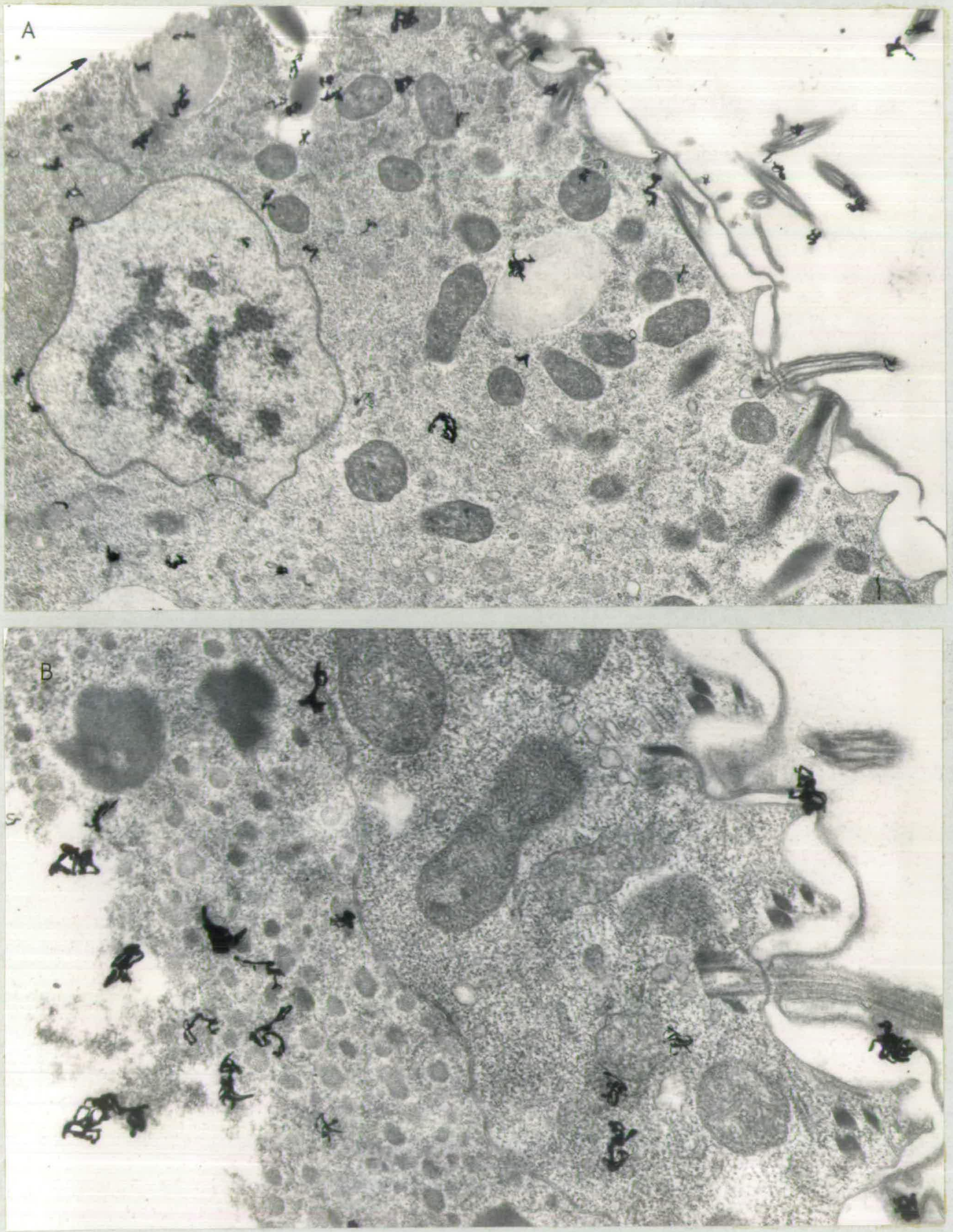

A
B

PLATE 7

A. E.M. autoradiograph of a frozen section of a cell (serotype 168G) labelled with iodine^{125} conjugated anti-168G globulin. The specific reaction of the pellicle and cilia, and heavy cytoplasmic labelling is apparent. The conjugated antibody can be seen to have penetrated deep into the section. The cut surface of the frozen section is indicated by the arrow. Mag. x 10,000.

B. High power micrograph of the same material. A globulin 'fuzz' is seen on the outer surface of the pellicle, the superimposition of some autoradiographic grains on this globulin layer indicates the high resolution possible with this antibody marker. Mag. x 25,000.

Immunological relationship between globulin and frozen sections	Pellicle	Trichocysts	Mitochondria	Macronucleus	Gullet	Contractile vacuole	Food vacuole	Cytoplasm
Homologous	3.902	1.095	1.697	0	-	0	0	0.774
Heterologous	1.572	1.032	1.421	0	0	-	0	0.970
Non-specific	1.398	0.953	0.110	3.714	0	1.514	0	1.261
Homologous	2.464	1.476	0.966	-	-	0	0.327	1.093
Heterologous	0.340	1.191	0.386	3.606	-	-	-	0.808
Homologous	2.737	1.549	1.351	0	-	0	0.600	0.603
Heterologous	0.630	1.297	0.385	-	-	-	-	0.781
Non-specific	1.294	1.257	0.880	2.083	0	-	0	0.801
Homologous	2.645	1.712	0.706	0.108	1.347	0	0.283	1.125
Heterologous	0.714	1.547	0.348	0.115	-	0.336	-	0.890
Non-specific	0.928	2.611	0.746	0	-	-	2.526	0.809
Non-specific	1.820	1.599	0	0	-	-	-	0.687
Homologous	2.952	1.403	1.098	0	-	-	0.778	0.908
Heterologous	1.035	1.535	0.883	-	-	-	-	0.736

<u>Table 10</u> The relative density of iodine^{125} labelled globulins from normal rabbit serum (non-specific reaction) and anti-168G serum in the various organelles of frozen sections of paramecia of serotype 168G (homologous reaction) or 168D (heterologous reaction). Each value was estimated from ten random electron micrographs as described in the materials and methods sections page 24
- represents a sample in which the particular organelle was not present in any of the micrographs taken
[indicates frozen sections of different serotypes treated with the same conjugated antibody preparation.

Immunological relationship between globulin & Frozen section	Pellicle	Trichocyst	Mitochondria	Macronucleus	Gullet	Contractile vacuole	Food vacuole	Cytoplasm
Homologous	2.940	1.447	1.160	0.027	1.347	0	0.398	0.901
	±0.235	±0.101	±0.166	±0.027			±0.134	±0.098
Heterologous	0.858	1.320	0.685	1.240	0	0.336	0	0.837
	±0.209	±0.099	±0.209	±1.183				±0.042
Non-specific	1.360	1.284	0.434	1.449	0	1.514	0.842	0.890
	±0.183	±0.425	±0.221	±0.900			±0.842	±0.127

TABLE 11 Mean values (± standard errors) of the relative density of iodine[125] labelled globulins in each of the organelles of paramecia listed in table 10. Significantly greater relative densities of isotope in the homologous organelles were demonstrated only between the following groups:

a. Homologous and heterologous reactions on the pellicle ($t = 6.366$, $p < 0.001$)

b. Homologous and non-specific reactions on the pellicle ($t = 5.113$, $p < 0.005$)

c. Homologous and non-specific reactions in the mitochondria ($t = 2.640$, $p < 0.050$)

Comparing the homologous and non-specific reactions (Plate 7 and plate 8b respectively), significant differences were found with regard to two types of structure 1) Mitochondria and 2) the pellicle and cilia.

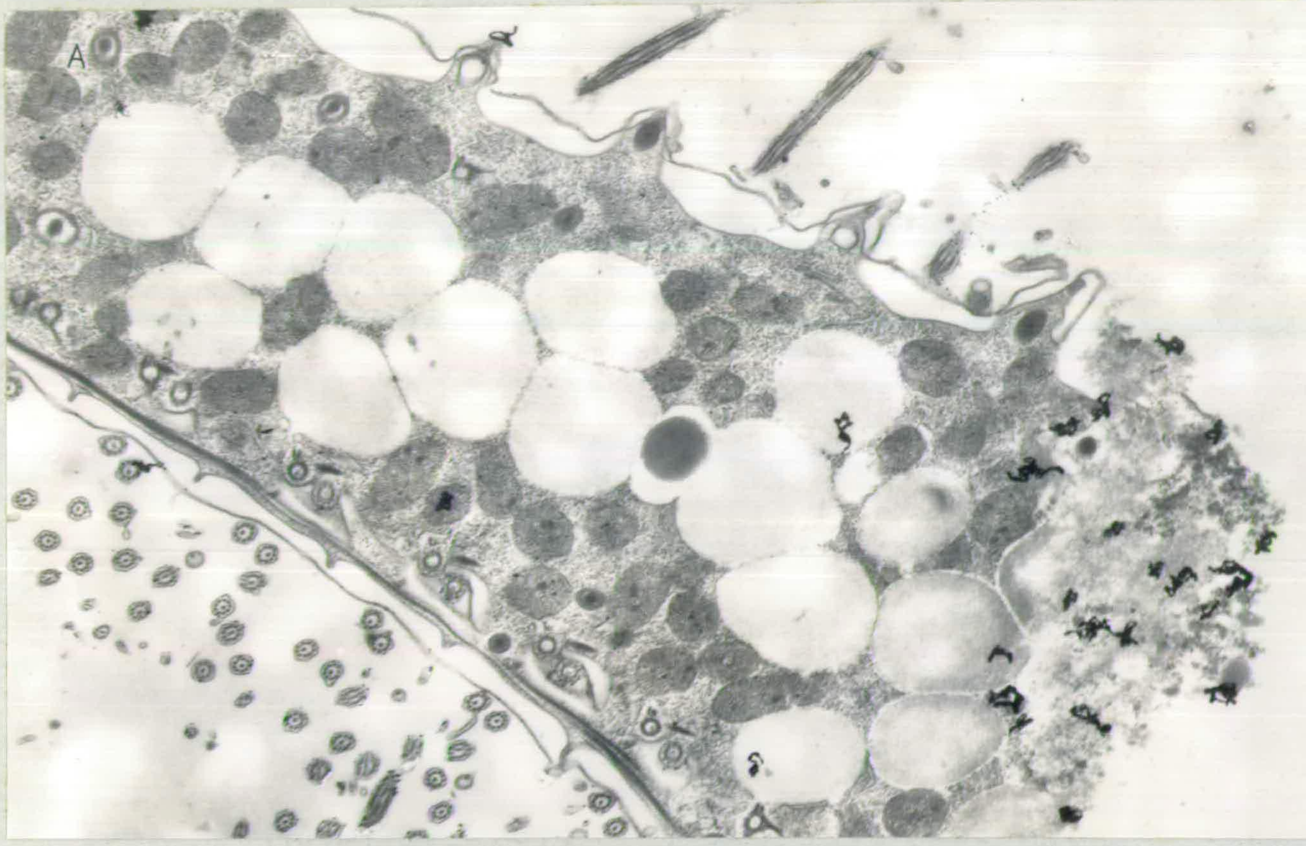
Finally comparing the homologous and heterologous reactions (Plate 7 and 8a respectively), only one statistical difference is found in the relative distribution of the iodine^{125}-conjugated antibody. This, the most significant of all the differences found in the distribution of the antibody was shown by the pellicle and cilia.

The overall reaction of the cytoplasm of frozen sections of serotypes 168G and 168D with iodine-labelled globulin from anti-168G serum, were compared in the following way. A given mass of whole cells, and a similar mass of frozen sections of each serotype were incubated with equal volumes of the same antibody preparation. After incubation and washing, samples were taken of each preparation and the radioactivity measured by scintillation counting. Other samples were taken and weighed and from these results the retention of radioactivity per unit dry weight was calculated. In the preparation of whole cells only the reaction of the outer surface of the pellicle and cilia was measured, whereas both the surface and the cytoplasmic reactions were measured in the frozen sections. The difference in the reactions of these groups of material indicates the total cytoplasmic reaction. The results are given in table 12, from which it can be seen that the reactions of both homologous and heterologous cytoplasms are almost identical.

It is concluded therefore that the only demonstrable immobilization antigen <u>in situ</u> is that found on the surface of the pellicle and cilia. The investigations on cell homogenates using ferritin-conjugated globulins

Serotype of tissue	Material labelled	Radioactivity retained Total counts (5 samples)	Counts per mgm dry weight	Cytoplasmic counts counts/mgm dry wt.
168G (Homologous)	Whole cells	440.4 ± 21.0	146.8	
	Frozen sections	1683.4 ± 49.1	1045.6	898.8
168D (Heterologous)	Whole cells	351.4 ± 20.4	78.9	
	Frozen sections	2387.4 ± 80.0	994.8	915.9

TABLE 12 Retention of iodine[125] labelled anti-168G globulin by the cytoplasm of frozen sections - shown by the difference in retention of radioactivity by whole cells and frozen sections of paramecia of serotypes 168G and 168D. Equal masses of each group of material were incubated with 2.5 ml of the same preparation of labelled globulin for two hours at 18°C, washed in veronal acetate buffer and dehydrated in ascending alcohols. All measurements were made in the manner described in the materials and methods section.


A

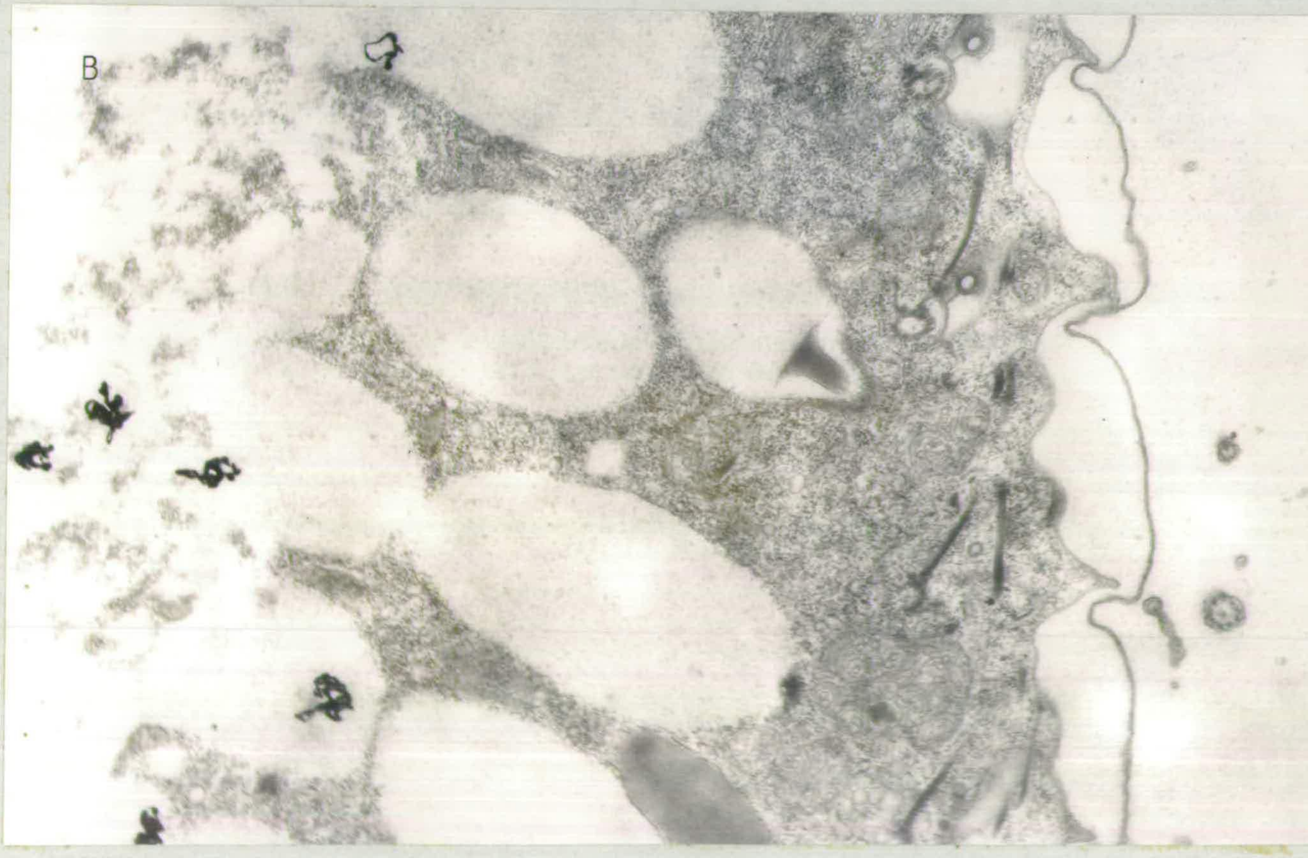

B

PLATE 8

A. E.M. autoradiograph of a frozen section of paramecium (serotype 168D) treated with $iodine^{125}$-conjugated anti-168G globulin. Trace labelling of the pellicle and cilia can be seen. As for plate 7 there is intense cytoplasmic labelling. The cilia at the bottom of the picture are in the gullet. Mag. x 10,000.

B. Frozen section of paramecium (serotype 168G) treated with $iodine^{125}$-conjugated normal rabbit globulin, showing the non-specific reaction of the globulin with the cytoplasmic organelles. No radioactivity or globulin 'fuzz' is detected on the outer surface of the pellicle. Mag. x 20,000.

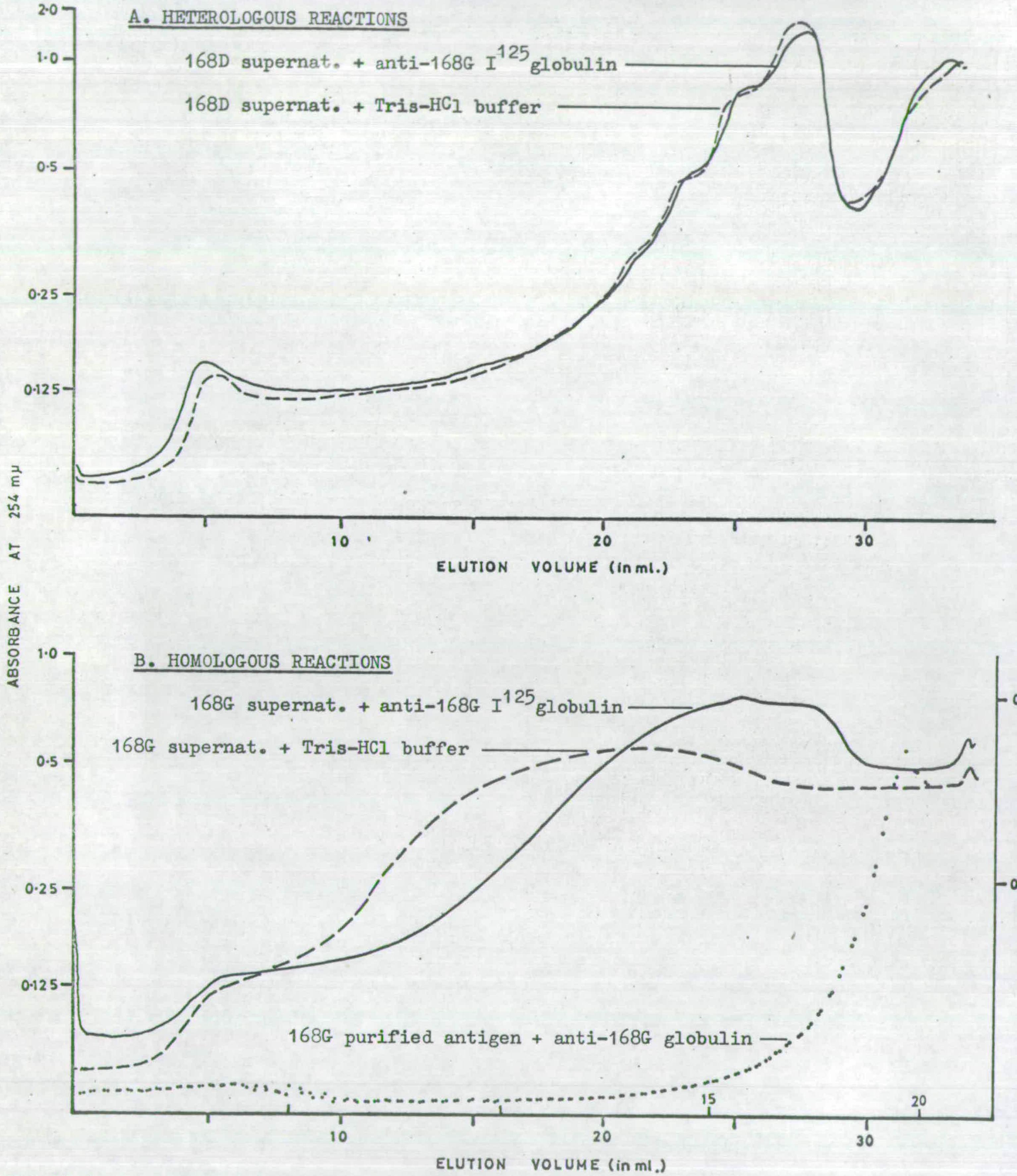


FIG.11 Sucrose gradient analysis of 25,000g supernatant of cell homogenates after incubation in the presence and absence of iodine[125] conjugated globulin. Gradient :- 60% sucrose in Tris-HCl 0.1M , pH 7.6 ,(6ml. cushion); 12.5 -25% sucrose gradient,(25ml.). Sucrose gradient analysis of purified antigen similarly incubated with unconjugated globulin.Gradient:- 4ml. cushion + 14ml. sucrose gradient . Techniques as described on page 48.

have shown that the inner surface of the pellicle, in contrast to the outer surface, reacts very non-specifically, indicate that the immobilization is probably confined to the outer surface of the pellicle. No immobilization antigen is detectable in the cytoplasm as a whole, or in any of the organelles studied here.

b) Selective Precipitation of Ribosomal material with iodine^{125} conjugated antibody

The results of in vivo and in vitro studies on the synthesis of immobilization antigen (Sommerville 1967a 1968), implicate polysomes as the site of synthesis of immunologically recognisable immobilization antigen. The in vivo incorporation experiments reported here also indicate that the synthetic pathway of immobilization antigen involves free or membrane associated ribosomes. These results therefore appear to contradict the findings described in the previous section, namely that there is no detectable antigen in situ in the cytoplasm. An attempt was made therefore to reconcile these apparently conflicting observations. This was achieved by using a modification of the selective polysome precipitation method of Allen and Terence (1968).

Paramecia of serotype 168G (homologous) and 168D (heterologous) were homogenised in Tris-HCl buffer 0.1M pH 7.6, and the homogenate was centrifuged at 25,000 for 3 minutes. On examination in the electron microscope, of material prepared in a similar manner, it was seen that the supernatant obtained after centrifugation contains only ribosomes and soluble material (Sommerville and Sinden 1968). All the large organelles and the majority of the material showing a non-specific reaction with the globulin had been

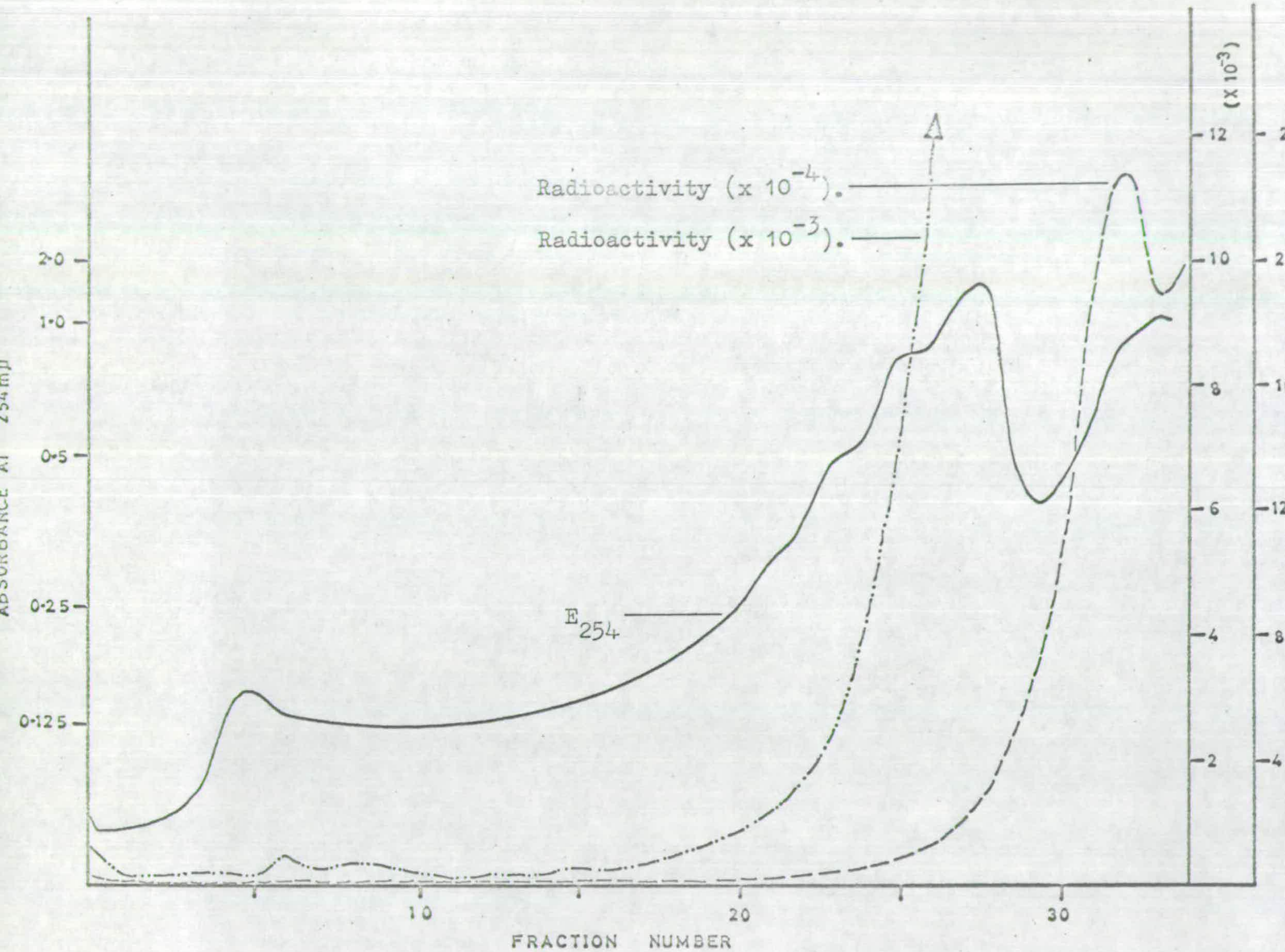


FIG.12 Distribution of radioactive iodine ^{125} in fractions taken after the sucrose gradient analysis of a 25,000g supernatant of paramecia serotype 168D - previously incubated with anti-168 G iodine^{125} conjugated globulin . Conditions of gradient as for fig.11. 0.1ml. of each fraction was added to 10ml. Brays solution and counted by liquid scintillation.

removed by the centrifugation. The decanted supernatant was divided into 1.0 ml aliquots, and each was incubated at 0°C for thirty minutes with 1.0 ml of iodine^{125} conjugated anti 168G globulin, or 1.0 ml of Tris-HCl buffer 0.1M pH 7.6. These mixtures were then placed on the top of a continuous gradient of 10-25% sucrose with a 60% sucrose cushion, and centrifuged at 45,000g for two and a half hours. The gradient was taken off and the E_{254} monitored continuously after which 1.0 ml fractions were collected. Samples of each fraction were monitored for radioactivity by liquid scintillation counting.

From a comparison of the E_{254} profiles of heterologous preparations incubated with buffer to those incubated with iodine conjugated globulin (Fig. 11a), it is readily apparent that no precipitation of ribosomes has occurred. However a similar comparison between homologous preparations incubated with buffer and those incubated with iodine^{125} conjugated globulin, shows in the latter preparation a very marked peak of nucleic acid-rich material pelleted to the bottom of the tube.

The iodine-conjugated globulin has therefore selectively agglutinated a small fraction of the homologous ribosomal material, and this material subsequently sedimented through the gradient and the cushion when the preparation was centrifuged. Confirmation that this agglutinated material had bound antibody was provided by measuring the radioactivity present in different fractions. Figure 12 shows the distribution of iodine^{125} conjugated antibody throughout the density gradient of a heterologous preparation. The radioactive globulin is predominantly found at the top of the gradient, although there may be a low non-specific binding of globulin to the

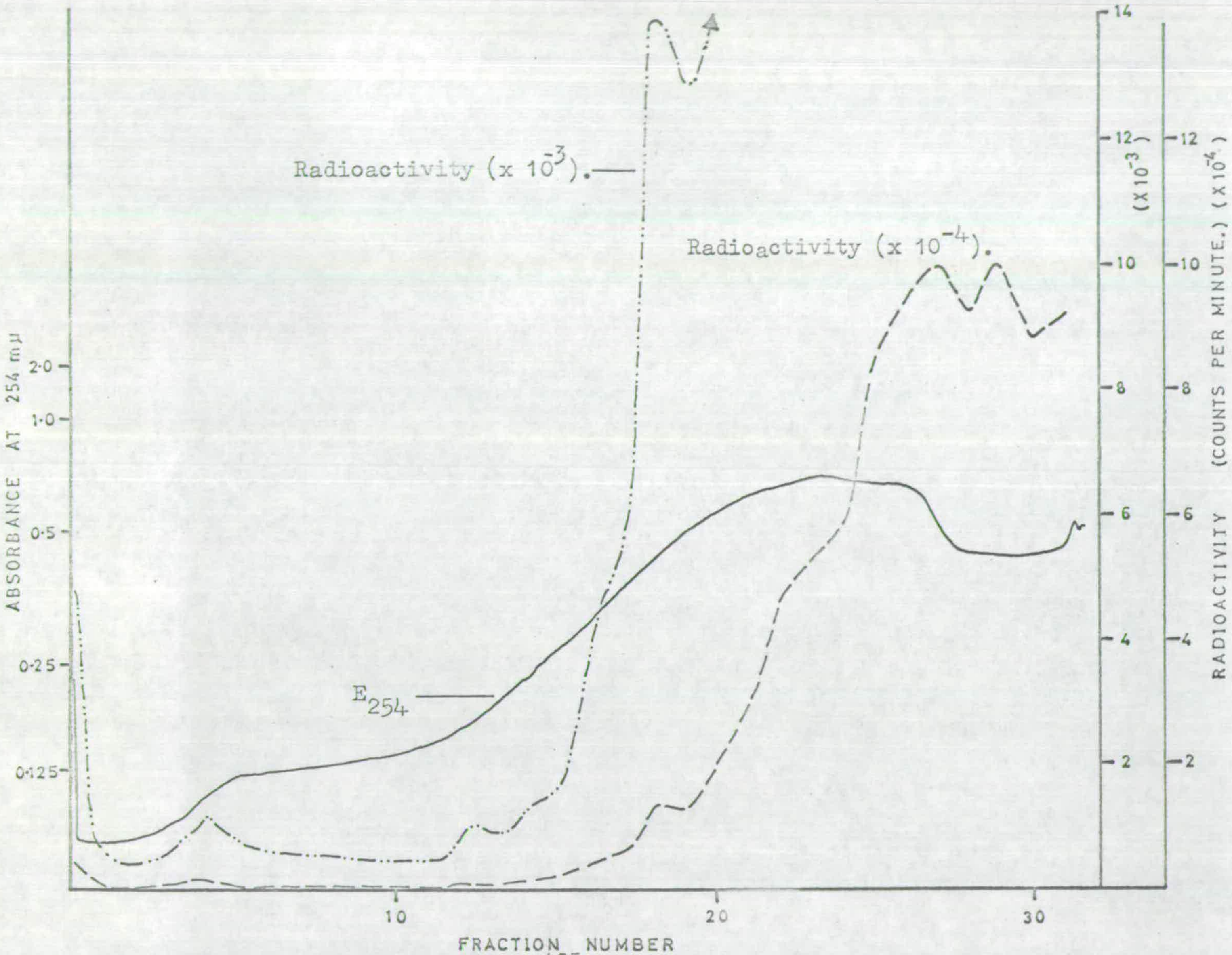


FIG.13 Distribution of radioactive iodine^{125} in fractions taken after the sucrose gradient analysis of a 25,000g supernatant of paramecia serotype 168G - previously incubated with anti-168G iodine^{125} conjugated globulin. Conditions of gradient and analysis as for fig.12.

sedimenting ribosomes. Only very small quantities of isotope are found pelleted to the cushion interface or the bottom of the tube. The profile of radioactivity shown by a homologous preparation similarly incubated with iodine-conjugated antibody is given in figure 13. Again most of the radioactivity is found at the top of the gradient, and globulin may be bound non-specifically to the ribosomal material. But in contrast to the results obtained from the heterologous preparation, in the homologous preparation there were significant quantities of radioactivity at the cushion interface and at the bottom of the tube. Since it was shown in control experiments that there was no precipitation of soluble antigen under similar conditions (Fig 11), the difference between the reactions of the homologous and heterologous ribosomal preparations is interpreted to show that a specific antibody-antigen reaction has occurred.

Non-specific adsorption of soluble protein onto the fractionated ribosomes (Petermann 1964) - between the immobilization antigens and paramecium ribosomes - has not been detected (MacIndoe and Reisner 1967, Reisner, Rose and MacIndoe 1968, Sommerville 1967a). It is therefore concluded that the ribosomal material precipitated by the iodine^{125}-conjugated globulin, was associated with the immobilization antigen in the living cell.

The in vivo incorporation of isotopes from labelled bacteria, into paramecia

A. The Incorporation of sulphur^{35} into Paramecia of stable serotype

The studies, described in the preceeding section, on the structural localization of immobilization antigen using conjugated antibody techniques, although informative, do not provide any information about the rate of synthesis of the antigens. Nor do they tell us anything about the metabolism of the antigens in relation to the general metabolism of the cell, or more particularly, the metabolism of the pellicle. To investigate these problems the incorporation of radioisotopes into the organelles and into the immobilization antigens of paramecium, from labelled bacteria, was followed. Sulphur^{35} was thought to be the most suitable radioactive tracer because when incorporated into bacteria approximately 95% of the isotope is incorporated into methionine, cysteine and cystine (Roberts et al. 1955). As it has been shown that the immobilization antigen has a very high cysteine content c.a. 10% of the amino acid content of the antigen (Steers 1965, Jones 1965a), the incorporation of sulphur^{35} into the immobilisation antigen is expected to be higher than into most other proteins. The incorporation of this isotope into the pellicle may therefore be expected to reflect the pathway of immobilization antigen synthesis.

The synthesis of the immobilization antigens was followed in cultures of serotype 168G at 18°C, and serotype 168D at 35°C. Both types of culture showed similar patterns of incorporation of isotope into all organelles, although the total ingestion of bacteria and incorporation of isotope was greater in paramecia of serotype 168D than 168G. This was due presumably. to the higher temperature at which the 168D serotype cells were grown.

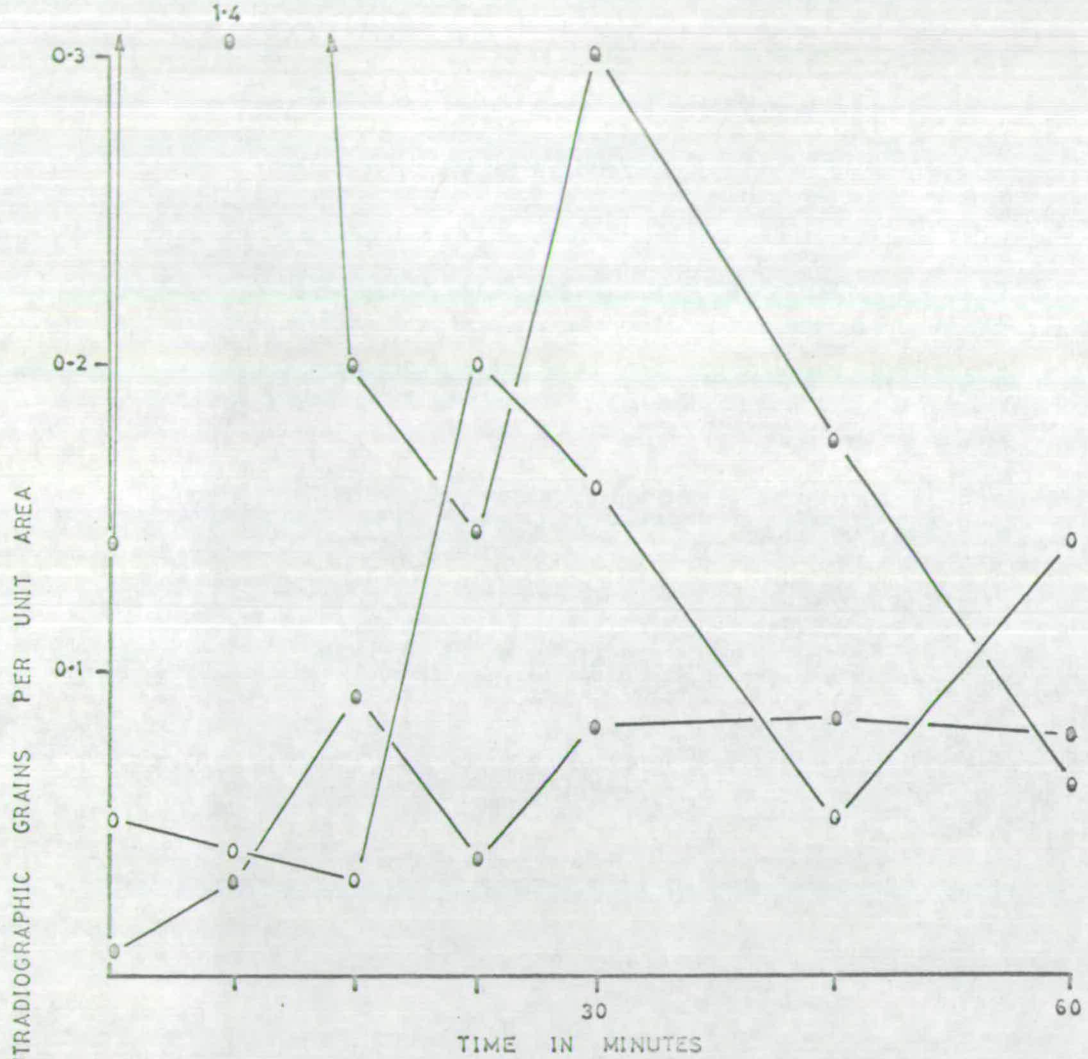


FIG.14 The concentration of radioactivity in the food-vacuoles, ●—●; cytoplasm, ●—●; and pellicle, O—O; after feeding paramecia on sulphur ^{35} labelled Aerobacter aerogenes. Conditions of culture as for fig.15.

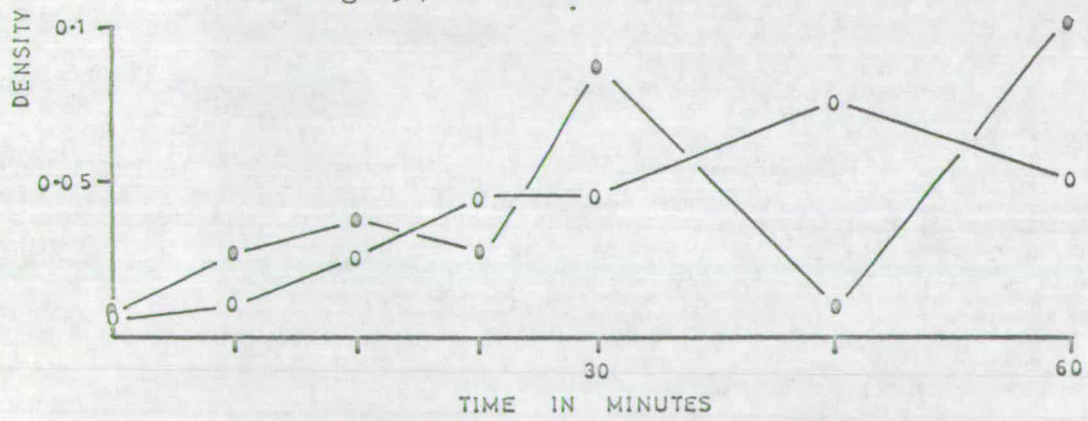


FIG.15 The concentration of radioactivity in the mitochondria, ●—●; and trichocysts, O—O, after feeding paramecia of stable serotype (168G) with sulphur labelled Aerobacter aerogenes between 0 and 15 minutes. Techniques described in the materials and methods , pages 23 and 27.

Both types of culture will therefore be regarded as similar in the following section.

For these experiments the synthesis of radioactive antigen was followed both by autoradiography (van Furth 1967, Sommerville 1967a) and scintillation counting of specific antigen-antibody precipitin bands produced on immunophoresis of whole cell homogenates. The ultrastructural localization of the sulphur^{35} was followed, in aliquots of the same samples, by electron microscope autoradiography.

1) The <u>in vivo</u> incorporation of sulphur^{35}

The density of isotope in the different organelles of paramecium at various times after the pulse labelling began, calculated as described in the materials and methods section, is shown in figures 14 and 15. There was a very rapid ingestion of bacteria into the food vacuoles (Fig. 14, Plate 9a,b). The release of isotope from the food vacuole appears to occur in two phases: an initial very rapid release of isotope for the first fifteen minutes of the experiment, followed by a relatively slow and very prolonged loss of isotope throughout the remainder of the experimental period. Berger & Kimball (1964) have also described an extended digestion period (2.5 hours) for bacterial precursors. It will be shown later in this section, that the incorporation of isotope into most organelles and into one protein in particular may also occur in two phases. It is believed therefore that the bacteria used in these experiments have provided two different sources of isotope one of which was rapidly assimilated, the other was incorporated over a longer period. The increase in the density of isotope in the food vacuole thirty minutes after the beginning of the experiment was repeatedly observed, and was assumed to be due

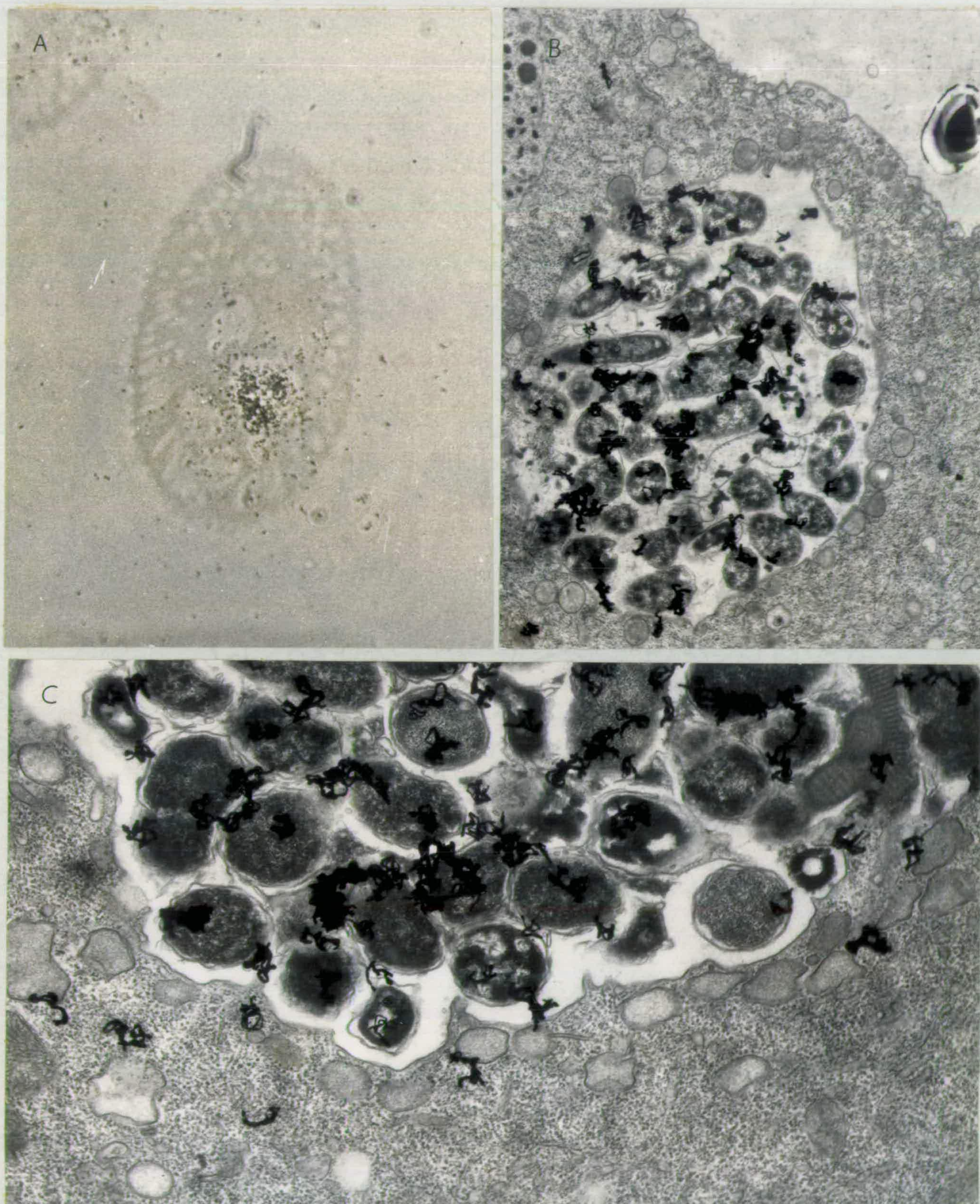

A
B
C

PLATE 9

A. Light microscope autoradiograph of paramecium fixed immediately after feeding with sulphur^{35} labelled bacteria. Paramecia of serotype 168G were cultured at 18°C. Radioactivity is concentrated within the food vacuole. Mag. x 320.

B. E.M. autoradiograph of paramecium fed with sulphur^{35} labelled bacteria and fixed immediately. Culture serotype 168D grown at 35°C. Radioactivity is localized in the bacteria within the food vacuole, some isotope is detected in the cytoplasm. Mag. x 10,000.

C. E.M. autoradiograph of food vacuole from a cell grown for 7.5 minutes at 18°C with sulphur^{35} labelled bacteria. Isotope is seen in the cytoplasm associated with free ribosomes and small vesicles. Mag. x 20,000.

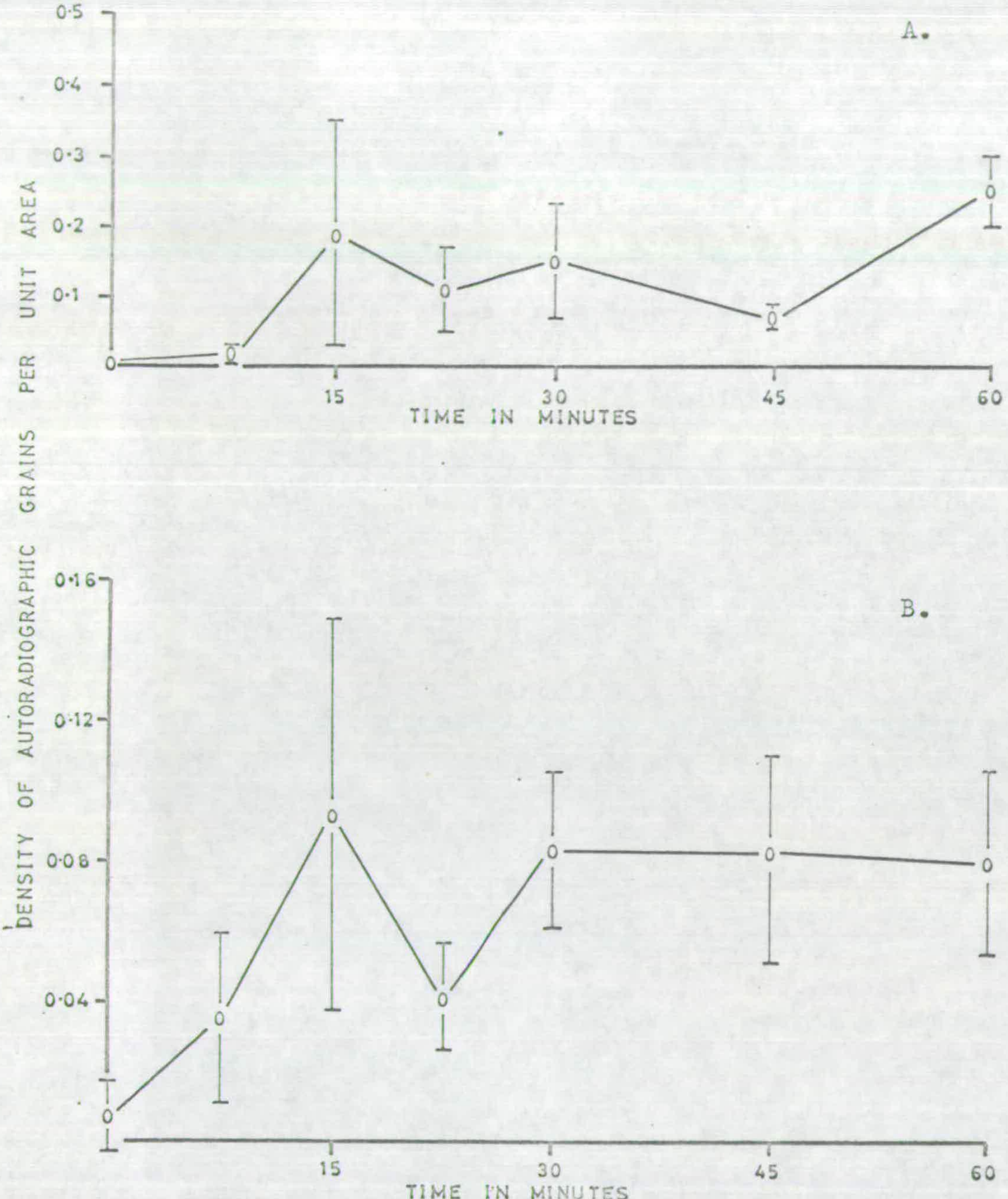


FIG.16 The concentration of radioactivity in the cytoplasm (∓ two standard errors) in preparations of paramecia of stable serotype:-

A. Serotype 168D cultured at 35°C.

B. Serotype 168G cultured at 18°C.

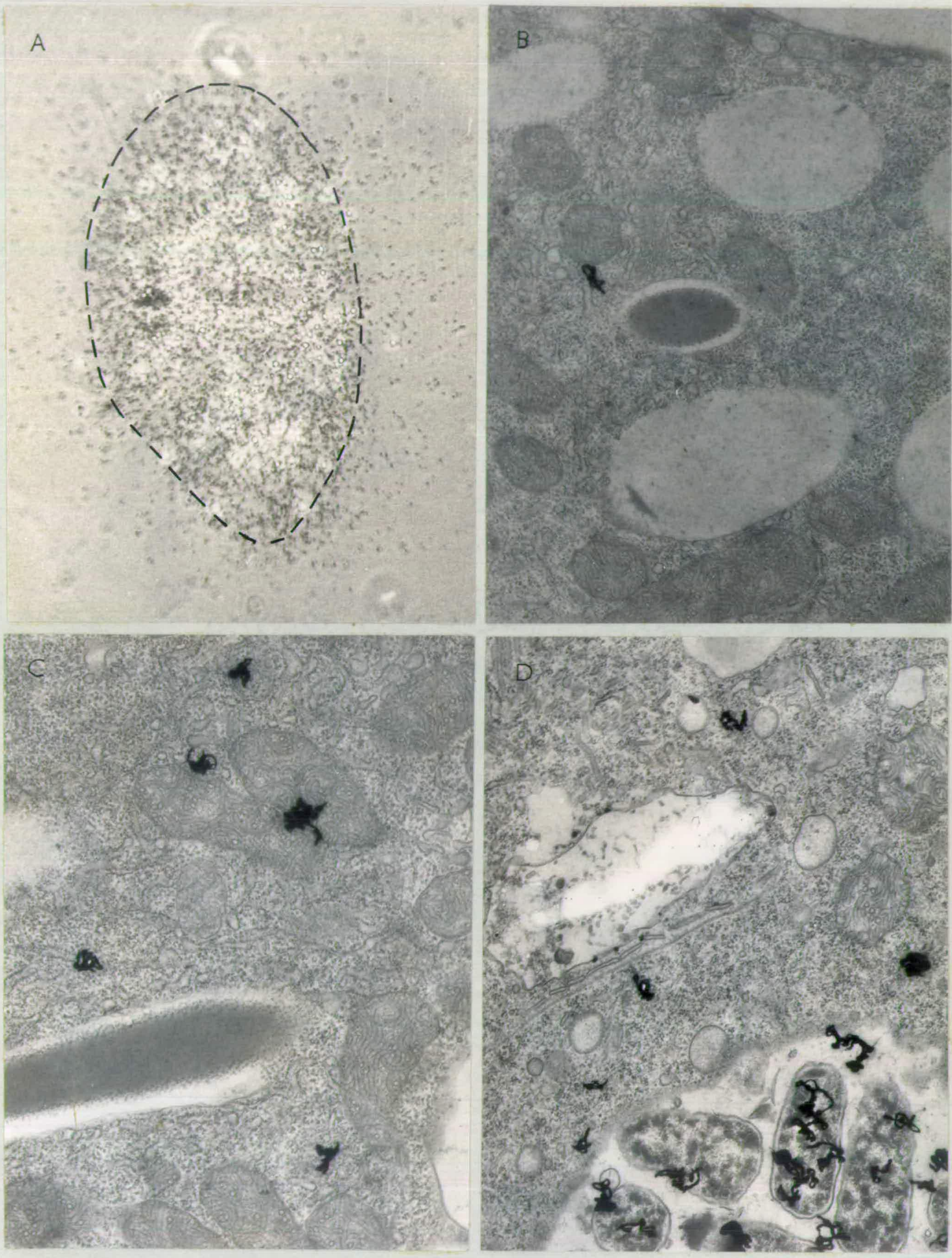
Conditions of labelling and analysis as described for fig. 15.

to a reduction in the volume of the vacuole, possibly due to the extraction of water. Changes in the volume of the food vacuole have been described previously (Jurand 1961).

Isotope detected in the cytoplasm (Fig.16) in early samples is often associated with ribosomal clusters and vesicles close to the food vacuoles. These vesicles, which are 0.2 to 0.5μ in diameter (Plate 9c), are similar in size and location, to vesicles described as secondary food vacuoles which have been formed by pinocytosis from the primary food vacuole (Jurand 1961). With increasing time the isotope became very diffuse throughout the cytoplasm (Plate 10a), and may be associated with both free and membrane bound ribosomes (Plate 10b-d). Unlike most of the vertebrate material which have been studied by similar methods (Ross and Benditt 1965, Caro & Palade 1964), here there are no large regions containing endoplasmic reticulum or free polysomes, and it has not been possible to state that the isotope is associated predominantly with either class of ribosome in any of the samples analysed.

The incorporation of isotope into the trichocysts occurred at the same rate in both developing (juvenile) and mature forms (Plate 11b,a), and increased steadily throughout the experiment (Fig. 15). There was however, a very marked incorporation into pretrichocysts (trichocysts at the earliest recognisable stage of development), (Plates 11c,d).

Macronuclear incorporation of $sulphur^{35}$ in early samples was almost exclusively into large bodies (irregular structures of electron dense material approximately 0.5μ in diameter which are believed to be nucleoli (Jurand and Jacob 1969) see plate 12) and in later samples the isotope


A
B
C
D

<u>PLATE 10</u>

A. Light microscope autoradiograph of a cell cultured at 18°C for two hours after the beginning of pulse feeding with $sulphur^{35}$ labelled bacteria. Isotope is uniformly distributed throughout the paramecium (outlined). Mag. x 320.

B. E.M. autoradiograph of a paramecium cultured for 15 minutes at 18°C with labelled bacteria. Isotope is associated with endoplasmic reticulum. Mag. x 20,000.

C. As for plate 10B. Paramecia cultured for 22.5 minutes at 18°C after the beginning of pulse feeding with labelled bacteria. Isotope is found in the mitochondria, endoplasmic reticulum and free ribosomes. Mag. x 20,000.

D. As for plate 10B. Paramecia cultured for 22.5 minutes at 35°C after the beginning of pulse feeding with labelled bacteria. Isotope is associated with free ribosomes. Mag. x 20,000.

became more diffuse throughout the macronucleus. Incorporation of isotope into the micronucleus (plate 12a) was confined to the matrix.

The incorporation of sulphur^{35} into the pellicle (Plate 13) occurred in two distinct phases (Fig. 18); an initial rapid increase in the density of isotope was observed between fifteen and twenty two minutes after the beginning of the experiment, and a second slower incorporation of isotope began after forty five minutes.

The success of electron microscope autoradiographic techniques in detecting a flow of isotope from one structure to the next, has been shown to be dependent on the time over which the isotope is available for assimilation (Ross and Benditt 1965). Obviously using radioactively labelled bacteria as a source of isotope, it was not possible to supply a discrete pulse of sulphur^{35} to the paramecia. However if the first phase of isotope incorporation from the food vacuoles is considered separately, a movement of isotope from one structure to the next can be resolved (Fig. 14.) It can be seen that the peak density of labelling in the food vacuoles occurred seven minutes after the beginning of the labelling. The cytoplasm showed a peak density of isotope after fifteen minutes, and the pellicle after twenty two minutes. This pattern of incorporation of the isotope is compatible with a precursor-product relationship between the food vacuole and the cytoplasm, also between the cytoplasm and the pellicle (Nadler 1963, Warshawsky et al. 1963, Zilversmidt et al. 1943). The pathway of incorporation of sulphur^{35} between the bacteria and the pellicle is therefore believed to be as follows:-

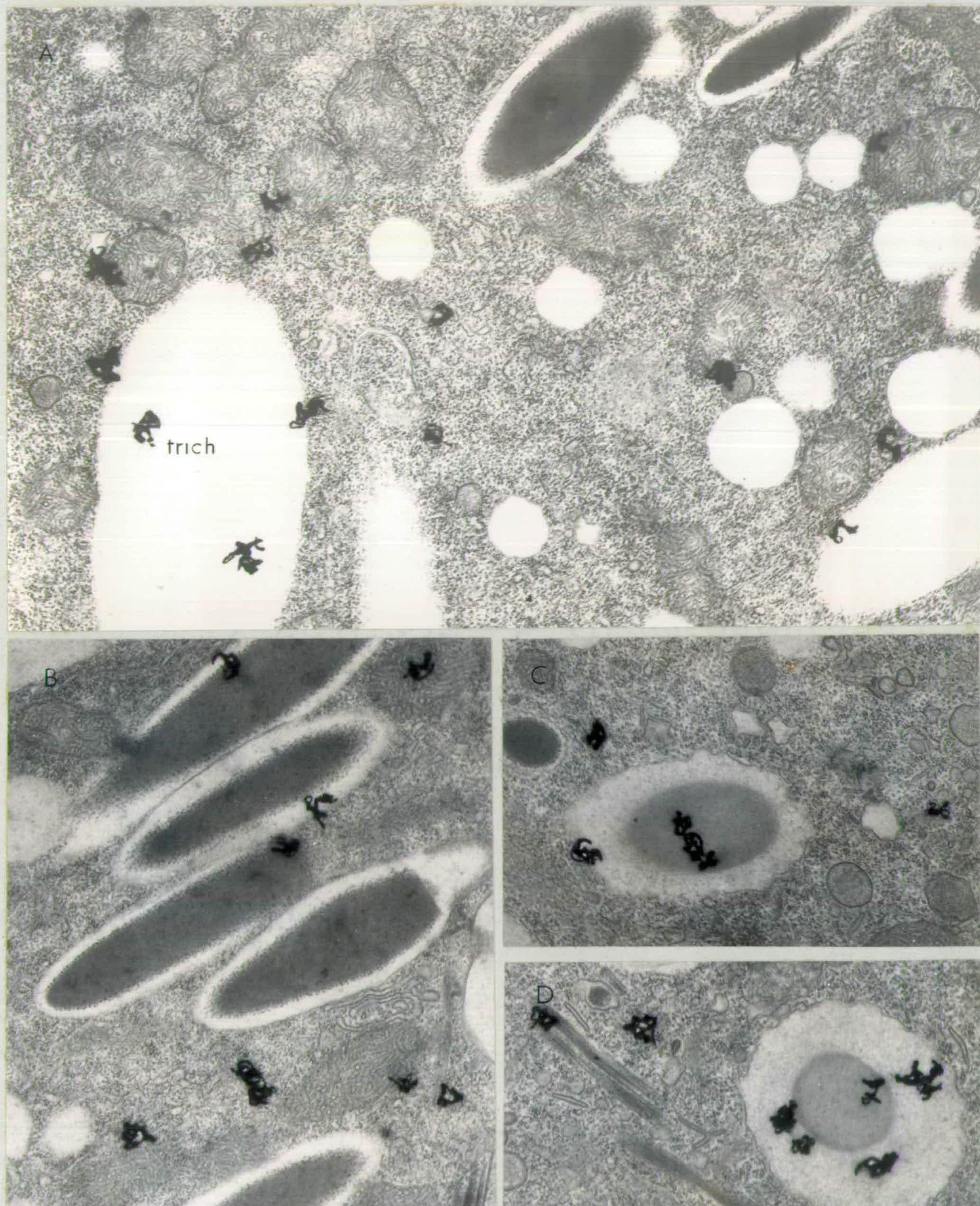

A
trich
B
C
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PLATE 11

A. E.M. autoradiograph of the cytoplasm of a cell cultured for 60 minutes at 18°C after the beginning of pulse feeding with $sulphur^{35}$ labelled bacteria, showing the incorporation of isotope into mature trichocysts (trich). Mag. x 20,000.

B. As for plate 11A, showing the incorporation of isotope into the bodies of juvenile trichocysts. Paramecia cultured for 30 minutes at 18°C after the beginning of pulse feeding. Mag. x 20,000.

C.-D. As for plate 11A. Paramecia cultured for 45 minutes at 18°C after the beginning of pulse feeding, demonstrating the very marked incorporation of isotope into the pretrichocysts. Mag. x 20,000.

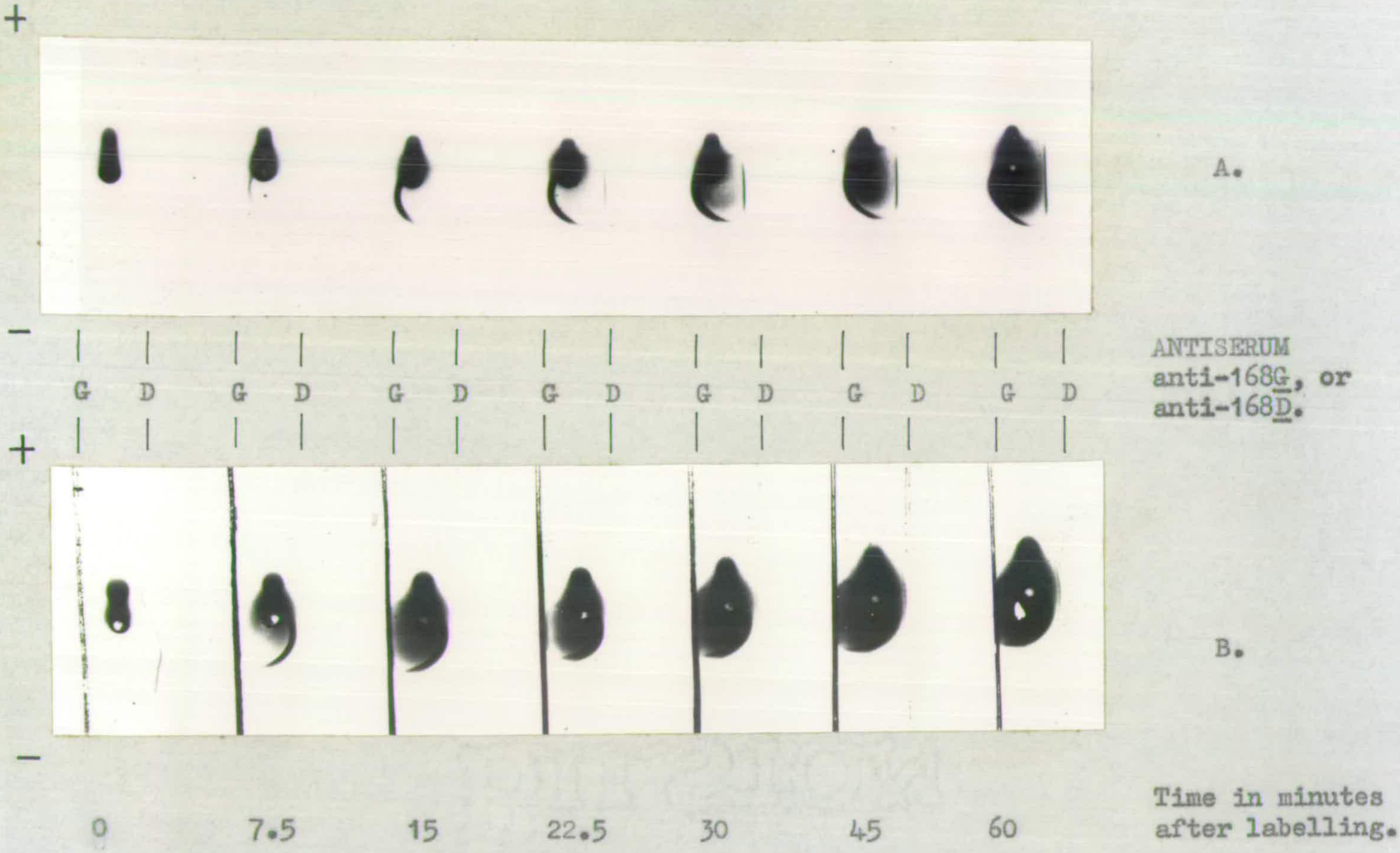
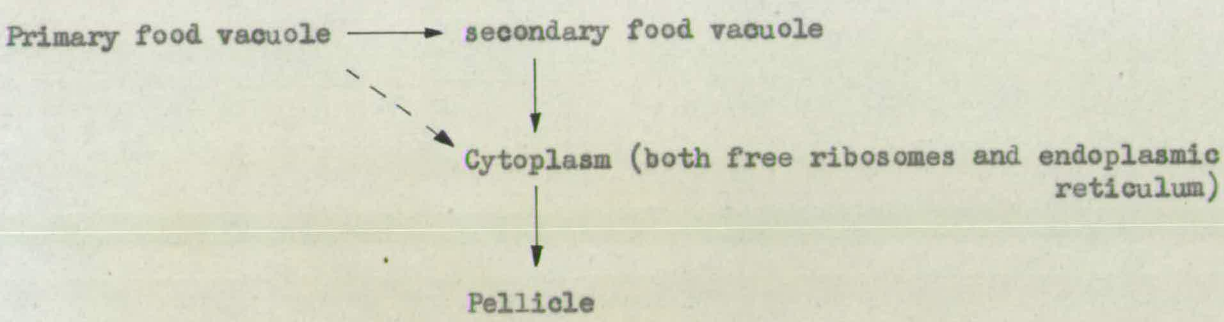


FIG.17 Autoradiographs showing the incorporation of sulphur^{35} into precipitated immobilization antigens - as revealed by immunophoretic analysis of 10µl. aliquots of packed cells , prepared from paramecia fed on radioactively labelled Aerobacter aerogenes . Conditions of labelling as for fig.15. Techniques as described in the materials and methods pages 12 and 24.

A. Paramecia of serotype 168G cultured at 18°C.
B. Paramecia of serotype 168D cultured at 35°C.



2) The incorporation of sulphur^{35} into the immobilization antigen

From the autoradiographs of the specific immobilization antigen-antibody precipitin arcs (Figs 17 a,b), it is evident that immobilization antigen was synthesised more rapidly in cells of serotype 168D at 35°C, than 168G cells at 18°C. Liquid scintillation counting of the specific precipitin arcs (Fig. 18), revealed that small amounts of radioactive antigen were synthesised by both cultures in samples taken immediately after the labelling period began. (It should be noted however that there was a delay of approximately four minutes between the time at which the sample was taken and the time of freezing (or fixation) of the pelleted paramecia). The incorporation of sulphur^{35} into both 168D and 168G antigens was approximately exponential over the period of the experiment, as would be expected from a growing culture of paramecium (Kimball et al. 1959). However, of particular interest in this investigation, was the slightly increased rate of incorporation of isotope into the immobilization antigens, shown by both cultures, at approximately the same time as the initial increase in density of isotope in the pellicle. (fig. 18). It has already been shown that the majority of the immobilization antigen is located on the pellicle and cilia where it is believed to represent a substantial fraction of the total protein. Also, the choice of sulphur^{35} as the isotope for incorporation

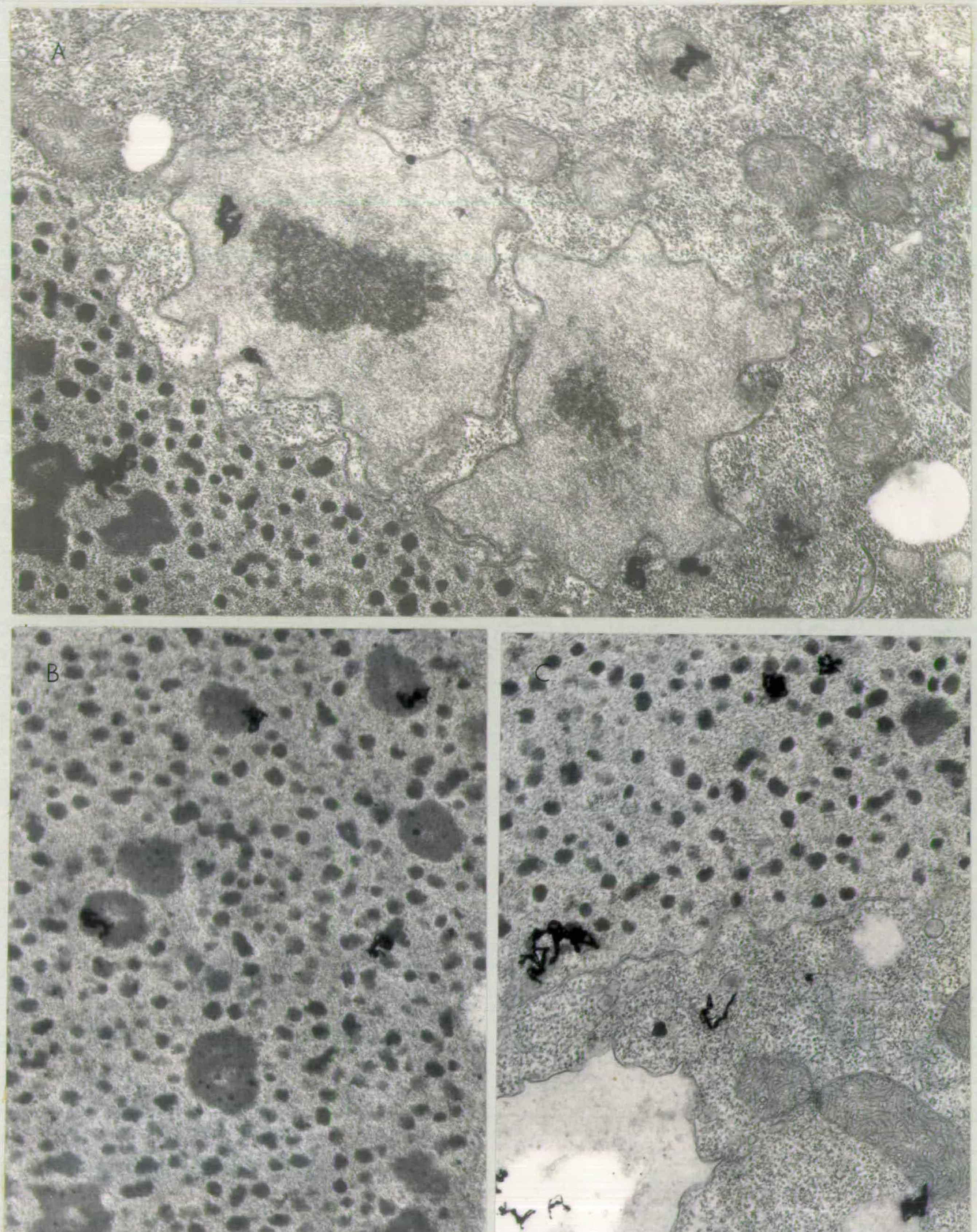

A
B
C

PLATE 12

A. E.M. autoradiograph of the paired micronuclei and macronucleus of a cell cultured at 18°C for 30 minutes after the beginning of pulse feeding with sulphur^{35} labelled bacteria. Isotope is associated with the large bodies of the macronucleus and the matrix of the micronucleus. Mag. x 20,000.

B. The macronucleus of a cell cultured at 18°C for 15 minutes after the beginning of pulse feeding with radioactive bacteria. Isotope is localized exclusively in the large bodies. Mag. x 20,000.

C. Macronucleus of a paramecium cultured for 45 minutes at 18°C after the beginning of pulse feeding with labelled bacteria. Isotope is detected in both large and small bodies. Mag. x 20,000.

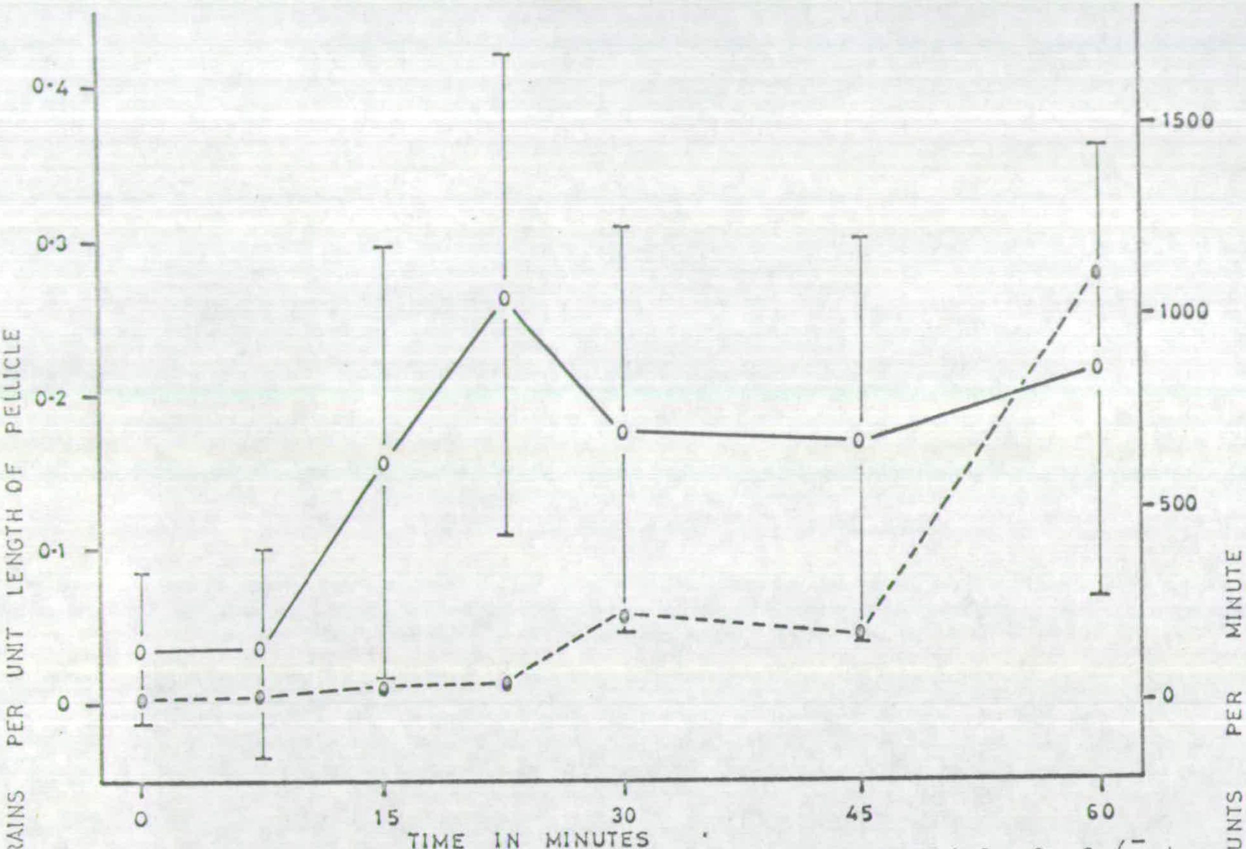


FIG.18a The concentration of radioactivity in the pellicle, 0—0 (∓ two standard errors), compared with the total incorporation of sulphur into precipitated immobilization antigen ●—●. The total incorporation of isotope was calculated by scintillation counting of specific immunophoretic precipitin arcs as described in the materials and methods pages 12 and 25. Conditions of labelling and analysis as described for fig. 15 . Paramecia serotype 168D cultured at 35°C

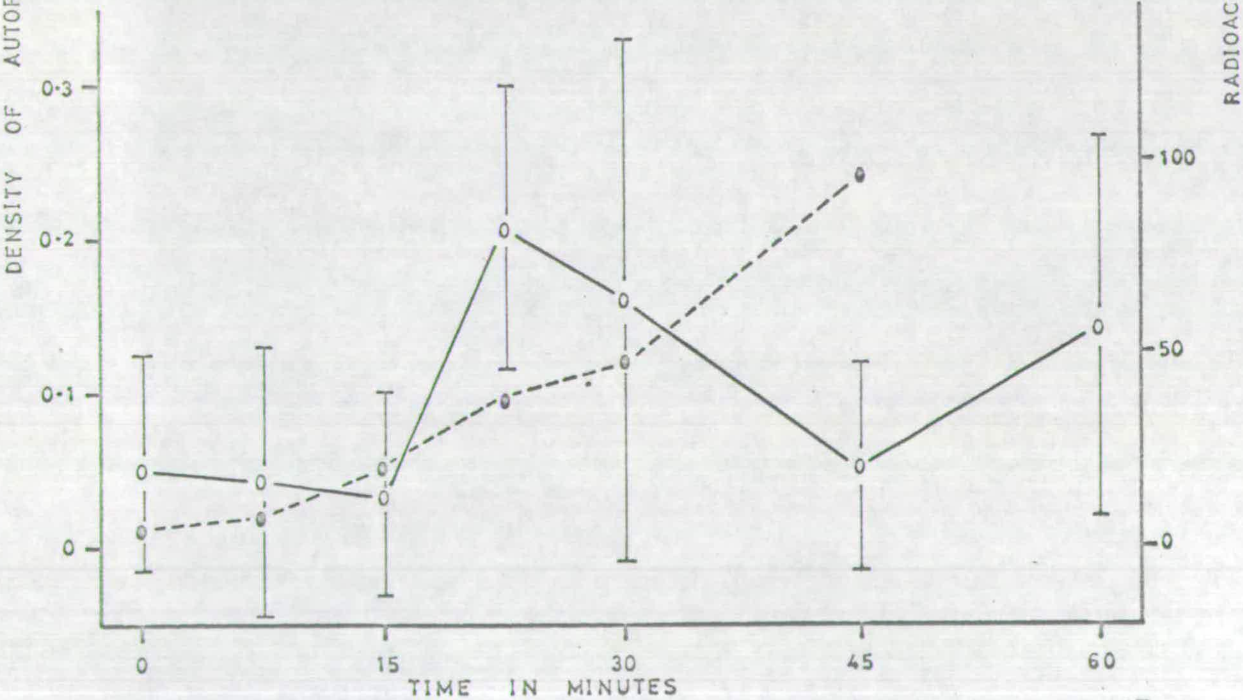


FIG.18b The concentration of radioactivity in the pellicle 0—0 (∓ two standarderrors) compared with the incorporation of sulphur^{35} into precipitated immobilization antigen ●--●. Conditions as for fig.18a. Paramecia of serotype 168G cultured at 18°C.

is believed to favour the incorporation of the radioactivity into the immobilization antigens. The probable correlation indicated between the total synthesis of radioactive antigen and the density of the $sulphur^{35}$ on the pellicle and cilia supports the conclusion that substantial quantities of the isotope incorporated onto the pellicle and cilia may also be incorporated into the immobilization antigens. Therefore it is believed that the pathway of incorporation of $sulphur^{35}$ onto the pellicle described above, also represents the pathway of synthesis of the immobilization antigen.

B <u>The in vivo Incorporation of Radioisotope into Paramecium during Serotype Transformation.</u>

Paramecia may, under stable conditions, synthesise only one immobilization antigen. Changes in the conditions of the culture, e.g. changes in the temperature or ionic strength of the medium may induce the cells to synthesise an alternative immobilization antigen. The process by which the change in the expression of the immobilization antigens is achieved is called serotype transformation.

Cultures of paramecium exclusively of serotype 168G were harvested and fed labelled bacteria in a manner similar to that used in the previous experiments. However at the same time as the bacteria - labelled with either $Mg\ S^{35}O_4$ or $C^{14}H_3COONa$ - were supplied the temperature of the culture was raised from $18^{\circ}C$ to $35^{\circ}C$. This temperature change is known to bring about a rapid transformation from serotype 168G to serotype 168D. Samples were taken from the culture at various times after the temperature increase, fixed in osmium tetroxide and then incubated with ferritin conjugated antibody

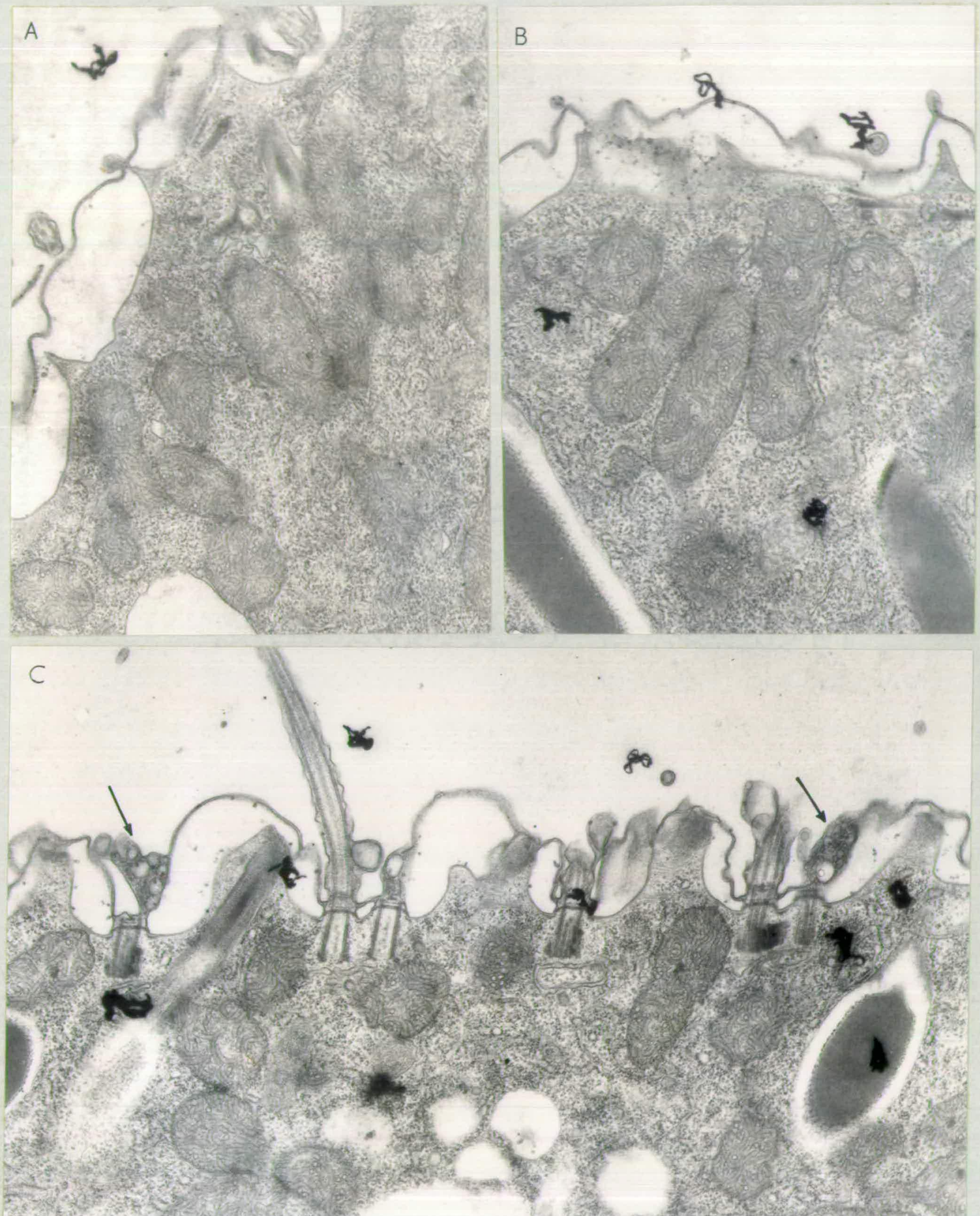

A
B
C

PLATE 13

A. E.M. autoradiograph of the pellicle and peripheral cytoplasm of a cell-cultured at 18°C, fixed immediately after feeding with sulphur^{35} labelled bacteria. Little isotope is detected in the pellicle and cilia. Mag. x 20,000.

B. As for plate 13A. Paramecia cultured at 18°C for 22.5 minutes after the beginning of pulse feeding. Isotope is associated with both the pellicle and peripheral cytoplasm. Mag. x 20,000.

C. As for plate 13A. Paramecia cultured at 18°C for 60 minutes after the beginning of pulse feeding. Arrows indicate regions of vesiculated (growing) membrane. Mag. x 20,000.

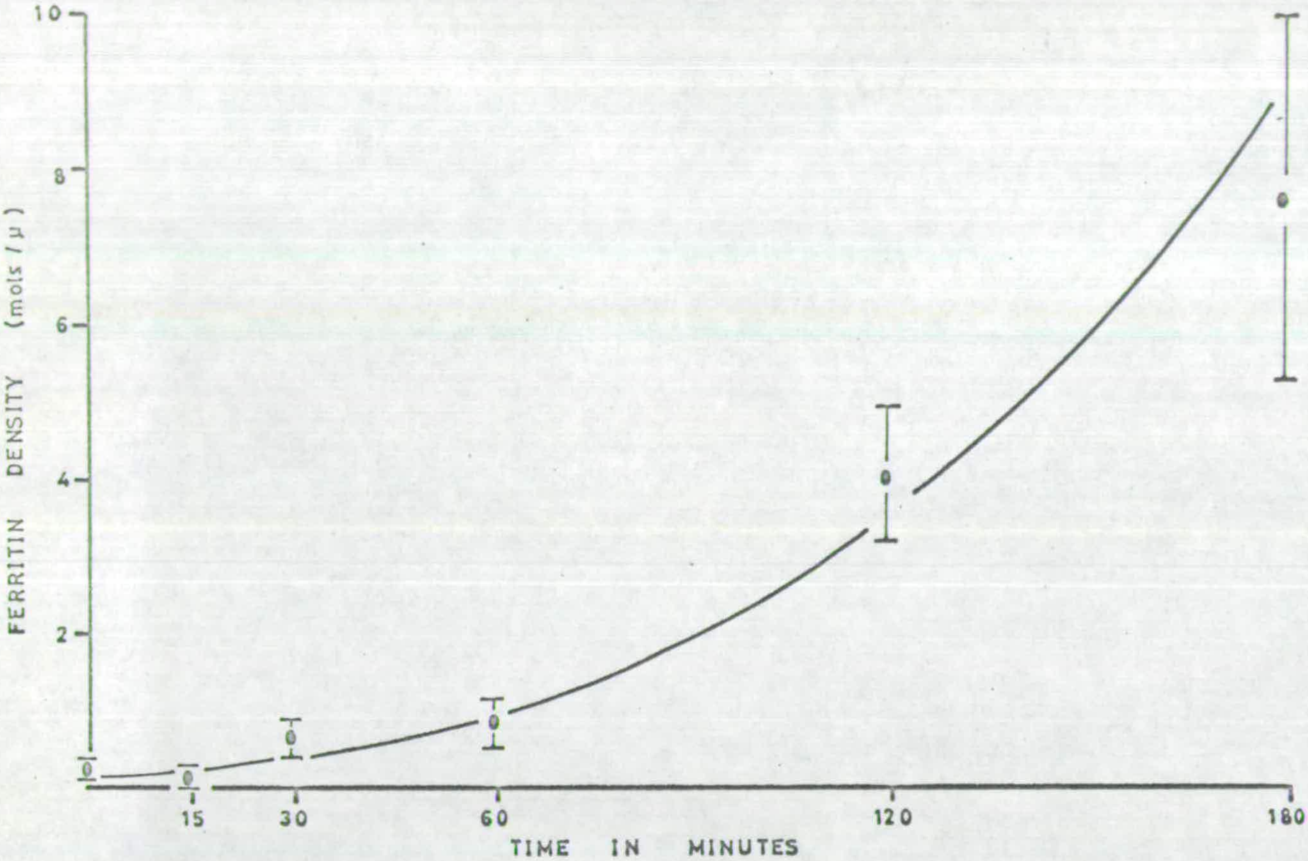


FIG.19 The density of ferritin - conjugated to anti-168D antibody - on the pellicle and cilia of cells taken from a culture of paramecia at different times during transformation from serotype 168G to 168D. The temperature of the culture was raised from 18°C to 35°C at time 0 minutes. Conditions of culture and labelling are described on page 56.

specific for 168D antigen. Electron microscope autoradiographs were prepared from each sample to localize the incorporated isotope, and each sample was examined for the presence of ferritin molecules on the pellicle and cilia. The following data were collected for each sample, from electron micrographs photographed at random as described in the methods section:-

a) The density of ferritin molecules which indicates the density of 168D antigen on the pellicle and cilia

b) The density of isotope, i.e. newly synthesised material, in the various organelles

c) The maximum isotope (autoradiographic grains) localized in each organelle, represented as a percentage of the total isotope incorporated into all organelles (A difference in this value, between S^{35} and C^{14} incorporation will indicate those structures synthesising products exclusive to the metabolic pathway of either isotope).

1) The incorporation of 168D antigen into the pellicle and cilia

The density of ferritin conjugated anti 168D antibody on the pellicle and cilia is shown in Fig. 19, the first significant increase in density was found thirty minutes after the temperature increase, before this time the ferritin density was no higher than the normal pellicle cross reaction (table 6). Between thirty minutes and three hours after the temperature increase the density of ferritin, and therefore 168D antigen, on the pellicle and cilia rose exponentially. When the rate of increase in the density of 168D antigen during each successive hour of the experiment was calculated (table 13) it was seen that after the second hour at the higher temperature that

Time in hours after temperature increase	Mean density of ferritin (molecules/μ pellicle and cilia surface)	Increase in mean density during each successive hour
0	0.208	
		0.59 (0-1)
1	0.801	
		3.19 (1-2)
2	3.994	
		3.57 (2-3)
3	7.567	

TABLE 13 Increase in mean density of ferritin, conjugated to anti-168D globulin, on the pellicle and cilia during transformation from serotype 168G to serotype 168D. Transformation was initiated by raising the temperature of the culture from 18°C to 35°C.

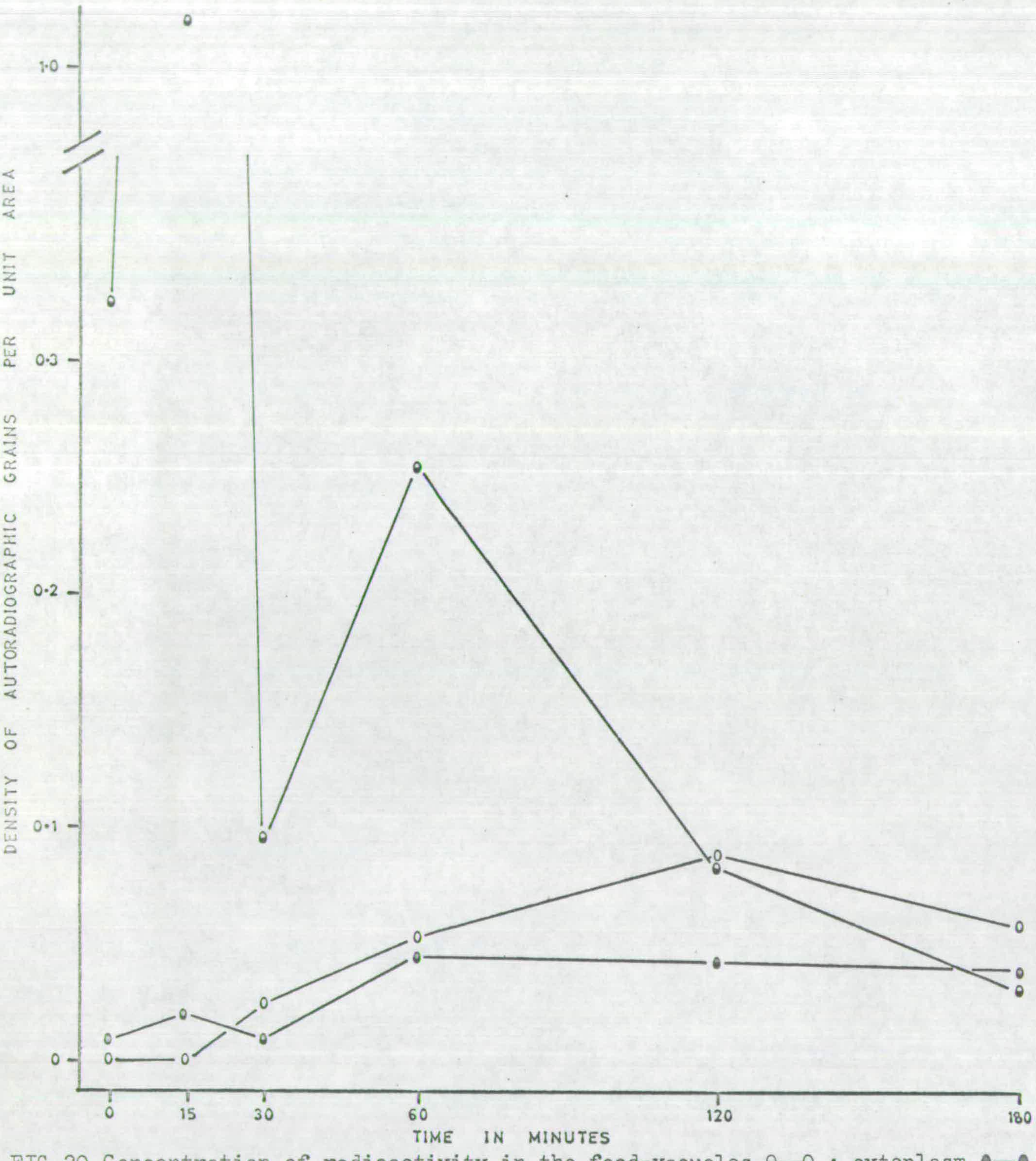


FIG.20 Concentration of radioactivity in the food-vacuoles ⊖—⊖ ; cytoplasm ●—● ; and pellicle O—O, of paramecia fed with sulphur^{35} labelled Aerobacter aerogenes . Paramecia of serotype 168G - previously cultured at 18°C - were raised from 18°C to 35°C at the same time (0 minutes), as the radioactive bacteria were supplied . Analysis of electron-microscope autoradiographs described for fig. 15.

the rate of synthesis of 168D antigen may be approaching a maximal value.

2) The incorporation of $sulphur^{35}$ into paramecium.

During serotype transformation the incorporation of $sulphur^{35}$ into paramecium was slower than into non transforming cells at either 18°C or 35°C (cf. figs., 14, 15, 16, 18 with fig. 20). The incorporation of $sulphur^{35}$ into the pellicle was first detected thirty minutes after the temperature increase (Fig. 20). This coincided with the first appearance of 168D antigen as shown by the observations with ferritin conjugated antibody (Fig. 19). The synchronous incorporation of 168D antigen and newly synthesised protein, could indicate that there was no pool of 168D antigen (as secondary antigen) within the cytoplasm of 168G cells, and that all the 168D antigen was synthesised after the temperature increase. Neither the incorporation of the $sulphur^{35}$, nor the localization of the ferritin conjugated antibody were found restricted to those regions of the pellicle containing organelles typically associated with membrane synthesis e.g. vesiculated membrane, microtubules and microvilli.

The structural association between the $sulphur^{35}$ and 168D antigen in the pellicle was investigated in the samples taken at 30 and 60 minutes after the temperature increase. The basic assumption for these experiments was that the structural coincidence of these two substances was demonstrated when the ferritin molecules and autoradiographic silver grains could not be resolved as separate: i.e. more than 0.3μ apart (Plate 16). A minimum of 100 ferritin molecules or 50 autoradiographic grains were examined for each group. The times chosen for the samples were determined by two factors. First the ferritin density is so high after two hours at the higher

Time in minutes after increase in temperature from 18°C to 35°C	Percentage of total observed autoradiographic grains associated with ferritin molecules	
30	74.42	
		Mean 71.0
60	67.75	

(A) Coincidence of ferritin conjugated anti-168D antibody with autoradiographic grains i.e. the percentage of radioactivity detected as 168D antigen.

Time in minutes after increase in temperature from 18°C to 35°C	Percentage of total observed ferritin molecules associated with autoradiographic grains	
30	7.03	
		Mean 5.8
60	4.66	

(B) Coincidence of autoradiographic grains with ferritin conjugated anti-168D antibody i.e. the percentage of 168D antigen detected as radioactive.

TABLE 14. The structural association of newly synthesised (sulphur^{35} labelled) protein and new serotype antigen (168D), on the pellicle and cilia of paramecia during transformation from serotype 168G to serotype 168D. Techniques as described on page 58.

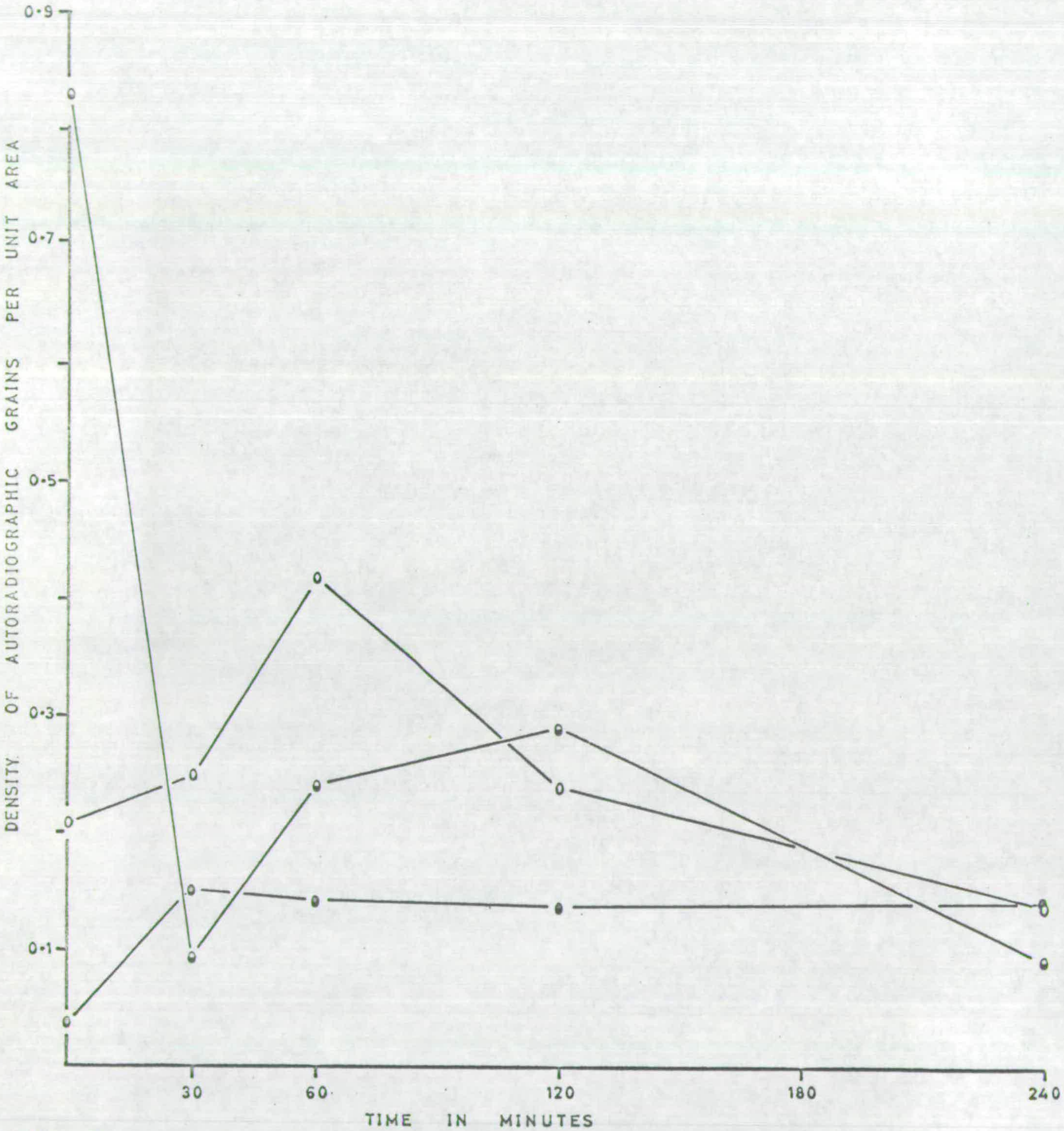


FIG.21 Concentration of radioactivity in the food-vacuoles ϴ—ϴ ; cytoplasm ●—● ; and pellicle O—O , of paramecia fed with carbon^{14}-acetate labelled Aerobacter aerogenes . Conditions of labelling and analysis as for fig 20.

temperature that the coincidence of ferritin and any autoradiographic grain on the pellicle is inevitable, therefore all the estimates of coincidence between the ferritin and autoradiographic grains must be made on samples taken before this time. Second the isotope only reaches the pellicle after 30 minutes at the higher temperature.

The results are given in table 14. The mean values indicate that while the majority (ca. 70%) of the incorporated radioactivity is associated with the incorporation of 168D antigen, only ca 6% of the antigen may itself be radioactive. The latter value is not surprising in view of the small proportion of the total available food material represented in a radioactive form. However, as it has been shown that 168D antigen represents only a very small proportion of the total antigen synthesised during the first two hours of transformation (see below, and Sommerville 1969 in Press), the high correlation between the incorporation of isotope and 168D antigen, must indicate that all the newly synthesised protein is incorporated into the same small regions i.e. smaller than can be resolved by this technique, of the pellicle and cilia.

3) A comparison of the incorporation of isotope from bacteria labelled with either $C^{14}H_3COONa$ or $MgS^{35}O_4$, into paramecium.

The pattern of incorporation of $carbon^{14}$ from the C^{14} sodium acetate labelled bacteria was followed in a culture of paramecia treated in a similar manner to that described in the previous section. The movement of isotope from the bacteria to the pellicle was similar to that of $sulphur^{35}$, and was compatible with a flow from the food vacuole to the cytoplasm (Plate 15), and from the cytoplasm to the pellicle (Fig. 21).

A comparison of the relative distribution of the isotopes throughout

	Pellicle	Tricho-cysts	Mito-chondria	Macro-nucleus	Gullet	Food Vacuole	Cyto-plasm
SULPHUR^{35}							
Percentage isotope	9.5	6.6	11.9	3.3	-	90.5	64.3
Time in minutes after adding labelled bacteria	180	30	180	30	-	0	180
CARBON^{14}							
Percentage isotope	7.0	2.7	18.7	15.1	2.9	66.6	66.8
Time in minutes after adding labelled bacteria	60	60	240	30	0	0	240

TABLE 15 The relative distribution of carbon^{14} and sulphur^{35} throughout the various organelles of paramecium. (Measured as the maximum percentage of the total isotope incorporated, located within a given organelle.) The isotopes were provided either as $MgS^{35}O_4$ or $C^{14}H_3COON_a$ labelled Aerobacter aerogenes. The temperature of the culture was raised from 18°C to 35°C at the same time as the labelled bacteria were supplied (0 minutes).

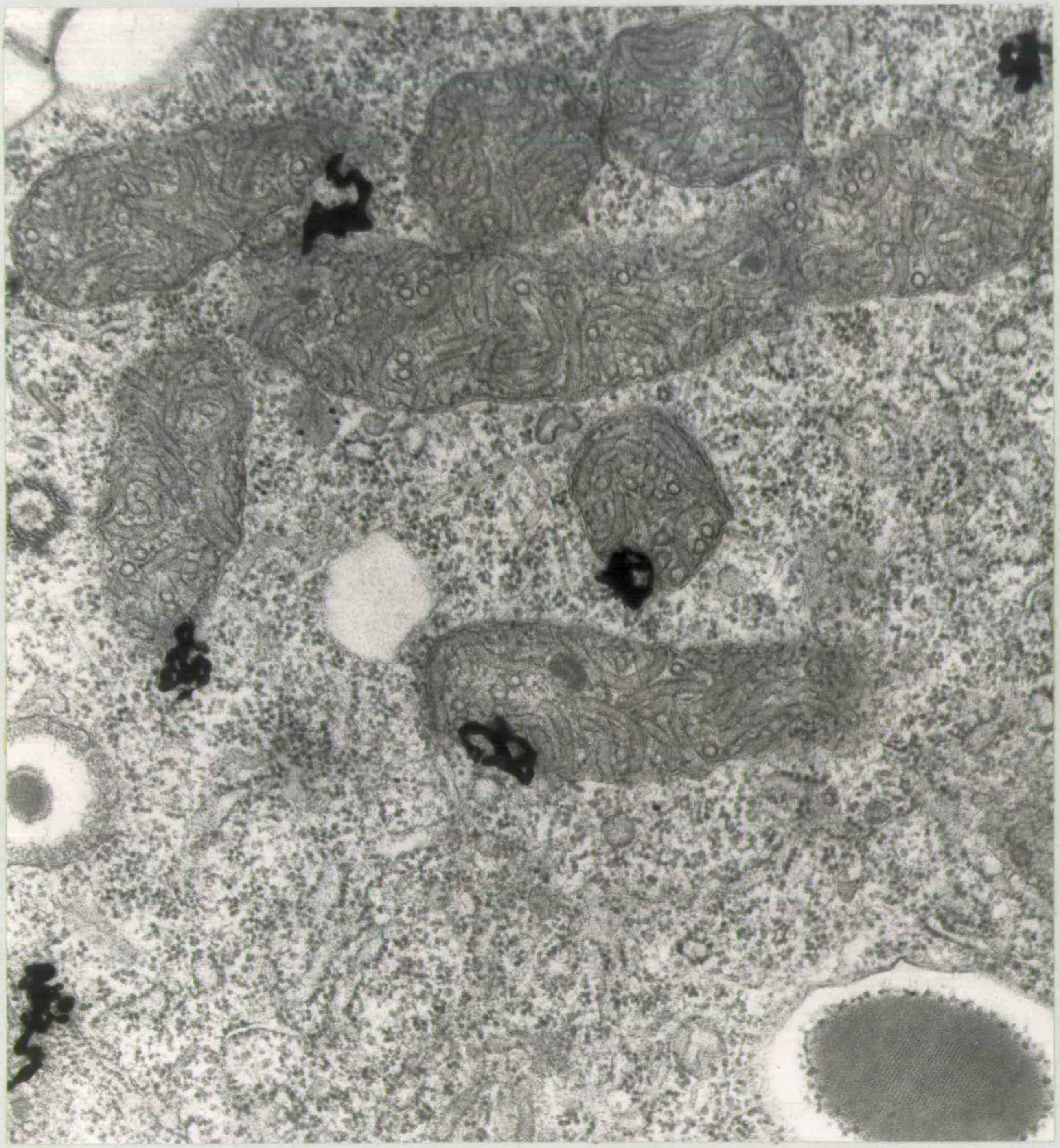


PLATE 14

Electron-microscope autoradiograph of the cytoplasm of *Paramecium* cultured for 60 minutes after the beginning of pulse feeding with $C^{14}H_3COON_a$ labelled *Aerobacter aerogenes*. Isotope is seen predominantly associated with the mitochondria, and also ribosomal material. Mag. x 50,000.

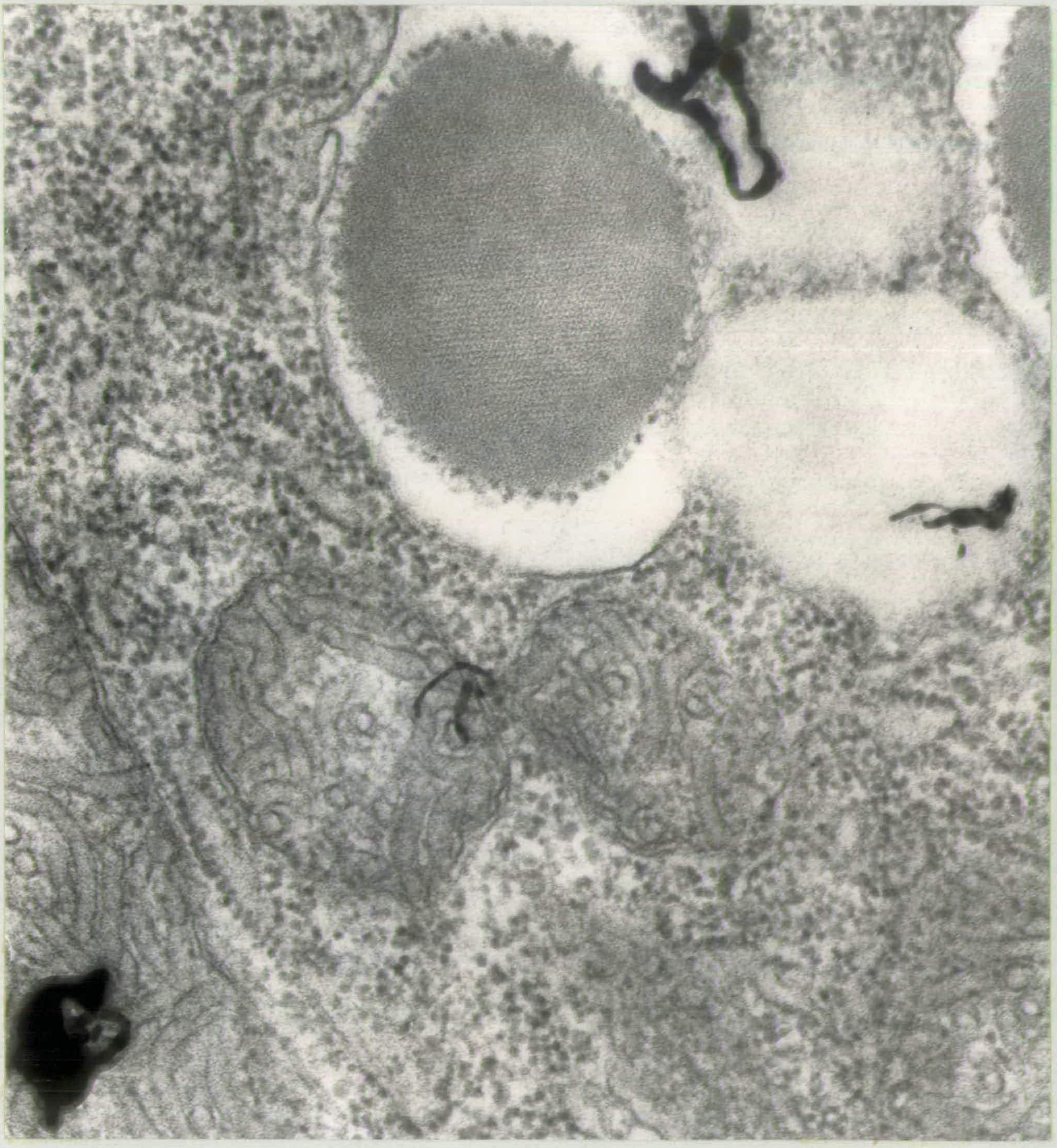


PLATE 15

Electron microscope autoradiograph of the cytoplasm of *Paramecium* cultured for 60 minutes after the beginning of pulse feeding with $C^{14}H_3COONa$ labelled bacteria. Isotope is associated with mitochondria and droplets (possibly lipid) in the cytoplasm. Mag. x 100,000.

the cell (Table 15), showed a greater proportional labelling by carbon^{14} than by sulphur^{35} of the macronucleus and the mitochondria (Plate 14). This agrees with the expected incorporation of carbon^{14} into end products exclusive to the above structures (Roberts et al. 1955, Fletcher and Sandai 1961). In contrast to the diverse end products associated with the incorporation of carbon^{14}, it is believed the incorporation of sulphur^{35} was wholly into proteins. The proportionately greater incorporation of sulphur^{35} than carbon^{14} into the trichocysts and pellicle is therefore interpreted as the synthesis of proteins in these structures.

4) The relationship between the synthesis of 168G and 168D antigens during transformation.

The process of serotype transformation involves the expression of two genetically determined antigens. It was shown that during transformation from serotype 168G to 168D, the expression of the new (168D) antigen on the pellicle and cilia began within 30 minutes of the temperature increase (page 58). Further investigations have been made to determine at what time the synthesis of the old (168G) antigen ceases. There may be either synchronous and complete 'switch' in the expression of the two antigens, or a period during which both antigens are expressed simultaneously - during which time there may be a gradual change in the relative proportions of old and new antigen synthesised. The synthesis and incorporation of both antigens has been studied by following the structural coincidence of either anti-168G or anti-168D ferritin-conjugated globulin with newly synthesised (radioactive) protein, on the pellicle and cilia, at various times during the transformation process.

A culture of 168G cells at 18°C was raised to 35°C by the addition of an equal volume of culture medium at 40°C and then maintained at 35°C in

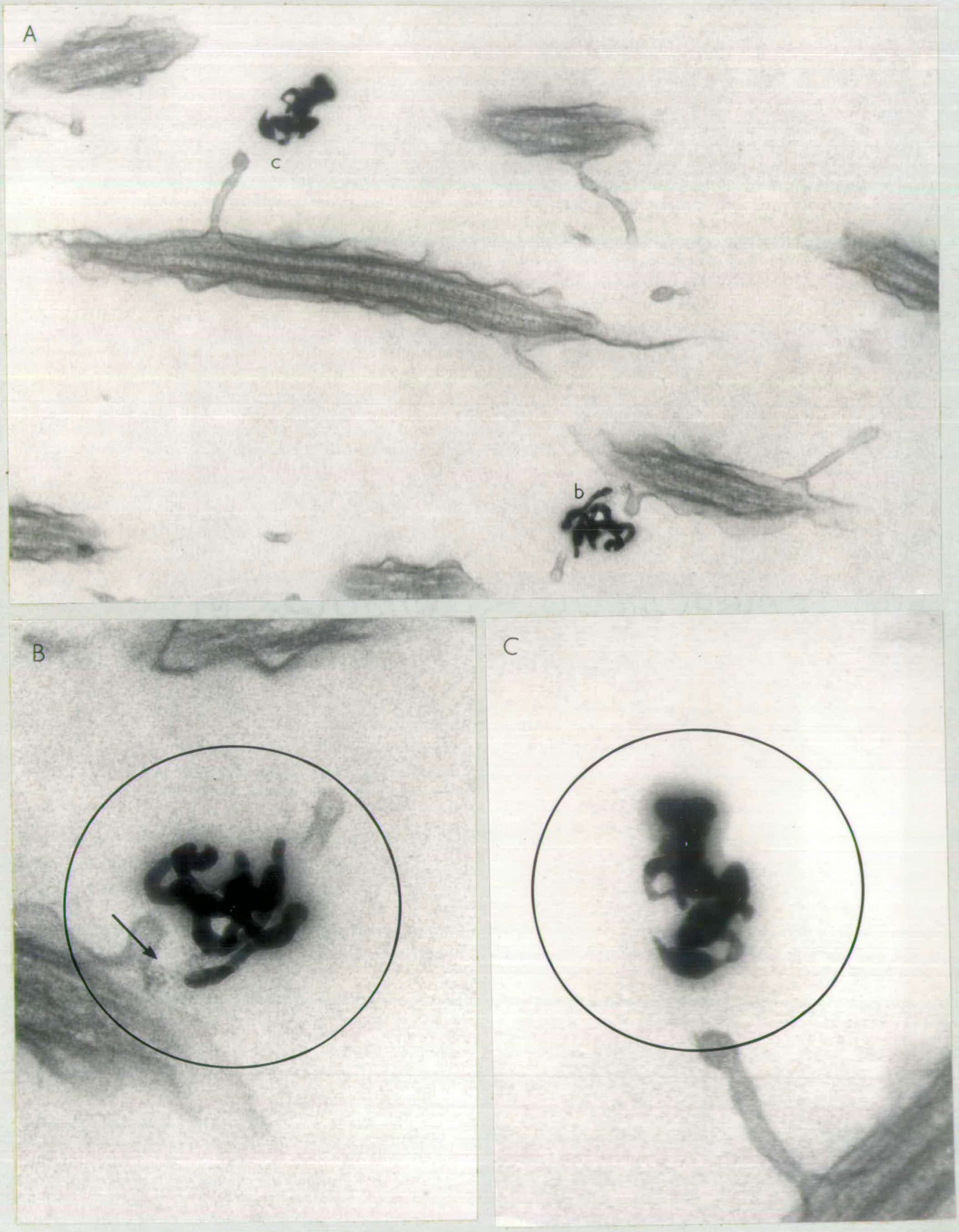

A
c
b
B
C

PLATE 16

A. E.M. autoradiograph showing cilia of paramecium-transforming from serotype 168G to 168D - fed with sulphur^{35} labelled bacteria. After fixation the sample was treated with ferritin-conjugated anti-168D globulin. Mag. x 50,000.

B. High power micrograph of region B, showing positive coincidence of autoradiographic grain and ferritin conjugated globulin. The circle limits the maximum projected distance (0.3μ) of the source from the developed grain. Ferritin (arrow) is located within this circle. Mag. x 100,000.

C. High power micrograph of region C, showing negative coincidence of autoradiographic grain and ferritin conjugated globulin. No ferritin is detected within the circle. Mag. x 100,000.

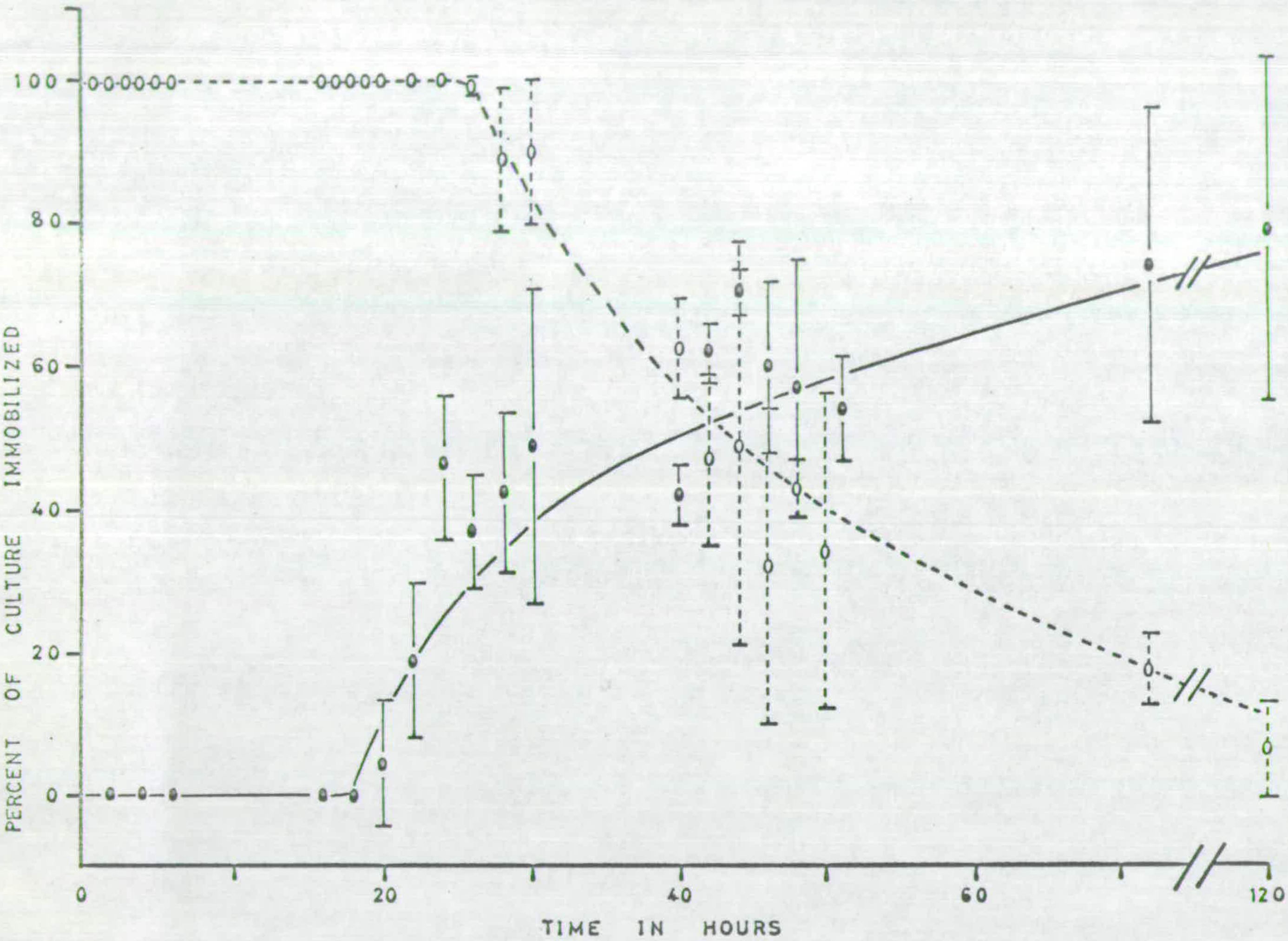


FIG.22 Expression of 168D and 168G antigens on cells of serotype 168G transforming to serotype 168D - as detected by the immobilization reaction (see page11.). Each test was scored as the percentage of the sample immobilized by each antiserum within two hours at 18°C. The conditions of culture are described on page 60.

O--O Reaction with anti-168G antibody.
●—● Reaction with anti-168D antibody.

a water bath. Samples were taken at various times and incubated for two hours with sulphur^{35} labelled bacteria, then fixed in osmium tetroxide and labelled with ferritin conjugated globulin from antibodies prepared against either 168G or 168D antigen.

Four pilot cultures were subjected to the temperature increase and assayed by the immobilization test, to find the most informative times at which to sample the cultures for the ferritin coincidence technique. The results were expressed as the percentage of a culture immobilized by either anti 168G or 168D antiserum. Since the results of the transformation experiments described above, indicated that a culture reacts homogenously during transformation (Fig. 19), the immobilization analysis used in these experiments therefore reflects the percentage of the total antigen on each cell represented by either 168D or 168G antigen. The results are summarized in Fig. 22. Thirty hours after the temperature increase the cells contain substantial quantities of 168D antigen, which is therefore assumed to be synthesised in excess of 168G antigen at that time. It was observed that if a culture exhausted its food supply the percentage of transformed cells remained stationary until a new food source was supplied, whereupon transformation continued, indicating that new synthesis was essential for transformation to proceed. The results of this immobilization analysis indicated that the significant period during transformation was between zero and thirty hours after the temperature increase. Consequently the synthesis of 168D and 168G antigens was investigated in the periods 0 to 2, 3 to 5, and 26 to 28 hours after the temperature increase.

The results of two separate experiments are given in table 16, the mean values indicate a higher coincidence of 168G antigen than 168D antigen with the newly synthesised protein, in the period 0 to 2 hours after the temperature increase. After 3 to 5 hours, the coincidence of 168G antigen

Time after temperature increase during which radioactive bacteria were supplied	Percentage of total autoradiographic grains associated with ferritin molecules.					
	Ferritin conjugated anti-168D globulin			Ferritin conjugated anti-168G globulin		
	Experiment A	B	Mean	Experiment A	B	Mean
0-2 hours	30	54	42	80	93	86
3-5 hours	82	45	63	72	88	81
26-28 hours	62	79	71	40	88	64

TABLE 16 The structural coincidence of 168D or 168G antigens with newly synthesised proteins on the pellicle and cilia of paramecia during transformation from serotype 168G to 168D. Newly synthesised (sulphur^{35} labelled) protein was detected by E.M. autoradiography, 168D and 168G antigens were detected by ferritin conjugated antibodies. Analysis was similar to that described for table 14a.

with the newly synthesised protein has fallen only slightly whereas that of 168D antigen has risen above 50% indicating that a substantial proportion of the immobilization antigen synthesised in this period is 168D antigen. Finally, after twenty-eight hours at the higher temperature more 168D than 168G antigen was being synthesised.

By the end of the experiment, i.e. after twenty eight hours at 35°C, the paramecia had passed, theoretically, through at least three cell fissions. Therefore only ca. 12% of the total immobilization antigen had been synthesised before the temperature increase. If the synthesis of 168G antigen had stopped at the same time as that of 168D started, i.e. within two hours of the temperature increase, the coincidence of 168G antigen with newly synthesised protein, due to random association, would have fallen after twenty eight hours to a value near 12%. The observed very high coincidence (64%), of the newly synthesised protein with ferritin conjugated anti 168G antibody, indicates that the synthesis of 168G antigen had continued, possibly for the complete period of the experiment. It is concluded that during serotype transformation there is a period, of up to twenty eight hours after the temperature increase, during which both antigens are synthesised simultaneously, with a gradual change from exclusively 168G antigen synthesis to exclusively 168D antigen synthesis.

DISCUSSION

1. The ultrastructural localization of the immobilization antigens in Paramecium aurelia

a) Site of final deposition

The observation that paramecia are immobilized due to the agglutination of the cilia - when suspended in homologous antibodies (Rossle 1905) - indicates the presence of specific antigenic substances on the surface of the cilia. Using fluorescein-conjugated antibodies Beale and Kacser (1957) demonstrated that these immobilization antigens were present not only on the cilia but also on the surface of the pellicle. The continuous distribution of the antigens over the whole of the surface of the cell, with the notable exception of the gullet region, was confirmed by the use of ferritin-conjugated antibodies (Mott 1964). However all the above observations were made on intact cells and therefore gave no information about the possible internal location of the antigens.

The pellicle and cilia have been shown to be the primary location of the immobilization antigens by two groups of workers. Preer and Preer (1959) using quantitative gel diffusion techniques found that most (ca. 68%) was associated with cell fractions containing the cilia and body wall (pellicle). Immobilization antigen was shown to represent 30% of the protein in isolated cilia (Preer 1968). Beale and Mott (1962), using fluorescein-conjugated antibodies to localize the immobilization antigens in frozen sections, were able to detect these proteins only on the pellicle and cilia.

In the work reported here using antibodies conjugated to fluorescein, ferritin, or iodine^{125}, the immobilization antigens were detected in situ, on the pellicle and cilia but could not be detected in any other part of the cell. Structures conspicuous by their lack of demonstrable association with conjugated antibody are ribosomes, endoplasmic reticulum and cytoplasmic vesicles - all components of the 'cytoplasmic' fraction (see table 11). Similarly the incorporation in vivo of $sulphur^{35}$ into the immobilization antigens could be correlated with the incorporation of isotope into the pellicle and cilia only. Both observations are consistent with the view that the pellicle and cilia contain the final sites of deposition of the immobilization antigens.

Earlier reports on the localization of the immobilization antigens have stated that there is no antigen on the gullet wall or cilia (Beale & Kacser 1957, Beale and Mott 1962). The results of the present investigation contrast with this earlier work in that when preparations of frozen sections were treated with fluorescein-conjugated antibody, the cilia and pellicle in the gullet were seen to react specifically with homologous globulins (Plate 6d). This discrepancy is believed to be the result of differences in experimental technique. Only in the current investigations was there effective contact between the antibody and the gullet cilia, combined with adequate removal of unreacted antibody to render visible a specific reaction.

Under normal conditions only a single type of immobilization antigen can be detected by the immobilization test on the surface of a given paramecium at one time (Sonneborn 1950b, Beale 1954). Quantitative analysis of the reactions of $iodine^{125}$ and ferritin-conjugated antibodies, has provided a technique for the detection of antigens on the cell surface which is more sensitive than the immobilization reaction. Using these quantitative techniques it was shown that the pellicle and cilia of paramecia of serotypes 168G and 168D react specifically with their respective homologous antibodies and do not contain any heterologous antigen.

cells was shed without any detectable damage to the paramecia. This observation is however questionable in the light of current experiments which have shown that the globulin molecules may cause a breakdown in the ultrastructure of the pellicle. Furthermore in the present work it was shown that substantial quantities of sulphur^{35} were assimilated into the immobilization antigens, however the incorporation of sulphur^{35} into the pellicle, as shown by light and electron-microscope autoradiography, was not seen to be restricted to any region of the pellicle where rapid membrane synthesis might be expected to occur, e.g. the fission-furrow , or to structures described as typical of growing membrane e.g. microtubules, microvilli or vesiculated membrane (Jurand and Selman 1969). Similarly Beale and Mott (1962) were unable to detect any specific incorporation of "new" 168D antigen into the region of the fission-furrow during transformation from serotype 168G to 168D. Finally Austin et al. (1956) have shown that transformation from serotype 51D to 51B can occur without cell division. Since it has been shown that the various components of mouse fibroblast membrane have the same rate of turnover (Warren and Glick 1968), the absence of any correlation between the incorporation of the immobilization antigens and membrane synthesis indicates that these proteins are not an integral part of the pellicle membrane.

Using ferritin-conjugated antibodies to reveal the incorporation of 168D antigen onto the pellicle of paramecia transforming from serotype 168G to 168D, Mott (1964) claimed that at early stages during the transformation process 168D antigen was found at widely separated areas of the pellicle, these later increasing in size until they coalesced. However after extensive examination of comparable experiments in the present investigation, it was concluded that "new" 168D antigen was randomly dispersed over the surface, although new antigen was first detected on the pellicle close to the cilia. Further evidence from this investigation indicated that during serotype transformation both 168G and 168D antigens are incorporated effectively within the same areas of the pellicle. The removal of large aggregates from the preparation of ferritin-conjugated globulin in the current investigation has prevented the labelling of single antigen molecules on the pellicle by such large masses of ferritin conjugated antibody - an occurrence which in Mott's work gave the false impression of intense antigen concentrations at discrete sites on the pellicle.

b) The site of immobilization antigen synthesis.

Electron microscope autoradiography was used to follow the incorporation in vivo of $sulphur^{35}$ and $carbon^{14}$ into paramecium from labelled Aerobacter aerogenes. The interpretation of such an analysis is influenced by two factors. The first is that no direct information is obtained about the biochemical nature of the substances into which the radioactivity has been incorporated. The second is that only polypeptides and proteins are bound to the tissues by the fixative. The possibility of non-specific binding of free radioactive amino-acids to the tissues (Peters and Ashley 1967, Bergeron and Droz 1968, Caro and Palade 1964), is believed to be unlikely because the radioactive material represented only a small proportion of the total available food supply (Caro & Palade 1964). Consequently any radioactive free amino acids will have been washed out of the paramecia during the preparation of the samples for electron microscopy. Therefore the observation that the peak density of radioactive labelling of the pellicle, unlike any other organelle, always follows the peak density of radioactivity within the cytoplasm (which for the purposes of this study consists of ribosomes, endoplasmic reticulum, vesicles and cell sap) indicates that the radioactivity is incorporated into large molecules within the cytoplasm before it is transported to the pellicle, i.e. the immobilization antigen is not synthesised on ribosomes in the pellicle.

Several workers using a variety of different techniques have found that material showing the same immunological reactions as the immobilization antigen is associated with isolated fractions of ribosomal material. The techniques include quantitative gel diffusion on cell fractions of various stocks of syngen 2 (Preer and Preer 1959); and antibody absorption techniques which have been used on stocks 72, 2, 30 and 83 of syngen 2 (Seed et al. (1964), and also on stock 51 of syngen 4 (Macindoe and Reisner 1967). The latter stated however that the immobilization antigen present in the ribosomal fraction is associated with "a particle distinct from the ribosome but not distinguishable from it on the electron-microscope". Finally Sommerville (1967a,b and unpublished) has found evidence for the synthesis <u>in vitro</u> of immobilization antigens by fractions of pure ribosomes and membrane bound ribosomes of stocks 90 and 168 of syngen 1.

These results contrast with the <u>in situ</u> labelling experiments (Beale and Mott 1962, and this investigation), which have not yielded any evidence for the presence of immobilization antigens in the cytoplasm. However when it is considered that more than 66% of the total antigen is associated with the pellicle, a structure representing ca. 5% of the cell volume (calculated as described in the materials and methods section), whereas less than 33% of the antigen is found in the cytoplasm which occupies ca. 40% - 50% of the cell volume, in view of the high background labelling encountered in the <u>in situ</u> labelling experiments it is not surprising an apparent conflict exists between these two groups of results. This was shown to be due to the technical limitations of the experimental procedures by using the same iodine^{125} conjugated globulins to detect the immobilization antigen on ribosomes *in vitro*, as were used for the *in situ* localization.

The technique of selective binding and precipitation of ribosomal material with antibodies has been used to show the association of myosin with chick muscle polysomes (Allen and Terence 1968); of albumin, glutamate dehydrogenase and catalase with ribosomes from beef liver (Duerre 1967); and of the L chain of gamma globulin with polysomes of rat spleen (Wust 1967, 1968), the latter investigation using iodine^{125} conjugated globulins.

The present investigation has demonstrated a specific reaction between free ribosomal material and homologous iodine^{125} conjugated globulin. The globulin not only agglutinated the ribosomal material causing it to sediment through the sucrose gradient, but also became bound to polysomes of specific sizes, the largest of which had a sedimentation coefficient of $\sim$320S. This value theoretically corresponds to a polysome containing 30 monosomes. Such polysomes would be capable of synthesising a polypeptide with a molecular weight of 100,000 (Staehelin et al. 1964). This is the proposed size of the immobilization antigen subunits (Steers 1965). These results are similar to those obtained for the size of polysome capable of synthesising immunologically recognisable immobilization antigen (Sommerville 1967a).

A plausible mechanism of immobilization antigen synthesis, based on results obtained from cell fractionation techniques, has been described by Sommerville (1967a). He proposed that the antigens are synthesised on free polysomes, the immunologically specific nascent polypeptide produced then causing the polysome units to aggregate on membranes - presumably of the endoplasmic reticulum. A similar mechanism has been described for the synthesis of collagen (Manner et al. 1967).

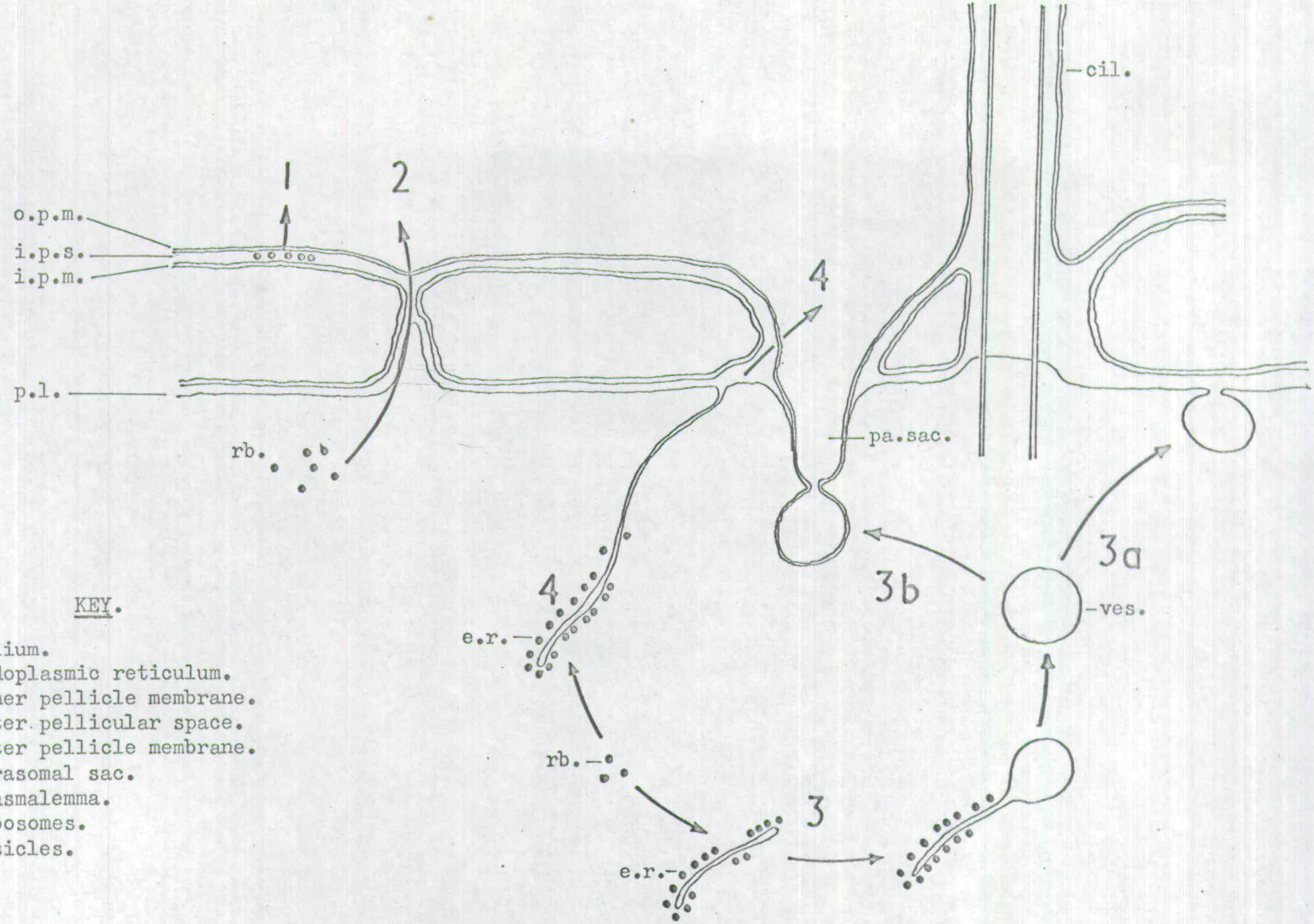
2. Transport of the immobilization antigens from their site of synthesis to the site of final deposition

Having established both the site of synthesis and the site of final deposition of these proteins the question remains as to how the immobilization antigens are transported from one site to another.

Since the antigens must presumably be carried through the plasmalemma, the transport of these proteins from the ribosomes to the cell surface is comparable with that of proteins in other secretory systems. Among these are the collagenous proteins in fibroblasts which have been traced directly from the endoplasmic reticulum to the cell surface (Ross and Benditt 1965, Goel 1969), and from the endoplasmic reticulum to the golgi apparatus, from where the proteins are transported to the cell surface (Revel and Hay 1963).

The golgi apparatus is apparently an indispensible organelle in the majority of secretory cells. Here, proteins which have been synthesised on the endoplasmic reticulum, may be complexed with carbohydrate e.g. tropocollagen (Revel and Hay 1963), non-collagenous protein (Ross and Benditt 1965, Goel 1969), and glycoprotein (Neutra and Leblond 1966); or condensed e.g. zymogen (Caro and Palade 1964, Jamieson and Palade 1967a,b, 1968a,b); or modified in some unspecified manner e.g. retinal rod protein (Young 1968, Young and Droz 1968).

Proteins released from the golgi apparatus and later secreted onto or through the cell surface, are almost invariably associated with smooth membraned vesicles which migrate to, and fuse with, the plasmalemma - a process found to be dependent upon respiratory energy, yet independent of protein synthesis in the pancreatic cell (Jamieson and Palade 1968a,b) - e.g. zymogen (Caro and Palade 1964, Jamieson and Palade 1967a,b), thyroglobulin (Nadler 1965),



KEY.

cil. - cilium.
e.r. - endoplasmic reticulum.
i.p.m. - inner pellicle membrane.
i.p.s. - inter pellicular space.
o.p.m. - outer pellicle membrane.
pa.sac. - parasomal sac.
p.l. - plasmalemma.
rb. - ribosomes.
ves. - vesicles.

FIG.23 The alternative hypothetical pathways for the transport of the immobilization antigens from ribosomes to the surface of the pellicle and cilia. For explanation see page 71.

tropocollagen (Revel and Hay 1963), pituitory hormone (Farquhar 1961), and milk (Wellings and Philip 1964). Some substances not released from the cell have also been associated with similar vesicles e.g. lipid (Stein and Stein 1967, 1968) and glycogen (Coimbra and Leblond 1966). Two possible mechanisms have been proposed for the formation of these vesicles: 1) they are actively pinched off from the endoplasmic reticulum (Jamieson and Palade 1968a,b), or 2) they result from the release of ribosomes from isolated pockets of endoplasmic reticulum (Coimbra and Leblond 1966).

Since _Paramecium aurelia_ has no ultrastructurally distinct golgi apparatus it has not as yet been possible to state that the synthesis of the immobilization antigens involves a golgi like structure. Also the limited resolution of electron microscope autoradiography - 0.1 to 0.3μ (Pelc 1963, Salpeter et al. 1969) - although adequate to localize incorporated sulphur^{35} and carbon^{14} to mitochondria (Bergeron and Droz 1969) is not adequate to assign the isotope to the abundant small vesicles in the cytoplasm. However the transport of the immobilization antigens from ribosomes to pellicle may be expected - on the basis of various mechanisms described for comparable proteins - to follow one of the alternative pathways listed below (see fig. 23):-

(i) The immobilization antigens are synthesised on ribosomes - located within regions of vesiculated membrane - in the pellicle, from where the antigen molecules move through the outer pellicle membrane.

(ii) Having been synthesised on free ribosomes in the cytoplasm the antigens diffuse to the pellicle, where they are transported through the membrane.

(iii) The antigens are synthesised on free polysomes which attach to the endoplasmic reticulum. The endoplasmic reticulum is modified to form vesicles which either:

a) fuse at random with the plasmalemma, or

b) fuse with the parasomal sac, (Plate 17).

(iv) The immobilization antigens are synthesised on free polysomes which associate with the endoplasmic reticulum, this being permanently connected to the plasmalemma (Plate 17c,d).

Pathway (i) is discounted, it has been concluded above that the immobilization antigens are not synthesised in the pellicle. Also there was no evidence for the incorporation of isotope into regions of vesiculated membrane. Pathway (ii) is also rejected being incompatible with the results obtained from either the <u>in situ</u> labelling of frozen sections with conjugated antibodies, or the very rapid <u>in vivo</u> incorporation of radioisotopes into the pellicle. There is no ultrastructural evidence to support pathway (iiia).

Pathways (iiib) and (iv) are however consistent with the immunological and biochemical observations on immobilization antigen synthesis. Furthermore the point at which antigen would be released onto the pellicle in both pathways is close to the base of the cilia (see fig. 23, plate 17c,d). It was observed in the present work that during the very early stages of transformation (between 30 and 60 minutes after the temperature increase) that "new" antigen was detected more frequently near the bases of the cilia than in any other region of the cell surface.

If transported to the pellicle via pathway (iv), immobilization antigen would be released into the interpellicular space. The localization of these proteins specifically on the outer pellicle membrane could be achieved by lipid carrier

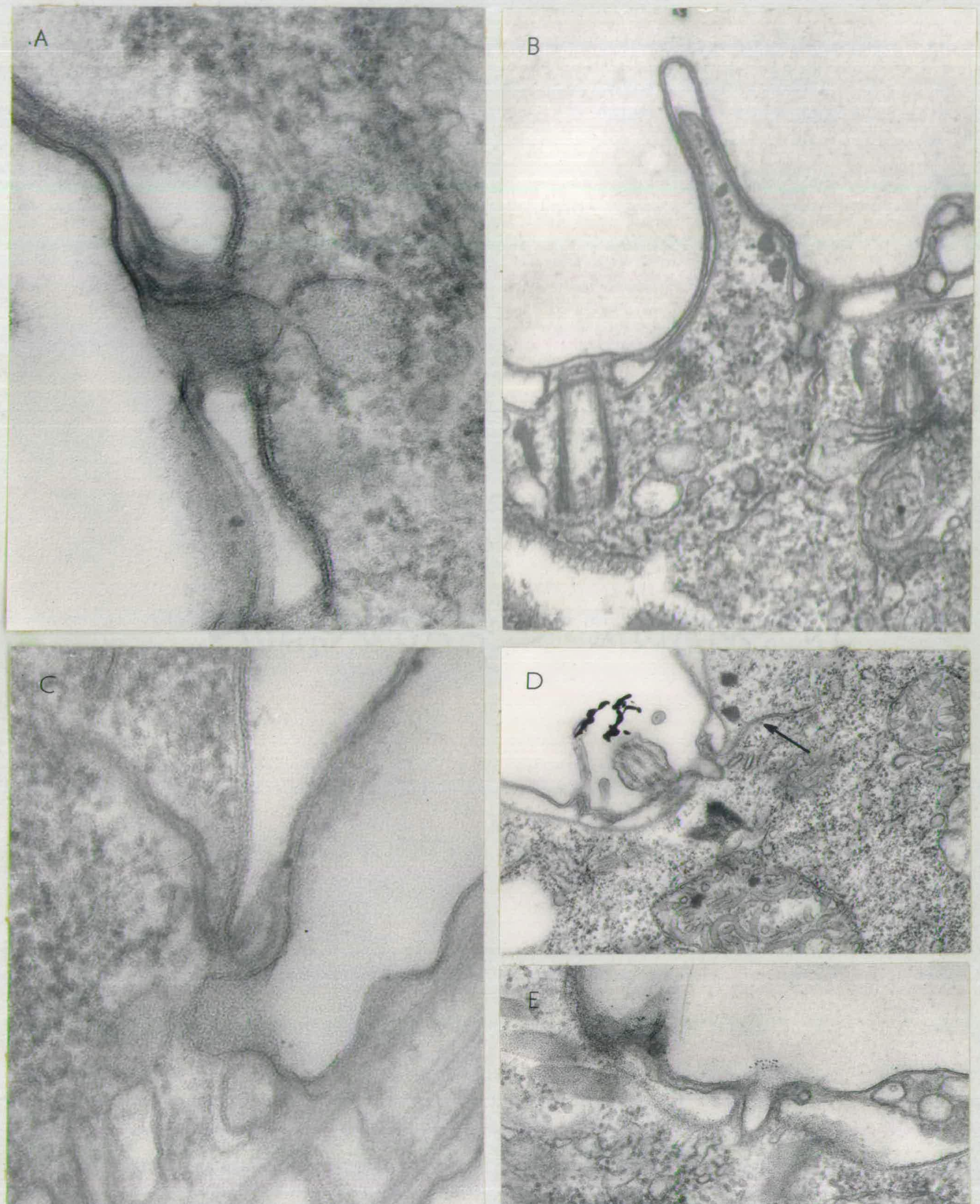

A
B
C
D
E

PLATE 17

A. Electron micrograph of the parasomal sac showing its connection with a smooth membraned vesicle in the cytoplasm. Mag. x 125,000.

B. Micrograph showing the fusion of a rough membraned vesicle with the parasomal sac. Mag. x 50,000.

C. Micrograph illustrating the location of the parasomal sac at the base of a cilium, also the diverticulum of the plasmalemma which is associated only with the parasomal sac. Mag. x 125,000.

D. As for plate 17C, showing the continuity of the plasmalemma diverticulum with the endoplasmic reticulum (Arrow). Mag. x 25,000.

E. Electron micrograph of the pellicle of paramecium (transforming from serotype 168G to 168D) after 3 hours growth at 35°C, treated with ferritin-conjugated anti-168D globulin. Note the ferritin associated with electron dense material at the pore of the parasomal sac. Mag. x 50,000.

molecules (Wright et al. 1967, Higashi et al. 1967), or transport proteins (Rothfield and Finkelstein 1968). Alternatively, as proposed for alkaline phosphatase in Salmonella typimurium "The transfer of newly formed protein from the cytoplasm to outside the cell membrane is a direct result of the unique chemical structure of the polypeptide chain (i.e. the amino acid sequence)". (Slesinger and Olsen 1968). These authors do not however propose any mechanism for the chemical basis of such a process. Only the latter mechanism is consistent with the expression of the immobilization antigens being independent of membrane synthesis. However there is, as yet, no evidence supporting this mechanism of incorporation of antigen on the pellicle.

All the available evidence on the localization and deposition of the immobilization antigen favours pathway (iiib). In this investigation antigen was frequently observed as electron dense material labelled with ferritin conjugated antibody, associated with the pore of the parasomal sac (Plate 17d) - a structure found close to the base of each cilium. Abundant vesicles (both rough and smooth membraned), which it is proposed carry the antigen from the endoplasmic reticulum to the pellicle, were found associated with, and fused to the parasomal sac (Plate 17a,b). The proposed occurrence of cytoplasmic antigen within vesicles is consistent with the results discussed in the previous section, in which significant quantities of soluble antigen have been detected in the cytoplasmic fractions of homogenised material, while none was detected in situ in frozen sections by conjugated antibody techniques. However there is no direct evidence for the presence of antigen within these vesicles because conjugated globulins cannot penetrate the vesicle membrane. The release of the antigens onto the outer surface of the pellicle by fusion of the vesicles with the parasomal sacs is in agreement with the expression of the antigens being independent of membrane synthesis, and readily explains the limited localization of the antigens on the outer surface of the pellicle.

The proposed mechanism of immobilization antigen synthesis is therefore summarised as follows:-

(i) Immunologically specific molecules are synthesised on free ribosomes in the cytoplasm.

(ii) The free polysomes associate with the endoplasmic reticulum and immobilization antigen is released into the cisternal space.

(iii) The endoplasmic reticulum is modified into vesicles which migrate to, and fuse with, the parasomal sac, from where the antigens are incorporated into the pellicle and cilia at random.

C. The control of immobilization antigen expression in Paramecium aurelia.

The immunological specificity of the immobilization (serotype) antigens has been shown to be controlled by single genes in two ciliate genera, namely Paramecium (Sonneborn 1948, Beale 1952), and Tetrahymena (Nanney and Dubert 1960). In Paramecium but apparently not in Tetrahymena the expression of these genes has been shown to be influenced by the 'cytoplasmic state' of the cell (Sonneborn and LeSuer 1948, Beale 1952), which is in turn modified by the environment. The suitability of the term 'cytoplasmic state' has been questioned (Preer et al. 1963, Preer 1968). Having demonstrated that there is a greater instability of antigen expression during periods of macronuclear re-organization, these authors believe that the expression of these proteins is controlled by the macronuclear rather than cytoplasmic conditions. This observation has been interpreted as an indication that a link exists between the transcription and translation mechanisms controlling the expression of the immobilization antigens (Gibson 1969 in press).

In spite of the elegant analysis of gene control mechanisms in bacteria (Reviewed Epstein and Beckwith 1968), there has been little available data on the control of immobilization antigen expression which discriminated between any of three possible control mechanisms:-

(i) All antigens capable of being expressed by a cell are present in the cytoplasm but only one of these is expressed on the surface at one time.

(ii) The control of translation of the messenger RNA (see review Harris 1968).

(iii) The control of transcription of the DNA (Jacob and Monod 1961).

The results of this and the majority of other investigations on the expression of immobilization antigens in stable cultures of paramecium are as discussed above, incompatible with the first proposed control mechanism. This conclusion is supported by the results obtained from the investigation of the incorporation of protein into the pellicle and cilia of paramecia transforming from serotype 168G to 168D. The techniques used to detect this process artificially divided the protein into two classes either radioactively labelled protein (detected by E.M. autoradiography), or 168D antigen (detected by ferritin-conjugated antibody). It is probable however that much of the radioactive protein is present in the form of immobilization antigen. Since 168D antigen was not incorporated into the pellicle before the newly synthesised (radioactive) protein, it is inferred that all the 168D antigen was synthesised after the temperature increase, and was not already present within the cytoplasm of the 168G cells. This inference is supported by the following investigations on the effects of hyperthermic shock on Tetrahymena pyriformis. When Tetrahymena is raised from 28°C to 34°C it has been found that protein synthesis - as shown by the incorporation of C^{14}-serine into protein - is inhibited for a short period (Levy et al. 1969), and that there is a reduction in the rate of division of the cells for a similar period (Rooney et al. 1969). However at the same time there was no detectable reduction on the rate of protein turnover, neither was there any reduction in the respiration rate. There was a loss of ATP (19%) and protein (17%) due to the temperature shock. The only metabolic process shown to be inhibited by the temperature increase is the de novo synthesis of protein. This would indicate - if the situation is the same in Paramecium as in Tetrahymena - that the transport of existing cytoplasmic antigens in paramecium from the cytoplasm to the pellicle would remain unimpaired by the increase in temperature, while the de novo synthesis of antigens would be delayed.

New (168D) antigen is expressed on the pellicle very rapidly - within 30 minutes of the temperature increase. This was before any reduction in the density of the old antigen was detected, and this relationship was also observed using the immobilization test, (fig. 22). A similar pattern of antigen expression was reported by Sonneborn and Whallon (1950).

The density of the new antigen on the pellicle as shown by the ferritin conjugated antibody was seen to rise exponentially after the first thirty minute delay. This result compares favourably with those of Balbinder and Preer (1959) who found an exponential increase in the quantity of new antigen after onset of transformation of stock 28 from serotype G to E. They also found, using quantitative gel diffusion techniques, no evidence for the presence of the new antigen in the original culture before transformation began. The observed exponential increase in the quantity of antigen is in accord with a controlled rate of antigen synthesis within cells of a growing culture (Kimball et al. 1959). In the present work a controlled rate of antigen synthesis was indicated by the quantitative analysis of the antigen density on the pellicle.

The delay in incorporation of $sulphur^{35}$ into the pellicle during the initial stages of serotype transformation was not accompanied by any visible breakdown in the ultrastructure of the endoplasmic reticulum as described for Tetrahymena (Levy et al 1969). The cause of the impaired protein synthesis is not as yet known, however Paramecia caused to transform as the result of homologous antiserum treatment have shown a reduced retention of H^3-uridine labelled RNA in both the macronucleus and micronucleus (Pasternak 1967). Also Tetrahymena, on a change in medium, has also shown a loss of RNA (Cline 1966). A similar loss in the total RNA content of paramecia during transformation induced by a temperature increase, would readily explain the observed reduced rate of protein synthesis (Kimball et al. 1959).

During transformation from serotype 168G to 168D the synthesis of the 168G antigen, shown by this investigation to continue for a period of possibly twenty to thirty hours (two to four cell fissions) after the onset of synthesis of 168D antigen can be interpreted in either of two ways:-

(i) the mechanism for switching-off the synthesis of the "old" antigen is not activated for many (ca. 20) hours after the switching-on of the "new" antigen. or

(ii) the messenger coding for the structure of the immobilization antigens has a life of up to four cell generations.

Using DNA/RNA hybridization techniques Gibson (1969) has demonstrated that some of the RNA synthesised within 60G cells ceases to be synthesised between one and six hours after an increase in the culture temperature, indicating the first of the two explanations given is perhaps unacceptable. Support for the second alternative is provided by two investigations. Kimball and Prescott (1964) studying the effects of 'genetic enucleation' of paramecia have reported that messenger RNA coding for total protein synthesis has a has a life of many hours. Also Sommerville (1969 in press), who has followed the incorporation of $sulphur^{35}$ into both "old" (168G) and "new" (168D) antigens under similar experimental conditions to those used in the present work, and has confirmed the continued synthesis of the "old" (168G) antigen for a period of sixteen to thirty two hours after the synthesis of the "new" (168D) antigen has begun. Furthermore it was shown that both antigens were synthesised for a similar period in the presence of Actinomycin D. From these experiments it was inferred that the messenger RNA coding for the immobilization antigens has a long half life.

Observations based on the effects of Actinomycin D should however be considered with caution in view of the results of Austin et al. (1967a) and Pasternak(1967), which indicate that the antibiotic only inhibits DNA dependent RNA synthesis by between 60 and 90%. Austin et al. (1967a,b) studied the effects of various inhibitors of protein and RNA synthesis - namely Actinomycin D, chloramphenicol and puromycin - on the rate of transformation of paramecia from serotype 51D to 51B. From these experiments they concluded that serotype transformation is dependant not only upon <u>de novo</u> protein synthesis, but also on <u>de novo</u> RNA synthesis - a conclusion consistent only with the control of immobilization antigen expression at the transcription level.

Only two published observations have made only direct measurements on the synthesis of RNA during serotype transformation in <u>P. aurelia</u>. The first, using autoradiographic techniques to assay the incorporation of H^3-uridine into RNA (Pasternak 1967) has shown that after treatment of paramecia serotype 51D with homologous antiserum or patulin there was an increased rate of RNA synthesis in both the macro- and micronuclei for the first eight hours after the stimulus. This preceeded the expression of the new (51B) antigen as detected by the immobilization reaction. The second investigation used

DNA/RNA hybridisation techniques to detect differences in the composition of the total RNA present in cells of stock 60 during transformation from serotype G to D, at different stages of the transformation process (Gibson 1969 in Press). Throughout the experimental period (60 hours) a constant spectrum of RNA was synthesised. However RNA of a new specificity was detected one hour after the stimulus to transform was given. Whilst in neither of these two experiments is it possible to state that any of the RNA synthesis observed is related to the expression of the immobilization antigens, the results of Gibson are promising in showing that RNA of a new specificity i.e. not present in 60G cells, is synthesised as a result of the increase in the temperature of the culture medium.

The available data, such as they are, on the control of immobilization antigen expression is therefore consistent with a control mechanism requiring both de novo RNA synthesis and de novo protein synthesis for the expression of a different antigen determining gene during serotype transformation.

4. The prospects of future work on the immobilization antigens in Paramecium aurelia

Experimental proof of the proposed pathway of transport of the antigens from the ribosomes to the pellicle may be provided by the use of various metabolic inhibitors e.g. cycloheximide - an inhibitor of protein synthesis, Antimycin-A - a respiratory inhibitor, or oligomycin - an inhibitor of oxidative phosphorylation, to block the incorporation of radioactive precursors (from axenic media) at various stages from the ribosome to the pellicle. The accumulated isotope may then be localized by E.M. autoradiography. This technique has been used successfully to localize the pathway of zymogen synthesis in the pancreas (Jamieson and Palade 1968a,b).

It is expected that the successful isolation of ribosomal material with antibody prepared specifically against immobilization antigen will permit the characterization of the isolated material in the electron microscope, as described for myosin synthesising polysomes in chick muscle (Allen and Terence 1968). The results would be informative in terms of the size of polypeptide synthesised *in vivo* (Staehelin et al. 1964), and the assembly of the immobilization antigen molecule (which is believed to contain a complex arrangement of sub-units (Steers 1965).

The more exciting possibility arising from the specific isolation of polysomes synthesising a single protein is that the messenger RNA coding the immobilization antigen may be isolated simultaneously within the polysome - from where it may be isolated (Heywood & Nwagwu 1968). Therefore by providing RNA precursors (H^3-uridine) to a transforming culture of paramecia, and analysing the incorporation of the isotope into the RNA coprecipitated with polysomes by antisera against either the "old" or the "new" antigen, the relationship between the synthesis of the messengers coding for both "old" and "new" antigen may be directly followed.

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