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STUDY OF THE IMMOBILIZATION ANTIGENS
OF PARAMECIUM AURELIA

by

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1. INTRODUCTION

The ciliate, Paramecium aurelia, contains excellent antigens, so that when suspensions of the broken animals are injected into a rabbit, immune antibodies quickly develop. When live animals of the same type as those injected are placed in dilute antiserum, a characteristic clumping of the cilia takes place, causing immobilization of the organisms and, with stronger treatment, eventual death of the animals.

The location of these immobilization antigens in the animal is the purpose of the study to be described herein, knowledge of the position of the final antigen in the cell being of value in the study of the mechanisms of its production.

The antigen-antibody reaction of immobilization is highly specific and therefore differentiates paramecia which in other respects appear similar. (Sonneborn, 1948, 1950; Beale, 1952, 1954, 1957, 1959). There are a great many antigenic types found in nature and a large number of stocks, e.g., stock 60, stock 90 etc., each derived from a single animal collected at a particular place, have been examined for antigenic types. (Sonneborn, 1950; Beale, 1954). Every stock of paramecia can develop a series of serologically unrelated antigenic types, e.g. 60D, 60G, 60S. Different stocks have corresponding series of antigenic types, e.g., 90D, 90G, 90S, in stock 90, c.f. 103D, 103G, 103S in stock 103. The antigen system has been shown to

be influenced by the interaction of three factors - the gene, the cytoplasmic state and the environment. (Sonneborn and Beale, 1949; Beale, 1952, 1954, 1958). The specificity of each antigen is controlled by a single gene, and all genes for possible antigens are present, though generally only one is active. Different stocks contain different alleles of the antigen - determining genes, thereby giving the corresponding series of antigenic types between stocks mentioned above. These series may or may not be similar serologically, that is, exhibit cross-reaction.

The cytoplasmic factor, which is controlled partly by the gene and partly by the environment, can exist in a number of mutually exclusive states which determine which gene shall be expressed and therefore which antigen formed. Variation of the cytoplasmic states therefore gives antigenic variation within a stock, e.g., 60D, 60G, 60S in stock 60.

Important to the study of the antigen system is the chemistry of the various antigens. Soluble antigens can be prepared and purified in quantities of approximately 100 mg. (van Wagendonk, van Tijn, Litman, Reisner and Young, 1956; Finger, 1956, 1957; Preer, 1959b; Bishop and Beale, 1960; Bishop, 1961). The purified antigens can then be subjected to various tests, such as chromatographic and electrophoretic separation, various immunological tests (Preer, 1959a, 1959b, 1959c, 1959d; Balbinder and Preer, 1959; Bishop, 1963) and finger printing (Ingram, 1959) for peptide differences. The antigens have been shown to be proteins of molecular weight of approximately 250,000 (Preer, 1959c; Bishop, 1961) and can be separated chromatographically (Bishop and Beale, 1960). All antigens are found to differ in peptide pattern, but the pattern of those antigens, e.g., 60D, 90D, controlled by alleles at the same locus $d^{60} d^{90}$, were of the same

general form, which was different from the peptide patterns of antigens controlled by alleles at a different locus. (Steers, 1962; Jones and Beale, 1963). A single gene difference may give as many as 20 peptide differences and even immunologically similar antigens can be chemically quite different (Jones and Beale, 1963).

The location of the immobilization antigens, forming the subject of this work, is of value in the study of the mechanisms of the interaction of the nucleo-cytoplasmic components of the antigen system, - a system which could provide a model for the understanding of cell differentiation generally. (Sonneborn 1947; Beale, 1952).

In previous work on whole paramecia, (Beale and Kacser, 1957), the immobilization antigens were shown to be located on the surface of both pellicle and cilia, and could be extruded into the medium under certain conditions. The position of the antigen on the live or fixed paramecium was identified by the use of labelled antibody, in this case the label being the fluorescent dye, fluorescein, (Coons, Creech, Jones and Berliner, 1942; Coons and Kaplan, 1950; Coons, 1956). The antibody was absorbed into the specific antigen in the antigen-antibody reaction, and the antigen then located by the label fluorescing under the ultra-violet light. Live animals were immobilized when exposed to fluorescent labelled specific antisera, and fluorescence was seen over the entire surface of the animal, and was seen to be particularly at the tips of the clumped cilia. When fixed animals were similarly treated, the entire surface of the pellicle and cilia fluoresced brightly, with no clumping of the cilia or concentration of fluorescence at the tips. Until the present investigation was undertaken, nothing was known about the internal contents of the cell as it was highly probable

that the fluorescent labelled antibody was not able to penetrate the cell wall of the intact animal.

The problem of gaining access to the interior of the cell and the use of these fluorescence methods, was therefore made the subject of a special study to be described in detail below. In this study, fixed paramecia were sectioned to expose the internal contents and then treated with the fluorescent labelled antibody. Double staining for the location of two different antigens in the same preparation with the use of two contrasting fluorescent dyes, (Silverstein, 1957; Chadwick, McEntegart and Nairn, 1958; Nairn, 1961) fluorescein, and lissamine rhodamine B 200, giving respectively green and orange fluorescence in ultra-violet light, was particularly useful in the study of transformation (Beale, 1954, 1957) from one antigenic type to another.

It was then felt that higher magnifications were necessary to study the exact location of the antigen and other details of the membranes on which the antigen was thought to be located. Paramecia were therefore studied with the use of the electron microscope, both for the ultrastructure of the paramecium, and for the detailed location of the antigen. It was now necessary to label the antibody with an electron dense material, such as the heavy metals, mercury (Pepe, 1961) or iron in the form of ferritin (Singer, 1959; Singer and Schick, 1961). In the study presented here, the γ globulins of the specific antisera were therefore labelled with the electron dense iron-containing substance, ferritin. Ferritin is a naturally occurring protein of M.W. 747,000 (Harrison, 1959) with 23% iron in the form of ferric hydroxide-phosphate micelles, situated in a protein shell (Granick, 1946; Harrison, 1959). This iron confers electron-scattering power on the substance and appears in the electron microscope as

a dense granule of 55A size. The iron is surrounded by the protein apoferritin, having a M.W. of 460,000 and a total diameter of 110A, which however, is not seen in unstained sections. The presence of the granules of ferritin when conjugated to specific antibody, indicated the location of the antigen.

It was then considered desirable to study molecules of purified immobilization antigen in the electron microscope, using high resolution techniques, and it is hoped later to identify these molecules in the external membranes of the paramecium. Small particles down to approximately 10A diameter can be resolved in the electron microscope, but only with the help of some method giving increased contrast. (Brenner and Horne, 1959).

One method of gaining increased contrast is the positive staining of the particle with an electron dense stain such as potassium permanganate or uranyl acetate, similar to the method described for the ultrastructure of the paramecium, but this method does not give good resolution of the structure of the particle.

A second technique is that of negative staining, in which the particle is embedded in an electron-dense material, such as phosphotungstic acid, the particle remaining unstained and electron transparent. The external form can be studied and sometimes the internal structure can be deduced if the phosphotungstic acid can gain access to any cavities in the particle. These facts were first recognised by Hall (1955) and Huxley (1956), who noted that to get negative rather than positive staining of the particles, the concentration of the phosphotungstic acid (PTA) must be low and the pH high. Horne and Brenner (Horne and Brenner, 1958; Brenner and Horne, 1959) developed the technique of negative staining using 1% PTA, adjusted to pH 7.5 with N.KOH.

That this method also preserves the true structure of the particles has been demonstrated by the fact that virus particles remain infective after treatment with negative stain (Horne and Nagington, 1959).

A third technique to give increased contrast to small particles is that of shadow-casting (Williams and Wyckoff, 1944, 1946). A thin layer of electron dense metal is deposited at an angle onto the particles which are supported on a smooth surface. The particles thereby cast an electron-transparent shadow and can also show some external structure. From the shadow-to-height ratio determined by the shadow cast by particles of known diameter, the height of the particle above the surface of the metal can be deduced.

These last two techniques of negative staining and metal shadowing were used in the remainder of this work for the study of the molecules of immobilization antigen.

From the results of these studies some possible mechanisms, and sites, for formation of immobilization antigen are suggested.

2. MATERIAL AND METHODS

I. GENERAL

1. Stocks and serotypes of Paramecium aurelia.

Stock 168, variety 1, serotypes G and D was used. Type 168G is stable at 25°C, type 168D at temperatures above 30°C. To get transformation from type 168G to type 168D, 100 ml. of culture of 168G, at temperature 25° were placed in 300 ml. of culture medium preheated to 35°, and incubated at 35°, the culture taking approximately 20 minutes to come to the higher temperature. Samples of animals were taken at intervals and examined for the stage of antigenic change. After 18 - 20 hours of growth at 35°, (Beale, 1948) the majority of the animals had transformed to type 168D.

2. General culturing techniques.

The paramecia were grown according to the general methods for culture of Sonneborn (1950) using dried lettuce infusions, with Aerobacter aerogenes, at a pH 6.8 - 7.4, giving approximately five fissions in twenty-four hours. Conjugation of the organisms was brought about by the method of Sonneborn (1950).

3. Maintenance solution. (M.S.)

A non-nutrient medium was used for washing the animals, consisting of - 0.013 M NaCl, 0.0003 M KCl, 0.0003 M CaCl₂, 0.004 M Na K phosphate buffer, pH 7.

4. Preparation of antisera.

Antisera were made against whole paramecia according to the method of Sonneborn (1950), by injecting homogenates of the whole animals into rabbits. The serum was incubated at 56° to inactivate complement, dialysed against M.S. and stored frozen in small aliquots. Antisera were developed against antigens 168D and 168G, and also a "heterologous" antiserum, i.e., one not reacting with the immobilization antigens 168G and 168D, was developed against stock 192X.

5. Immobilization.

The serotype of the animals is based on this test. Live paramecia when placed in a dilution of homologous antiserum, become immobilized and the immobilization time, which is somewhat arbitrary, is taken as the average of the times animals take to cease to move in any direction.

6. Determination of titre of antiserum.

The titre of the serum is taken as the dilution in M.S. of the serum which will immobilize live, healthy paramecia in two hours.

II. METHODS USED FOR STUDIES INVOLVING FLUORESCENT ANTIBODIES.

1. Preparation of fluorescent labelled antibody.

a. Fluorescein.

The labelling of the antisera with fluorescein was first done by the original method of Coons and Kaplan (1950) and later by the isothiocyanate (Riggs, 1957) modified method of Marshall, Eveland and Smith (1958), using whole sera. One volume of whole serum was added to 5.3 volumes of 0.15 M NaCl and 0.7 volumes of 0.5 M carbonate-bicarbonate buffer, pH 9.0, at 2°C, with stirring. 5 mg. of fluorescein isothiocyanate, prepared by Dr. R. Alexander, was added with stirring in the cold overnight. Free dye was removed by passage through a column of De-acidite "F.F.", buffered with phosphate buffer at pH 7. The solution was finally dialysed against M.S. to give a final serum dilution of 1/8. The method of preparation resulted in the immobilization titre being reduced to half the original activity, allowing for dilution. The labelled antisera were stored at -20°C in small aliquots. As the fluorescence was very bright with the fluorescein-labelled whole antisera, it was unnecessary to concentrate them by precipitating the γ globulin, as with rhodamine-labelled antisera.

b. Rhodamine B.200.

The labelling of the antisera with lissamine rhodamine B.200 (R.B. 200) was done by the method described by Chadwick, McEntegart and Nairn (1958). Initially whole serum was used, but as the resultant preparation gave poor fluorescence, in later work the γ globulins were precipitated in half-

saturated ammonium sulphate, after conjugation with rhodamine. One volume of whole serum was added to one volume of 0.15 M NaCl + one volume of 0.5 M carbonate-bicarbonate buffer, pH 9.0 at -2°C . 0.1 volumes of an acetone solution of the sulphonyl chloride of RB 200, prepared by Dr. R. Alexander, were slowly added with stirring, the stirring continuing in the cold overnight. The rhodamine-labelled γ globulin was then precipitated with half-saturated ammonium sulphate in the cold, centrifuged briefly at approximately 22,000 r.p.m. and re-dissolved in a small volume of M.S. After dialysing against M.S., the labelled globulin was passed through a column of De-acidite to remove any remaining free rhodamine. The volume was finally adjusted with M.S. to be the same as the original volume, the loss of antibody titre being again about one-half. These labelled globulins also were stored at -20°C in small aliquots.

2. General procedure

Animals were fixed, embedded and sectioned. The sections were then treated with antibody, and examined under ultra-violet light and the sites of fluorescence noted. These procedures are described in detail in the following sections.

3. Embedding and sectioning of paramecia.

The animals were concentrated by gentle centrifugation, fixed for 20 mins. in 1% osmic acid in M.S., and washed five times in M.S. The fixed animals were embedded in gelatin of a standard consistency obtained by autoclaving 7% gelatin in M.S. at 25 lb. per sq. inch for 20 mins.

The gelatin-suspensions of paramecia were transferred to P.V.C. tubes (The ink sacs of Parker pens) and frozen at -20°C with occasional shaking while solidifying to keep the animals evenly distributed. The block was stored at -20°C.

The frozen gelatin block was sectioned at about 10μ thickness in a freezing cabinet at -8°C. Individual sections were unrolled on the knife and transferred to an albuminized slide, gently flattened and left overnight at -8°C to flatten completely.

4. Treatment with antibody.

Slides were removed from the freezing cabinet, gently warmed to melt the gelatin to cause the sections to stick to the slides. Staining and washing was done by carefully pipetting solutions onto the slides. The following treatments were used:

- a. Fluorescein alone, dil. 1/32, for 2 hours in the cold;
- b. Rhodamine alone, full strength for 3 hours in the cold;
- c. Double staining, first with fluorescein, then rhodamine;
- d. Any of the above, preceded by treatment with non-fluorescent, heterologous (anti-192X) serum dil. 1/2, overnight in the cold.

The slides were well washed in M.S. between procedures, and finally mounted in M.S., ringing the coverslip with vaseline to prevent evaporation. Fresh mounts gave the best results, but preparations did not fade for several weeks or even months if kept cold and in the dark.

5. Fluorescent microscopy.

The sections were examined under ultra violet (U.V.) radiation, with a dark ground condenser in the microscope (Beale and Kacser, 1957), and a seal of non-fluorescent immersion oil between condenser and the underside of the slide. Ordinary glass optics and slides were adequate, and for greatest magnification, (X 800), a special (Zeiss) oil immersion objective with iris diaphragm was used. Under these conditions, material treated with fluorescein-conjugate fluoresced much more brightly than material treated with rhodamine-conjugate.

6. Colour photography.

Great difficulty was experienced in getting adequate reproduction of the orange colour of rhodamine, especially in combination with fluorescein. High speed Ektachrome film ASA 160 was used with exposures of 20 minutes or longer. Various colour compensating filters were tried and also the film speed was increased to ASA 320, but without success.

III. METHODS USED FOR STUDIES INVOLVING THE USE OF FERRITIN-LABELLING AND COUNTERSTAINING THE ABSORBED GLOBULIN

1. Preparation of Ferritin-labelled γ globulin.

The γ globulins of the antisera were precipitated with half-saturated ammonium sulphate, the final precipitated globulins being made up in M.S., and dialysed against M.S. to give a final globulin concentration of 20 mg. γ globulin per ml. M.S. Some of this globulin from each antiserum was retained unlabelled, and the remainder labelled with ferritin. Ferritin preparations from two sources have been used, the first, prepared by Dr. P.M. Harrison was obtained from Dr. R.M. Clayton, and the second was obtained from Calbiochem, Los Angeles, U.S.A., in a preparation in CdSO₄, crystalized X6.

Ferritin was coupled to the γ globulin by the use of the m. xylylene diisocyanate bifunctional coupling agent of Singer (1959) using the improved method of Singer and Schick (1961). 0.5 ml. ferritin (= 40mg. ferritin) solution was made up in 0.1 M borate-NaOH buffer, pH 9.5 to give 2.5 ml. of 1.6% ferritin in the buffer. The ferritin was first conjugated to the m. xylylene diisocyanate. 0.05 ml. m. xylylene diisocyanate were added dropwise with stirring, at 0°C to the 2.5 ml. of ferritin in borate-buffer, to form ferritin diisocyanate.

The ferritin-diisocyanate was centrifuged at approximately 7,000 r.p.m. for 30 minutes in the cold to precipitate free diisocyanate but not too much ferritin-diisocyanate. The supernatant was carefully removed and allowed to stand for one hour.

The ferritin-diisocyanate was then conjugated to the globulin. To 2.5 ml. of ferritin-diisocyanate was added 2.5 ml. (40 mg.) γ globulin dialysed against the borate-NaOH buffer pH 9.5, with stirring for two days in the cold. The resultant ferritin-conjugated globulin was dialysed against approximately 0.05 M - 0.1 M ammonium carbonate, pH 8.8, to destroy the unreacted isocyanate groups on the conjugate. The labelled globulin was then dialysed against M.S. and centrifuged at 10,000 r.p.m. for 30 minutes, giving a clear stable solution which was stored frozen in small aliquots at - 20°C. The loss of activity of the globulin during the conjugation procedure was about one third. As an alternative to freezing, some of the labelled globulin was sterilized by using "Hemmings" milipore filters, but this occasionally resulted in loss of activity and labelling and this method was discontinued.

2. General Procedure.

Animals were fixed, treated with antibody and then embedded and sectioned. The sections were then examined, either stained or unstained, in the electron microscope. The sites of ferritin granules were noted visually, and, in more detail, by means of photographs. These procedures are now described in detail in the following sections.

3. Treatment with antibody.

Animals were gently centrifuged, washed in M.S., and then fixed for 30 minutes, with occasional mixing, in 1% osmic acid in 0.014 M veronal-acetate buffer, pH 7.4. (Palade, 1952) containing 1.3% sucrose, (Caulfield, 1957). When the material was insufficient, wetted slides were used for the procedures, the animals being transferred by pipette to the various solutions.

The fixed animals were pre-treated first with unlabelled, heterologous (anti-192X) serum, as with the fluorescent technique, diluted 1/2, for one hour, and then treated with the specific ferritin-labelled γ globulin, undiluted, for 1 - 2 hours. The animals were well washed in M.S. between procedures.

4. Araldite embedding and sectioning.

Dehydration of the animals prior to embedding was carried out as quickly as possible to prevent leaching out of material, using ascending alcohol concentrations of 35%, 70% and 95%, each for five minutes, followed by absolute alcohol, with three changes, giving a total time in absolute alcohol of 15 minutes.

The dehydrated animals were embedded in the epoxy-resin Araldite M, obtained from CIBA Ltd. Basle, (Glauert and Glauert, 1958). For better penetration volatile 1 - 2 - epoxy propane was used with Araldite, (Luft, 1961), as follows: The animals were soaked, first in 50% absolute alcohol-epoxy propane mixture for 10 minutes, then in pure epoxy propane for 10 minutes, then in 50% epoxy propane-Araldite mixture for one hour. The Araldite mixture was prepared

in accordance with the technique of Glauert and Glauert (1958). To 10 ml. of Araldite CY212 were added 10 ml. of hardener D64B (dodeceny succinic anhydride). Both the Araldite and the hardener and measuring glassware were warmed to 48° to reduce the high viscosity, before measuring out the quantities. 1 ml. of plasticizer (dibutyl phthalate) and finally 0.43 ml. of accelerator (tridimethylaminomethylphenol) were added and the resultant mixture was well stirred, while warm, with an electric stirrer. This mixture was kept at room temperature to prevent excessive hardening, and warmed before use.

The animals in 50% epoxy-propane-Araldite mixture were then transferred to the final gelatin capsule. The capsule was centrifuged to sediment the animals and the 50% epoxy propane mixture replaced by Araldite mixture alone and left overnight at room temperature. Next morning, the capsule was warmed to 48° C and centrifuged to sediment the animals, and the Araldite mixture then polymerized at 48°C for two days.

After hardening, the Araldite block was trimmed to expose the animals in a rectangular block of roughly 0.25 - 0.5 m.m. Sections were cut on a Porter-Blum microtome, using glass knives, in the interference colour range of silver to pale yellow, giving section thickness of approximately 600^oA. The sections were floated on 10% acetone in the trough, xylol vapour was used to flatten the sections, and they were picked up from the acetone in the trough on copper supporting grids of 3 m.m. diameter, 200 mesh, with an electron-transparent supporting film of formvar and carbon. The formvar film was made by dipping clean slides in 0.2% formvar in dry chloroform, leaving the film to dry in the chloroform vapour, and floating it on a clean water surface. The grids were dropped matte side down onto the film, which was then picked up with newsprint and dried in the air. The formvar

film was stabilized by depositing on it a very light film of carbon, using a carbon-arc evaporating unit, until carbon is just seen to be deposited, using a drop of oil on white porcelain as a guide. (Bradley 1954).

5. Counterstaining the sections.

Some grids were left unstained to demonstrate ferritin granules in the sections. Other sections were counterstained by floating the grids, section downwards, on a combination of potassium permanganate and uranyl acetate, a modification of methods by Lawn (1960) and Watson (1958). The grids were floated for twenty minutes on 2.5% uranyl acetate in 1% potassium permanganate, and washed by floating on the surface of distilled water. The sections were then partly decolourised by floating, for thirty seconds only, on a citric acid wash of approximately 1 ml. of 5% citric acid to 30 ml. of distilled water, then again washed on distilled water.

6. Electron microscopy.

The sections were then examined on the grids in the electron microscope, using either a Philips E.M. 75 with plate magnifications of up to X 12,000 or in a Siemens Elmiskop - I, generally using a plate magnification of X 30,000. This latter high resolution microscope was necessary for examination for ferritin granules. Plates were generally enlarged X 3 to give prints of magnification X 90,000 for easy comparison. Some later examinations were made on a Philips E.M. 6.

IV. METHODS USED FOR ELECTRON
MICROSCOPE STUDIES OF THE MACROMOLECULES
OF PURIFIED IMMOBILIZATION ANTIGEN

1. Preparation of carbon film supports for the molecule preparations.

Early preparations of molecules were made on formvar films, stabilized with evaporated carbon, but for later preparations, supporting films of pure carbon were used.

Two methods were used to prepare carbon films. First, that of Watson M.L. (1955) and Bradley (1954) in which the carbon was evaporated onto grids covered with a formvar film, the formvar being later removed by carefully immersing the grids on a wire mesh in chloroform for several hours, and drying on the mesh. Care was taken with the preparation of films, torn films resulting in contamination of the electron microscope.

The second method used was that of ^{Hall}~~Horne~~ and Litt (1957), Brenner and Horne (1959) in which carbon was evaporated onto freshly cleared mica, which gives an atomically smooth surface, the carbon film being floated from the mica by carefully lowering the mica sheet at an angle into water, the surface of which had been brushed clean of floating dust with a glass rod. The water surface was slowly lowered, by syphoning, until the carbon film was deposited on electron microscope grids placed ready on a wire mesh below the original water level, the grids being left on the mesh to dry.

2. Evaporation of carbon.

The carbon was evaporated in a vacuum by the method of Watson M.L. (1955)^a from carbon rods of 99.8% of carbon, 0.5 mm diameter, obtained from Morgan Crucible Company, Church Lane, Battersea, London. Two rods were pointed in a pencil sharpener and the tips faced on carborundum paper to give one a diameter of 0.5 mm and the other a diameter of 1.5 mm, to give a fine tip, but not loss of contact during evaporation. The rods were placed under tension in an A.E.I. Metrovac Coating Unit, 10 cm above the freshly cleaned mica or formvar coated grids. The carbon was evaporated at 10 volts, with a Filament and High Voltage Control of 95-100 in a vacuum of 10⁻⁴ mm Hg for approximately 10 - 20 seconds or until the thickness of the C film was standardised by the method of Bradley (1955) using an oil drop on a piece of white porcelain. The evaporation was discontinued when the porcelain round the drop had a definite tan colour, of carbon approx. 150-200A thick, too thick carbon giving a definite brown colour.

3. Preparation of specimens for examination by negative staining and platinum shadowing

Protein solutions were made up to give a final concentration on the grids of 0.05 - 0.025 mg protein/ml. Polystyrene latex particles of known average diameter of 0.264 μ or 0.088 μ (obtained from the Dow Chemical Company, Midland, Michigan) were suspended in the solution at a final suspension of approximately 1 part in a Thousand, to act as markers for the presence of protein and as an aid to calibration.

4. Negative staining.

The phosphotungstic acid preparations were made according to the method

of Brenner and Horne (1959). A 2% solution of phosphotungstic acid (PTA) was adjusted to pH 7.4 with NaOH, and left at room temperature for two days to precipitate out any crystals. The pH was then re-adjusted, and the final solution kept at room temperature.

One volume of this 2% PTA was added to one volume of the protein solution plus polystyrene latex particles, and the resulting preparation placed on the coated grids either with a platinum loop, removing excess fluid by touching with filter paper, or by spraying with a commercial atomiser.

5. Metal shadow casting.

Solutions of protein were made up either in distilled water or ammonium acetate - ammonium carbonate buffer (Hall and Litt, 1957; Hall and Doty, 1958) with added polystyrene latex particles.

The preparation was either placed on coated grids with a platinum loop, removing excess fluid by touching with filter paper, or was sprayed in the cold onto the surface of freshly cleared mica (Hall and Doty, 1958) giving an atomically smooth surface for ⁱⁿmaximum background and easy stripping off later.

The mica strips or carbon coated grids supporting the specimen, were then shadowed (Williams and Wycoff (1946) with platinum at an angle 10 cm above the metal source. The angles selected were either $22^{\circ} - 30^{\circ}$ giving approximately $\tan^{-1} \frac{1}{2}$, i.e. a shadow-to-height ratio of 2:1, or $11^{\circ} - 15^{\circ}$, giving a $\tan^{-1} \frac{1}{5}$ ^{i.e.} a shadow-to-height ratio of 5:1 (Bradley, 1961) This shadow-to-height ratio was later checked in the area under examination in the electron microscope by measurement on the ~~photographic~~ prints of the

shadow cast by psl particles of known diameter, i.e. height. 2.5 cm of 0.1 mm diameter platinum wire (obtained from Johnson, Matthey and Co. Ltd. Hatton Garden, London) evaporated at 10v under vacuum in an A.E.I. Metrovac coating unit, supported on 2.5 cm. of 1.0 mm diameter cleaned and annealed tungsten wire (obtained from Associated Electrical Industries, Ltd. Rugby,) which had previously been cleaned by bringing to white heat under vacuum. The platinum coated mica sheets were then coated with a supporting layer of evaporated carbon, and the resulting film floated off on water and picked up on grids as described previously for the preparation of carbon films.

6. Preparations examined by negative staining and platinum shadow.

- a) Purified immobilization antigen.
- b) Antigen - ferritin labelled antibody complex.
- c) Ferritin labelled globulin and unlabelled globulin.
- d) Cilia and fragments of pellicle of paramecium.

These were prepared as follows:

a) Purified immobilization antigen.

Preparation of purified immobilization antigen.

The antigen was extracted by the method of Preer (1959b). Healthy, cleared cultures of paramecia of a single serotype were harvested, after straining through cheese-cloth, in a De Laval centrifugal separator, which was modified according to Preer and Preer (1959) with a flow of 600 ml/min. The live paramecia were further concentrated by centrifugation at approximately 200G for 2 minutes in a Baird and Tatlock electric oil - testing centrifuge

(Balbinder and Preer, 1959). The yield was approximately 1 ml of packed live cells (about 10^7 paramecia) per three litres of growing culture.

The soluble antigen was extracted ^{by} the salt/alcohol method of Preer (1959b). 1 volume of live, packed paramecia to 4 volumes of a mixture of one part 0.045% NaCl in 0.02 M sodium phosphate buffer, pH 6.8 and one part of 30% ethanol. After extracting for one hour in the cold, the precipitate was removed by centrifugation in a M.S.E. refrigerated centrifuge at 20,000 r.p.m. and the supernatant extract stored in the deep freeze until further purification. Dr. I.G. Jones kindly provided the final extract purified by ammonium sulphate precipitation and chromatography on carboxy-methyl cellulose. (Jones and Beale, 1963) The pure immobilizing antigen was freeze dried and stored in dry form in the cold, the yield being 1mg pure immobilizing antigen per ml of packed cells, approximately 10% of total surface protein. Solutions were freshly made up in concentrations of 0.1 - 0.05 mg protein/ml distilled water or ammonium acetate - ammonium carborate buffer.

b. Antigen- ferritin-labelled - antibody complex.

Equal volumes of the same concentrations of purified antigen and specific ferritin-labelled γ globulin were freshly mixed and allowed to react for 30 minutes, giving a final concentration of approximately 0.1 mg antigen-antibody complex / ml final solution.

c. Ferritin-labelled globulin and unlabelled globulin.

These solutions were prepared to give a concentration of 0.1 - 0.5 mg

protein/ml.

d. Cilia and fragments of pellicle of paramecium.

Fixed paramecia were broken by drawing through a fine needle in a syringe and the resulting cilia and fragments examined by negative staining and platinum shadowing, using a drop of preparation on grids with a carbon and formvar film, the formvar giving added support to the fragments.

7. High resolution operation of the electron microscope for small particles.

The grids were examined in both a Siemens Elmiskop I and a Philips E.M. 6 at 60 kv, with a double condenser lens system with a condenser aperture of 250μ and care was taken with alignment and freedom from contamination. A small illuminating beam (10 - 20 μ) was used for the examination of negative stained preparations, as PTA sublimates in a broad beam and with a single condenser system (Brenner and Horne, 1959). 3.05 mm diameter copper grids of small mesh were used to give good support and therefore lessen the contamination of the microscope due to broken films. Such grids were 200 mesh, 85μ hole size; and 400 mesh, 40μ hole size, (obtained from Polaron Instruments, Ltd. Headstone Drive, Wealdstone, Middlesex) and also grids with circular holes of 30μ diameter, (obtained from Smethurst High Light Ltd., Sidcot Heaton, Bolton, Lancs.)

Astigmatism of the objective lens and apertures was corrected, using a holey carbon test film strengthened with a deposit of gold palladium, until the Fresnel fringe of a selected hole was symmetrical. This was corrected on both the scanning 100μ and 50μ diameter objective apertures and the clean 50μ aperture was used solely for photography. Accurate focus of

the specimen was obtained by focusing on the Fresnel fringe of a hole in the supporting carbon film, a through focus series of photographs being taken of each specimen. Photographs were taken at plate magnifications of from 20,000 - 60,000X and prints were generally enlarged 3X or 10X for easy comparison. Reversed prints were made of shadowed negatives to give dark shadows and occasionally also of negatively stained preparations (Huxley and Zubray, 1960b, 1961) to give a dark stained effect to the protein, both these methods of printing giving a more realistic effect for better interpretation of fine structure.

3. RESULTS

i. LIGHT MICROSCOPY - FLUORESCENT LABELLING

1. Fixation in 1% Osmic acid.

Paramecia fixed in 1% osmic acid were found to retain their antigenic activity, whereas fixation in 2% Formalin was found to destroy the specific properties of the immobilizing antigens.

2. Specificity of the labelling technique.

The specificity of the fluorescent-labelling technique was demonstrated with mating pairs of paramecia of two different serotypes (168G and 168D). When sections of these fixed pairs were treated with fluorescein-labelled anti-168G serum, only one member of the pair fluoresced brightly on the pellicle and cilia. (Plate 1).

3. Location of antigen.

However, at the same time, the internal cytoplasm of both the homologous (168G) and non-homologous (168D) organisms fluoresced to a lesser, but very appreciable, extent, though the region of the macronucleus and gullet remained non-fluorescent. (Plate 1). Fluorescence of the cytoplasm was avoided by pre-treating sections of the same mating pair with a heterologous, non-fluorescent serum (anti-192X) containing no antibodies against G or D immobilization antigens. The sections were then treated as before with the fluorescein labelled anti-168G serum, and now only the immobilization antigens on the

the pellicle and cilia of the homologous 168G organism were fluorescent, the non-homologous member of the pair appearing as a faint "ghost" due to blue autofluorescence, the interiors of both cells remaining dark, and non-fluorescent. (Plate 2).

These results showed the immobilization antigens to be located only on the pellicle and cilia of the animal and not in the internal cytoplasm. Other antigens are known to be present in the interior of paramecia as shown by (Finger, 1956) and (Preer and Preer, 1959) and as our labelled antisera were made against whole animals, fluorescent antibodies against these antigens, as well as against the immobilization antigens, would be present, resulting in the fluorescence of the cytoplasm as well as the surface. The effect of pre-treatment with the heterologous anti-192-X serum in preventing this internal fluorescence, suggests that the internal antigens are relatively invariant in paramecia with different immobilization antigens, (Finger, 1956). The anti-192-X serum therefore contained non-fluorescent antibodies which neutralized the internal antigens of type 168D and 168G, leaving the immobilization antigens able to react with the fluorescent specific immobilization antibodies.

Double staining of further sections of the same mating pairs with fluorescein-labelled anti-168G serum and rhodamine-labelled anti-168D γ globulin after pre-treatment with non-fluorescent heterologous antiserum, showed one member of each pair with green fluorescent pellicle and cilia, and the other with orange pellicle and cilia. (Plate 3). Double labelling with its specificity for two different antigenic types, could therefore be used to detect the presence of two different antigens in the same cell.

4. Transformation experiments.

The double staining technique was then used to study the transformation from one antigenic type to another. Animals of type 168G were placed at 35°C, a temperature at which type 168D is stable, as described in the methods section, and samples of animals were taken at intervals during the process of transformation to type 168D. Some animals from each sample were fixed and sectioned as before. These sections were either double stained with fluorescein-labelled anti-168G serum and rhodamine-labelled anti-168D γ globulin, or treated with fluorescein only, or rhodamine only, after pretreatment as above. Live animals from each sample were also tested for immobilization by unlabelled anti-168G serum, and others by anti-168D serum. The average times of immobilization, when present, were noted, as there is some variation in the rates of transformation of individual organisms. The results of these tests are shown in Table 1. It is seen from this Table that each transforming organism gradually produced increasing amounts of the "new" 168D antigen, and decreasing amounts of the "old" 168G antigen, and that the "new" antigen was found to appear first on the pellicle and subsequently on the cilia.

The orange fluorescent marking the 168D antigen was first just detected, on the pellicle, at stage 2 (after 2 - 3 hours at 35°C), whereas the time of immobilization of animals in anti-168G serum was unaltered and the animals were only faintly retarded in anti-168D serum.

At stage 3, (after 7 hours) the fluorescence of the pellicle was mainly orange, indicating predominantly 168D antigen, whereas the cilia still had the original green fluorescence indicating 168G antigen. The animals were immobilized in anti-168G serum almost as rapidly as at the start, and were only slightly retarded by the anti-168D serum.

At stage 4 (after 8 hours) the mainly green fluorescence of the cilia still indicated predominantly 168G antigen, though the orange fluorescence of the 168D complex was just beginning to show. At the same time the immobilization test with anti-168G serum gave the first definite indication of the appearance of the "new" antigen, although the increase in the immobilization time was relatively small, and this was consistent with the finding by the fluorescence method of the first indication of the new antigen on the cilia. Therefore the fluorescent labelling proved to be a more informative test, since it recognises antigen both on pellicle and cilia, whereas the immobilization test indicates antigen on cilia, and not on the pellicle.

Also it was interesting to note that at this stage, though the cilia still had a very high proportion of 168G antigen and the animals were immobilized in the anti-168G serum as noted above, when the animals were left in the immobilizing serum at room temperature they were found to have recovered after 24 hours. Previous to this stage, animals had been found to be dead the following day. Therefore it is presumed that the process of transformation continued even after the animals had been immobilized, and that the 168G antigen-antibody complex on the tips of the cilia was shed and replaced by 168D antigen, unless the preponderance of 168G antigen had been too large and death intervened. These findings conform with the findings of Beale (1958) that once transformation has begun, it carries through to completion, even though the conditions, in this case the lower temperature, were not those at which the new serotype is stable.

To bear this out further it was found that though animals placed in 168D antiserum at this stage were at first relatively unaffected, they

were eventually immobilized and found to be dead after 24 hours. On the other hand prior to this stage 4, animals placed in the 168D antiserum were relatively unaffected and had completely recovered after 24 hours. This does not fit the previous findings, unless the presence of the then mainly non-homologous 168D antiserum retarded the production of its own serotype when not much antigen had been produced.

It was interesting to note that the first fission of the animals after the beginning of the experiment also took place at this stage, but from concurrent fluorescent labelling of whole animals, no evidence was shown that there was production of new antigen on sites of new cilia formation. This was consistent with the results of Beale and Kacser (1957) that change of antigen was found to occur uniformly over the animal. Also, comparison of cilia at later stages of transformation by treatment of animals with single and double stains and by viewing under both U.V. radiation and visible light, indicated that a single cilium contained both antigens.

To return to the general study of the stages of transformation, at stage 5 (after 11 hours), the pellicle was seen to have only orange fluorescence and therefore to have 168D antigen, whereas the cilia contained roughly equal proportions of both antigens and therefore the immobilization in both antisera was found to be much the same. The study of the stages of transformation was continued and after about 18 - 20 hours, the animals were seen to fluoresce only orange, and they were rapidly immobilized in 168D antiserum and unaffected in 168G antiserum, indicating a complete transformation to 168D serotype.

From the experiments described it was concluded that the

immobilizing antigens were present on the pellicle and cilia of the animal and not in the internal cytoplasm, and that new antigen appeared first on the pellicle and later on the cilia. It was also concluded that non-immobilization antigens in the interior of the cell are relatively invariant in paramecia, whatever the immobilization antigen present.

However, the magnification obtained even with the high (X 100) oil immersion lens was insufficient to decide the exact location of the antigen on the pellicle and cilia and therefore it was decided to use the higher resolution of the electron microscope. This study forms the second part of this work.

Plate 1.

Section through conjugating pair of paramecia, antigenic types 168G and 168D, treated with fluorescein-conjugated anti-168G serum. Note fluorescent pellicle and cilia on conjugant at left (presumed 168G) and fluorescent cytoplasm in both conjugants. X 510.

Plate 2.

As in Plate 1. but pretreated with non-fluorescent anti-192X serum to prevent reaction of cytoplasmic antigens with fluorescent antibody. Only the immobilization antigen, on pellicle and cilia of 168G cell, now shows the green fluorescence of fluorescein. X1,280.

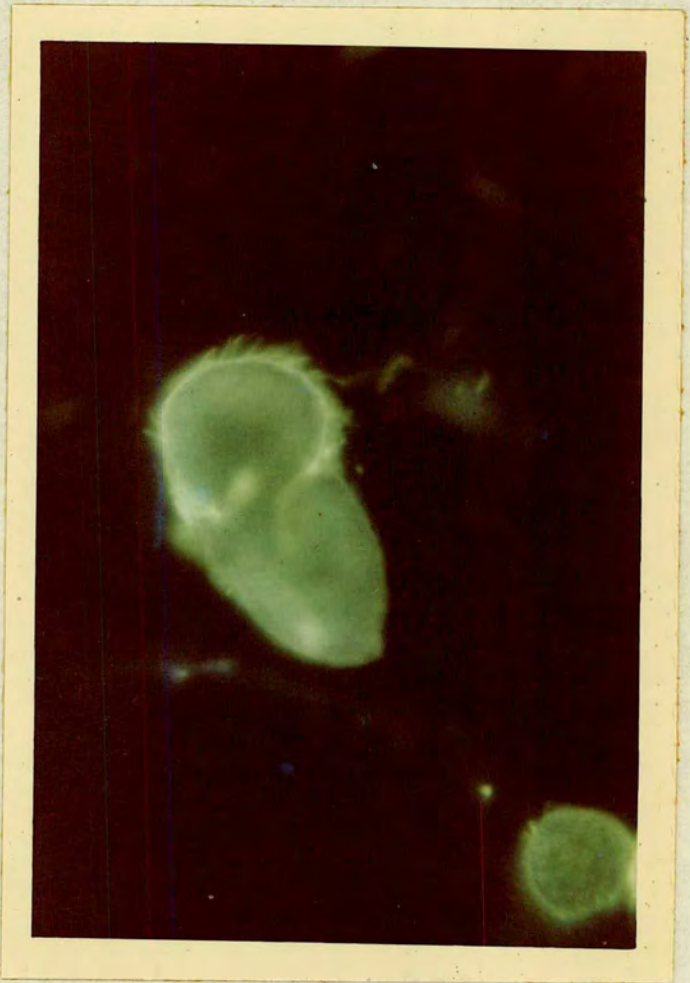


Plate 1.

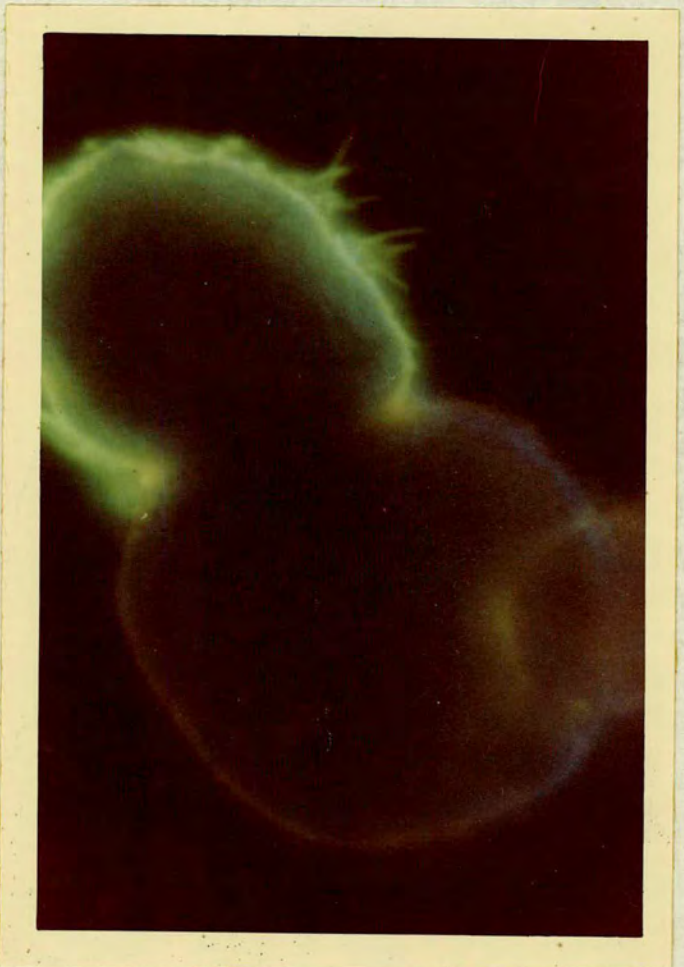
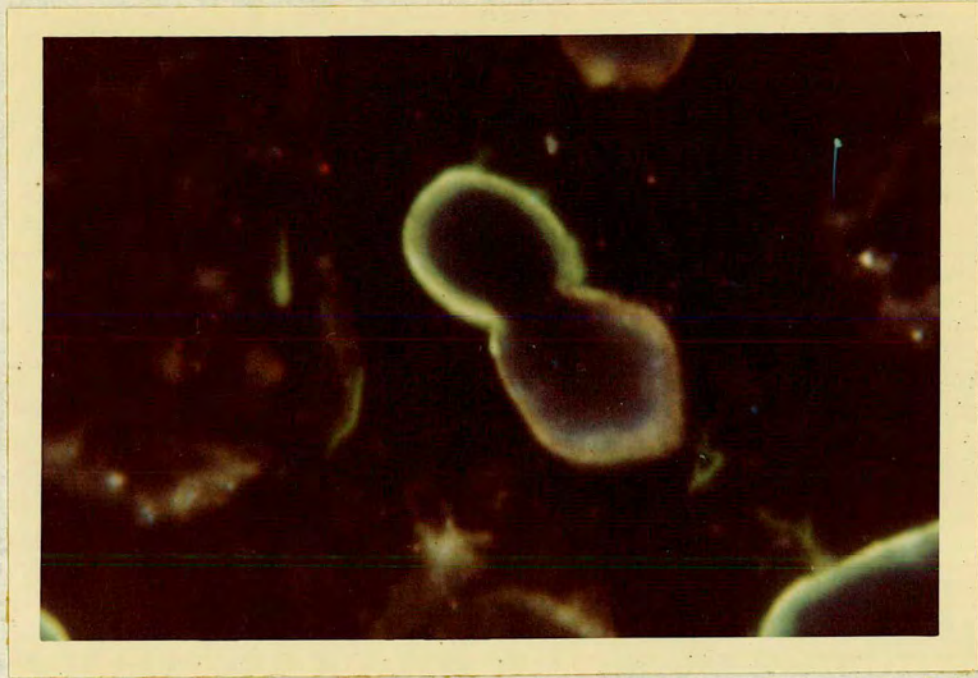


Plate 2.

Plate 3.

Section through pair of conjugants 168G and 168D, pretreated with non-fluorescent anti-192X serum, then with fluorescein-conjugated anti-168G serum and finally rhodamine - conjugated anti-168D γ globulin. X 510.



NO. 15700

Plate 3.

Plate 4.

Low power (X 6,700) section of Paramecium. On the cell surface, are cilia in longitudinal section, arising from the pellicle between the peribasal spaces, and above the surface are numerous cilia in cross section. In the cytoplasm adjacent to the surface are dark staining mitochondria and a single trichocyst, most trichocysts having been extruded on fixing live animals in O_3O_4 . In the lower half of the plate are two contractile vacuoles containing bacteria at various stages of digestion, and also part of the macronucleus. To the right is part of the gullet in section.

Plate 5.

Higher power (X 36,000) section of Paramecium. Numerous microsomes are seen in the cytoplasm.

KEY TO PLATES

- c = cilium
- cf = central fibrils
- cs = circumciliary space
- csc = cross section of cilium
- cv = contractile vacuole
- f = fold
- g = gullet
- h = homogeneous layer
- k = kinetosome
- kf = kinetodesmal fibres
- lsc = longitudinal section of cilium
- m = microsome
- mn = macronucleus
- mt = mitochondrion
- pbm = peribasal membrane
- pbs = peribasal space
- pf = peripheral fibrils
- plm = pellicular membrane
- ps = portion of parasomal sac
- s = septum
- t = trichocyst

when some sections were eventually obtained and treated with ferritin-labelled antibody, the ferritin was against deposited non-specifically, as with Araldite.

That ferritin labelling of the interior of the cell is feasible was shown by the fact that one animal on sectioning was found to have ferritin associated with structures in the cytoplasm, (plate 18). It was presumed that the animal had been accidentally broken after fixation and before treatment with ferritin-conjugated antibody. The antibody, being made against whole animals, as noted before, contained ferritin-labelled antibodies to all the antigens present in the cell, and therefore the ferritin-labelling could possibly be specific for the internal antigens, whatever they may be. No ferritin granules were seen in the cilium, and this may have been due to the septum cutting off the interior of the cilia from contact with the ferritin-labelled antibody.

distributed homogeneously over the surface of the cilia (plate 17), although possibly less dense than that on the cilia of similarly treated, untransformed animals. On the pellicle the ferritin granules appeared to be very sparse and present at small, isolated sites. The immobilization times of live animals at this stage were much the same in antisera to each of the antigens, indicating a fair amount of "new" 168D antigen already present on the cilia. These studies suggest that there is possibly a general overall lessening of the initial antigen, until it is demonstrated with homologous labelled antibody at only a few isolated sites, before finally ceasing to be present.

Attempts to demonstrate with the electron microscope the two antigens present on the same animal at this stage of the transformation, were, unfortunately, unsuccessful. Fixed animals when treated with antibodies to both antigens present, one antibody being labelled with ferritin, showed ferritin granules absorbed non-specifically over the entire surface of the pellicle and cilia, possibly due to incomplete washing off of the ferritin labelled globulin due to the large amount of globulin fuzz present. However it is hoped to improve this technique in the future.

Since the ferritin-labelling and counterstaining proved to be a specific and sensitive technique, it was hoped to use it to examine the interior of the cells for the possible presence of immobilizing antigens. Unfortunately, all attempts failed to label Araldite sections of paramecia with ferritin-labelled antibody, ferritin being deposited over the entire surface in accordance with the findings of Baxandall, Perlmann and Afzelius (1962, 1963), possibly by an electrostatic charge. Embedding the animals in the water-soluble polyepoxide Durcupan (X133/2097) according to Staubli (1960) and Leduc and Bernhard (1962), was also a failure, as the blocks would neither set nor section satisfactorily, and

An animal examined under the electron microscope after 7 hours at 35°C, showed that the antigen was distributed homogeneously over the pellicle, but was still appearing only at isolated sites on the cilia. (Plate 15). These last two stages are compared in Plate 16. At this stage the fluorescence technique was showing a very strong fluorescent pellicle and faintly fluorescent cilia, and the immobilization tests were just beginning to show some definite effect.

At the final stage (18 - 24 hours) by electron-microscopy, the fuzz and ferritin granules were seen to occur homogeneously over the entire surface of pellicle and cilia, giving the same picture as seen previously with a completely homologous animal, (Plate 10). Both the fluorescence and immobilization tests also showed complete change to the new antigenic type.

From these studies, it was therefore found that antigen possibly appeared initially at isolated sites on pellicle and proximal ends of the cilia, the number of sites gradually increasing until the antigen completely covered first the pellicle and later the cilia. Comparison of the three concurrent tests showed that the ferritin-labelling technique was seen to be much more sensitive than the fluorescent-labelling technique, both being more sensitive than the immobilization test which only reveals ciliary antigen and then only when present in fairly large amount.

The transformation experiment was again carried out, this time treating fixed, transforming animals with ferritin-labelled antibody to the "old" antigen going out.

After 9 hours growth at 35°C, ferritin granules were seen to be still

were studied with the use of the electron microscope and ferritin labelling, and also with the light microscope and fluorescent labelling. At the same time, live samples were tested for immobilization with antisera to the initial and final antigens. The results of these three concurrent series of tests are shown in Table II.

From the electron-microscope study, globulin fuzzi and ferritin granules were seen to appear initially at isolated sites on the pellicle and the proximal ends of the cilia. This test was extremely sensitive, the antigen being located initially after an interval of only one hour of growth at 35°C, (Plate 13). A structure was seen in this section which could possibly be the opening of the parasomal sac. This did not appear to have any particular connection with the ferritin granules to suggest any production of antigen from this point. At this same stage neither the fluorescence or the immobilization tests gave any indication of the appearance of the new antigens.

On examination under the electron microscope it was seen that the antigen sites continued to increase on both the pellicle and cilia, and were fairly numerous after three hours growth at 35°C. At this stage the fluorescence test was just giving faint indication of the appearance of the new antigen, on the pellicle only, and the immobilization tests still giving practically negative results.

After six hours' growth at 35°C, the electron-microscope method showed many sites of new antigen formation on both pellicle and cilia, and the fluorescence technique showed a fairly strong fluorescent pellicle and faintly fluorescent cilia, the immobilization tests now showing a very slight effect, (plate 14).

findings using the fluorescence technique, when animals treated with fluorescent antibodies to all the possible antigens in a cell, both immobilizing antigens and others, showed complete lack of fluorescence in the gullet area. (Beale and Kacser, 1957 and Plate 1). The gullet appeared to have the usual "3 - ply" pellicular membranes, made up of layers of apparently the same width and density as those present over the rest of the animal.

Therefore, as no other antigen appeared to be present on the surface, or at least was revealed by the technique used, the non-immobilization antigens studied by Finger (1957) and Preer and Preer (1959) would seem to be located in the interior of the cell, in agreement with the findings of Preer.

4. Transformation experiment.

The counterstaining of any absorbed globulin proved to be very useful in the study of transformation of the antigens, for when transforming animals were treated with ferritin-conjugated antibody to the "new" antigen being formed, the successive stages could be quickly scanned for the first appearance of the globulin fuzz. This was especially so in the early stages, when little specific antigen had then been formed, for the faint globulin fuzz of the antigen-antibody reaction was easier to pick up than a few sparsely separated ferritin granules.

Live animals were exposed to the higher temperature environment favouring the formation of the new antigen 168D. Samples of animals were taken at intervals and their antigens studied by three methods. Fixed animals

quick scanning of preparations, whereas the ferritin granules would be undetected at these low magnifications. Sections were therefore generally counterstained and then examined in the electron microscope, both for ease of detection of the antigen, and for increase in the structural detail of the animal.

3. Location of the Antigen.

By examination of many electron micrographs of the surface of paramecia by the above techniques, the immobilization antigen was found to be present over the surface of the pellicle and cilia. The entire lack of globulin fuzz on the non-homologous animals showed not only that there was no specific antigen-antibody reaction, but also that pretreatment with heterologous antibody, as used in the fluorescent antibody technique with internal antigens as described in Results I part 2, was unnecessary for the study of the surface antigens. Any non-specific antigen-antibody reaction resulting from this pretreatment would have resulted in the staining of any globulin absorbed. (Plates 10 and 11).

From these studies the surface antigens of paramecia appear to be immobilization antigens only, which were found to be present over the entire external surface of the animal, with the exception of the gullet area. Here the pellicle and cilia were found to have no fuzz or ferritin granules when the animal was treated with ferritin-labelled antibody, homologous to the immobilization antigen on the rest of the animal. (Plate 12). It is concluded therefore that the gullet area has no immobilization antigens, and in fact, no antigens at all, for pretreatment with heterologous antiserum, which presumably contained antibodies to the relatively invariant non-immobilization antigens common to all serotypes, gave no absorbed globulin fuzz. This agreed with the

granules were seen on the pellicle and cilia of homologous animals only, (Plate 7) none at all being present on the non-homologous animals. This established the excellent specificity of the test. The distance of the ferritin granules from the surface membrane was seen to vary considerably, possibly due to the plane of section and to the varying positions of the antibody molecules.

Resolution of the ferritin granules (diameter 55^oA) was poor with the Philips E.M. 75, therefore it was necessary to examine the sections under the higher resolution Siemens microscope.

As mentioned earlier, the contrast of embedded material in Araldite is very low, resulting in poor structural detail of the organisms, particularly of the membranes, and therefore some sections were treated with the electron-dense counterstain of uranyl-acetate and potassium permanganate. It was of great interest to find that not only was the structural detail of the animals excellent, especially of the outer membranes, but that the counterstain also revealed the absorbed globulin of the specific antibody as a thick fuzz which covered the pellicle and cilia of homologous animals. In this fuzz could be seen the conjugated ferritin granules, the apoferritin protein shells of which also stain by this method, giving a slightly larger granule size compared to unstained sections. No such fuzz was present in non-homologous animals (Plates 8 and 9).

The complete lack of ferritin granules and globulin fuzz on the control animals established the specificity of the techniques of ferritin labelling and of counter-staining for demonstrating the presence of antigen. Moreover, the globulin fuzz could be easily detected at low magnifications of the electron microscope, and therefore was of use for

the usual arrangement of nine double peripheral fibrils with two single central fibrils (Metz, Pitelka and Westfall, 1953; Fawcett and Porter, 1954; Gibbons and Grimstone, 1960) of approximately 150A to 200A diameter. Nine secondary fibrils could sometimes also be seen, but only in cross sections. The peripheral fibrils were seen to pass through the transverse septum of each cilium and terminate below in the cytoplasm in the kinetosome, the central fibrils of the cilium however, stopping short a little above the septum, (see Plate 12 for cross sections of cilia and kinetosomes.) The cilia were seen to taper at the tips, with eventual lack of fibrillar structure, but it was difficult to decide when an actual tip was being sectioned. Microsomes (Palade and Siekevitz, 1956 a and b) were seen to occur thickly throughout the cytoplasm of the interior of the cell and also in the cilia. (Plate 6).

The significance of the various structures as seen from this electron microscopy study will be discussed later in connection with the probable site of synthesis of the immobilization antigens.

2. Demonstration of specificity of the labelling technique.

The osmic acid fixation used did not result in loss of antigenic activity, in agreement with the findings of Spendlove and Singer (1961) with a variety of antigens.

Fixed whole animals of two different serotypes, 168G and 168D, were separately treated with ferritin-labelled anti-168D γ globulin, after pretreatment with heterologous, unlabelled antiserum, as described in the Methods section, to block any non-specific antigens which might be present, as previously with the fluorescent antibody technique. After embedding in Araldite and sectioning as described in the Methods section, and then viewing the sections unstained under the electron microscope, ferritin

corpuscles alternate with the trichocysts. These various structures under the electron microscope make up the characteristic silver-line system of light microscopy, (Pitelka, 1963).

The size of the Paramecium, $150\mu \times 50\mu$, made it possible to examine the general structure under low power microscopy (at approximately X2,000 - X12,000 magnification) (Plates 4 and 5) and then to study the fine structure of the cilia and the external membranes in considerable detail (at approximately X30,000 magnification). The outer membranes of the paramecium were seen to consist of two distinct membranes, as previously noted (Stewart and Muir, 1963), (Plate 6). The outermost pellicular membrane was seen to be continuous over the entire surface of the pellicle and cilia, whereas the inner peribasal membrane formed a continuous lining around the peribasal space, (Sedar and Porter, 1955; Ehret and Powers, 1959). Over the peribasal space these two membranes were double, separated by various widths, the two together generally making a double membrane of approximately 250A width. Both these membranes were seen to consist of the classic unit membrane, the "3 - ply" sheet (Robertson, 1960) of approximately 80A width, made up of two dark-staining outer layers each about 20A thick, separated by a clear layer about 40A wide. No pores were seen in these membranes, but there was frequent overlap of the membrane layers.

Below the peribasal membrane, a dark-staining, thick, homogeneous layer or membrane was seen, approximately 100A wide, noted previously Pitelka (1961), in Tetrahymena. This appeared to be continuous with a septum across the base of the cilia. Openings of the parasomal sac connecting the interior cytoplasm to the exterior, can be seen in Plate 10, and Plate 13.

The cross-sections of the cilia of diameter approximately 0.27μ show

II. ELECTRON MICROSCOPY - FERRITIN LABELLING AND
COUNTERSTAINING OF ABSORBED GLOBULIN

1. Ultrastructure of Paramecium.

The fine structure of the paramecium was found to be very well preserved for electron microscope studies in the epoxy resin, Araldite (Glauert and Glauert, 1958) which gives little polymerization damage on hardening and sections of which are not degraded in the electron beam. This applied especially to the outer membranes, as previously noted by Stewart and Muir (1963), as there is little polymerization damage on hardening. A methacrylate embedding medium (Borysko, 1956) was also tried, (85% methyl methacrylate; 15% butyl methacrylate) but the structural detail was not so good due to the polymerization damage caused by shrinkage on hardening, (Glauert and Glauert, 1958). Counterstaining of the Araldite sections was necessary however, to bring up the fine detail of the membranes, etc., as the contrast of embedded material in Araldite is very low. Uranyl acetate and potassium permanganate, as described in the methods section, proved to be an excellent counterstain. Lead acetate was also tried, but was not successful.

The following details of the general ultrastructure of the paramecium were noted. The ciliary "corpuscle" in the terminology of Ehret and Powers (1959) consists of the cilium, arising from the kinetosome, from the centre of the pit of the circumciliary space, the walls of which enclose the peribasal space. The parasomal sac connects the internal cytoplasm of the animal with the exterior, (Figure 1). These ciliary

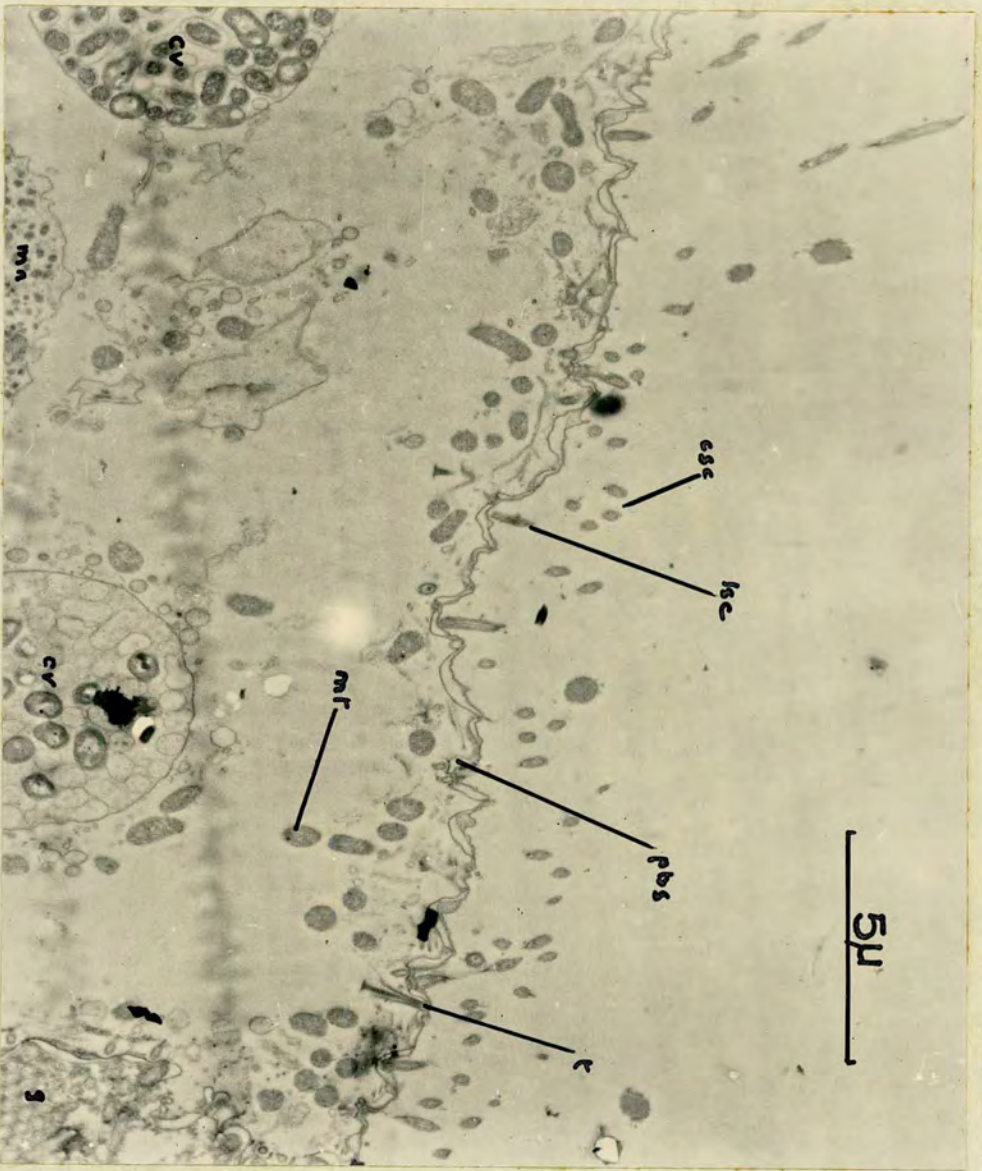
Table 1. Stages during transformation of Paramecium aurelia from antigenic type 168G to type 168D by growth at 35°

Stage	Time of growth at 35° (hr.)	Effect of antisera (dil. 1/50) on live organisms				Fluorescence following treatment with antibody conjugates (green, 168G; orange, 168D)	
		168G antiserum		168D antiserum			
		Immobilization time (min.)	Effect after 24 hr. at 20°	Immobilization time (min.)	Effect after 24 hr. at 20°	Pellicle	Cilia
1.	0 - 2	3	Dead	Unaffected	Alive	Green	Green
2.	2 - 3	3	Dead	R	Alive	Green (+ slight orange *)	Green
3.	7	4	Dead	R	Alive	Orange (+ slight green)	Green
→ 4.	8	* 6	Alive	R	Dead	Mainly orange	Green (+ slight orange)
5.	11	35	Alive	45	Dead	Orange	Green and orange
6.	12	R	Alive	20	Dead	Orange	Mainly orange
7.	13 - 16	R	Alive	3	Dead	Orange	Orange (+ slight green)
8.	18 - 20	Unaffected	Alive	3	Dead	Orange	Orange

→ indicates approximate time of first fission after beginning of experiment

R indicates retarded but not completely immobilized after 2 hr. in serum

* indicates first definite detection of "new" antigen by each method



45.

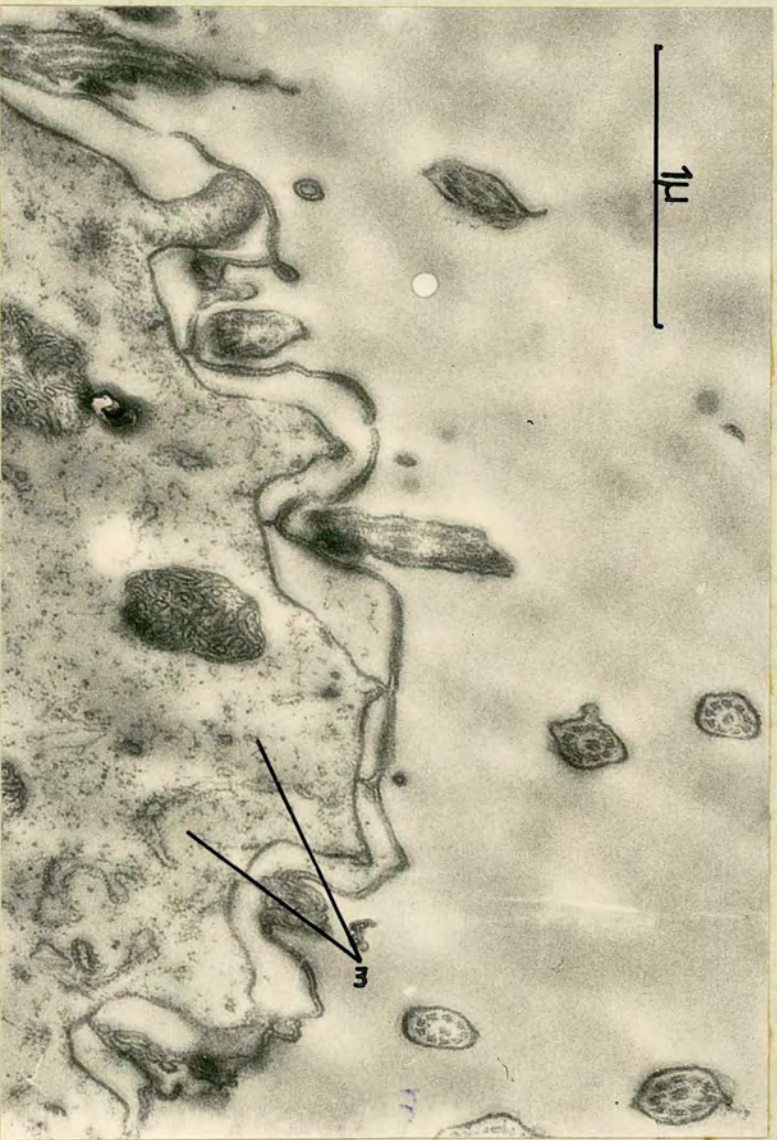


Figure 1.

Stereogram of the cortex of Paramecium, showing the ciliary corpuscles of Ehret and Powers. Each pair of cilia rises from the centre of a pit, formed by the pair of inflated peribasal spaces which are shown in section at the right. The Parasomal sacs are shown connecting the interior of the animal to the exterior, through the peribasal space. Below the cortex, in the interior of the animal, are seen the resting trichocysts alternating with the ciliary corpuscles, the kinetosomes from which the cilia arise, and also the kinetodesmal fibres.

(From Pitelka, 1963, from Ehret and Powers, 1959)

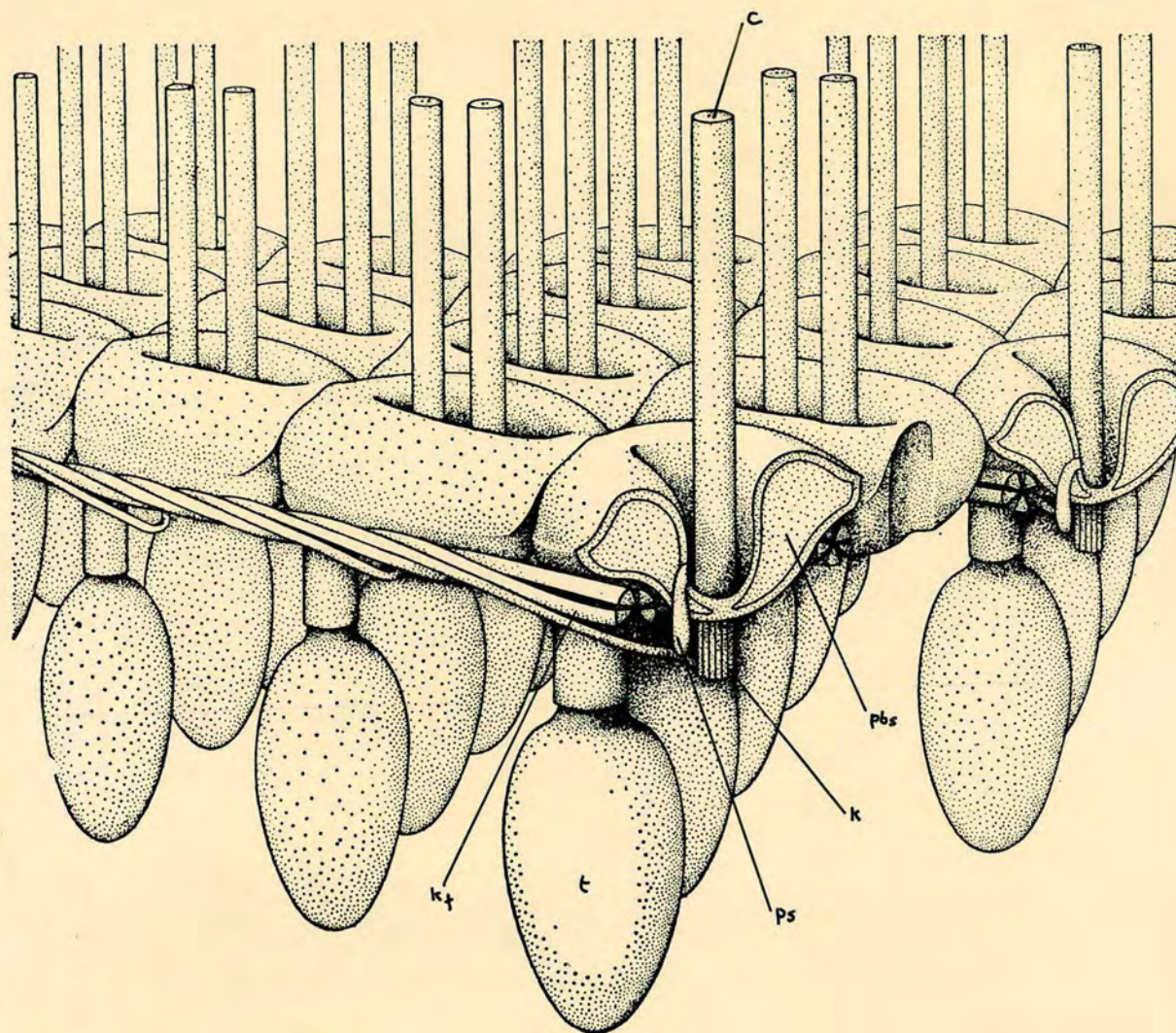


Figure 1.

Plate 6.

High power (X 108,000) section of a single cilium of Paramecium.

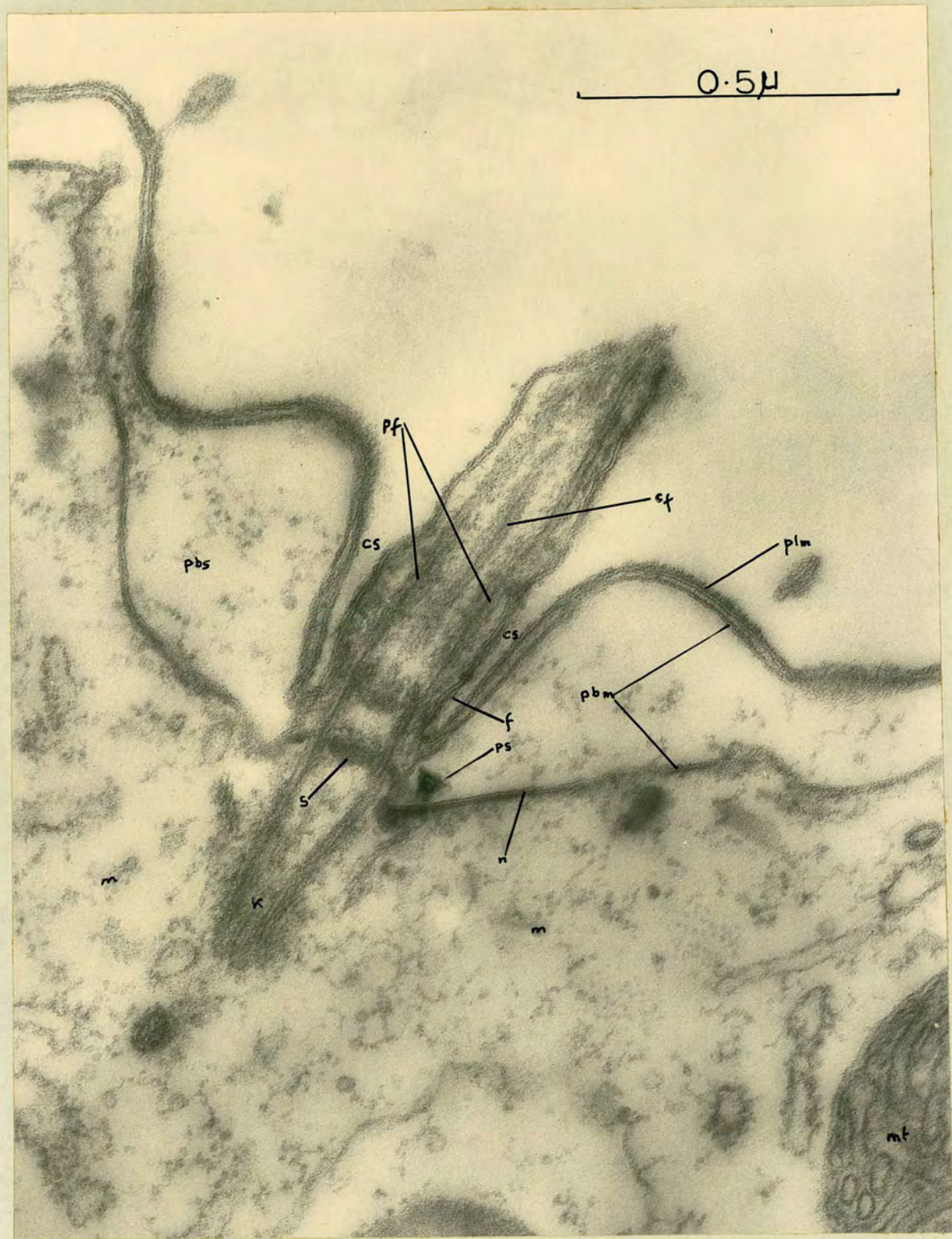


Plate 6.

Plate 7.

Unstained section of body wall of *Paramecium*, showing ferritin granules (arrow) in pellicle and cilia after treatment with ferritin-labelled homologous antibody. The cytoplasm is seen at the bottom left of the plate, with the pellicular membrane and cross sections of several cilia, above. Note the poor structural detail in this unstained section.



Plate 7.

Plate 8.

Counterstained section of pellicle of homologous animal treated with ferritin-labelled antibody, showing two "3-ply" membranes, plus globulin fuzz and ferritin granules (arrow).

Plate 9.

Counterstained section of pellicle of non-homologous control animal treated with ferritin-labelled antibody, showing two "3-ply" membranes, and no globulin fuzz or ferritin granules.



Plate 8.

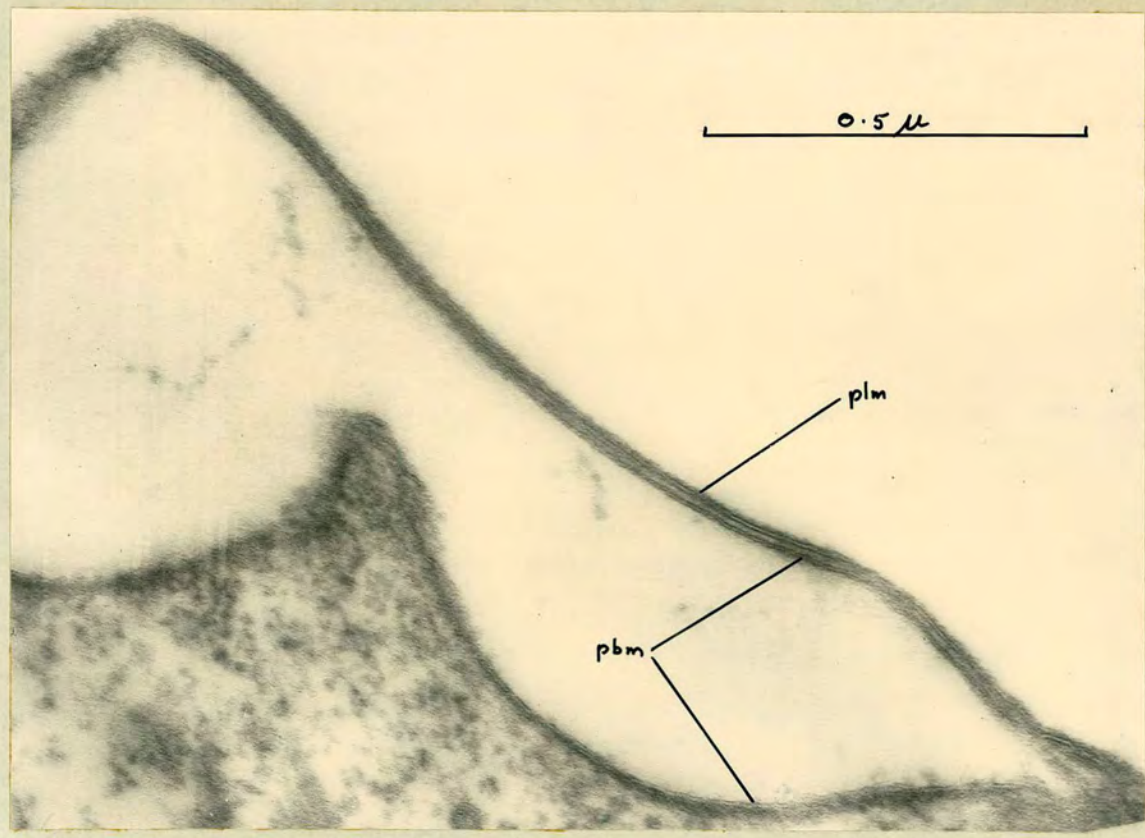


Plate 9.

Plate 10.

Counterstained section of a cilium of homologous animal treated with ferritin-labelled antibody, showing a single "3-ply" membrane around cilium, and double "3-ply" membrane over pellicle, plus globulin fuzz and ferritin granules (arrow).

Plate 11.

Counterstained section of a cilium of a non-homologous control animal treated with ferritin-labelled antibody, showing a single "3-ply" membrane around cilium, and double "3-ply" membrane over pellicle, and no fuzz or ferritin granules.

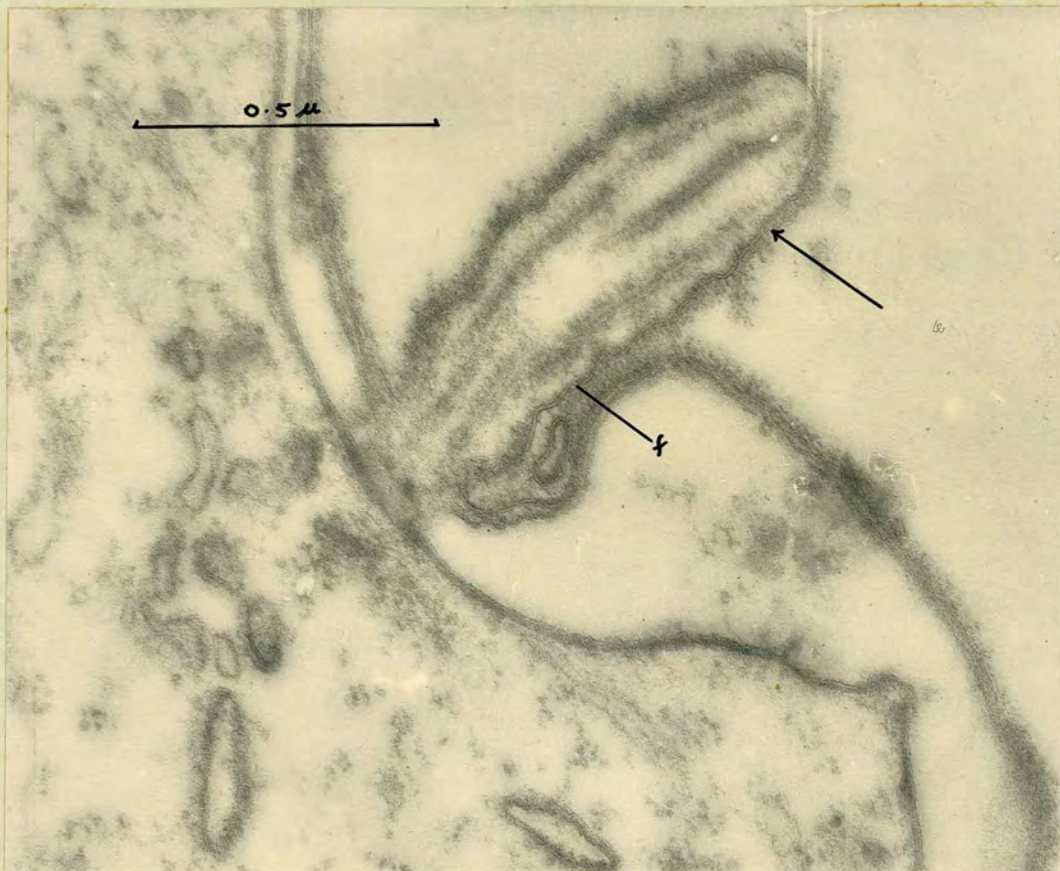


Plate 10.



Plate 11.

Plate 12.

Counterstained section of gullet area of an homologous animal treated with ferritin-labelled antibody, showing the usual "3-ply" membranes and no globulin fuzz or ferriting granules in this area. Cross sections of cilia and kinetosomes are seen.

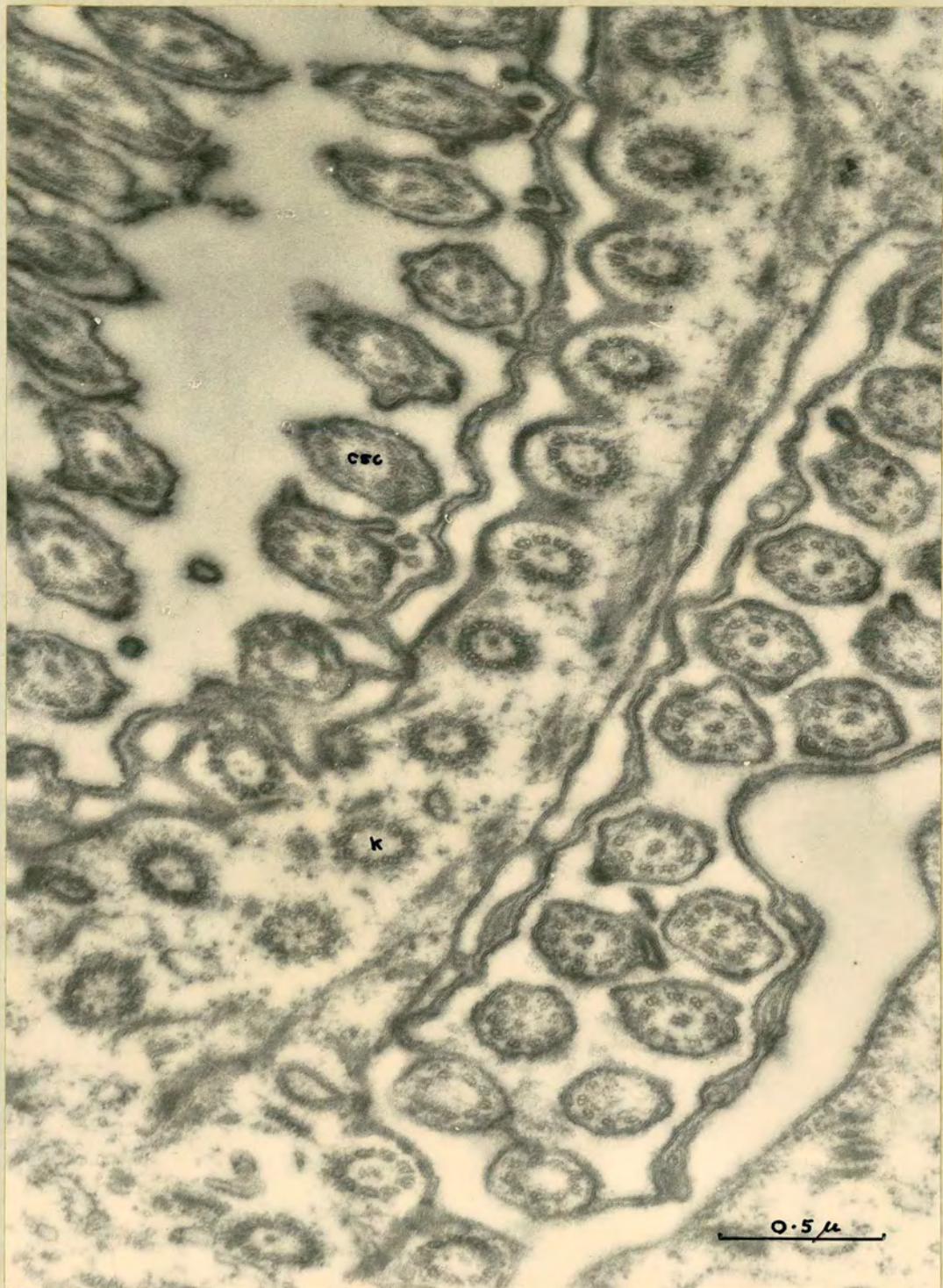


Plate 12.



Plate 13.

Counterstained section of pellicle and cilia of a paramecium, treated with ferritin-labelled antibody to the "new" antigen, after 1 hour of growth at 35°C, showing very few isolated sites of ferritin granules and faint globulin fuzz on pellicle (double arrow) and cilia (arrow). The possible opening of the parasomal sac is seen below the central cilium.

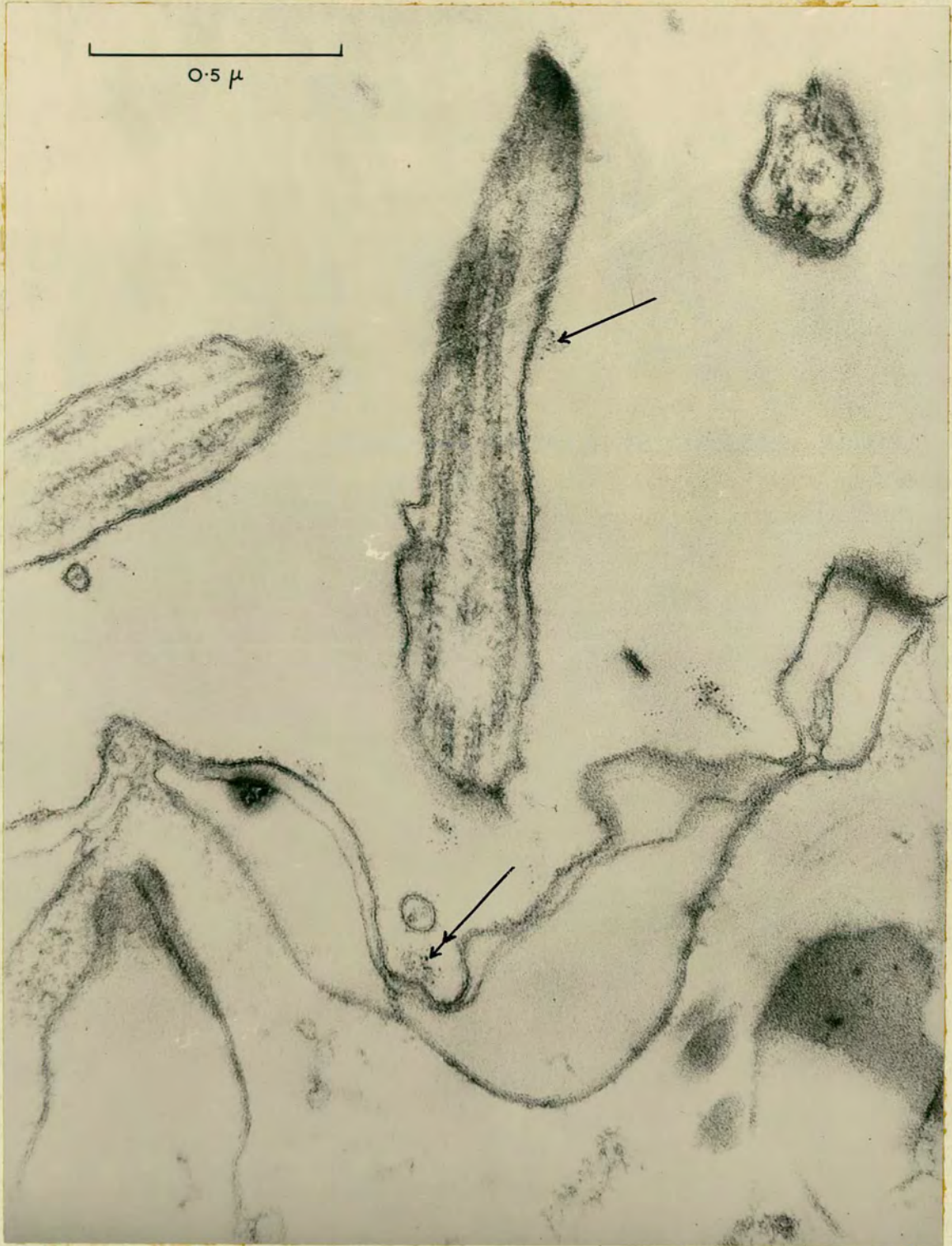


Plate 13.

Plate 14.

Counterstained section of pellicle and cilia of a paramecium, treated with ferritin-labelled antibody to the "new" antigen, after 6 hours growth at 35°C. The globulin fuzz and ferritin granules can be seen at a large number of separate sites on the pellicle (double arrow) and also in the longitudinal and cross sections of the cilia (arrow).

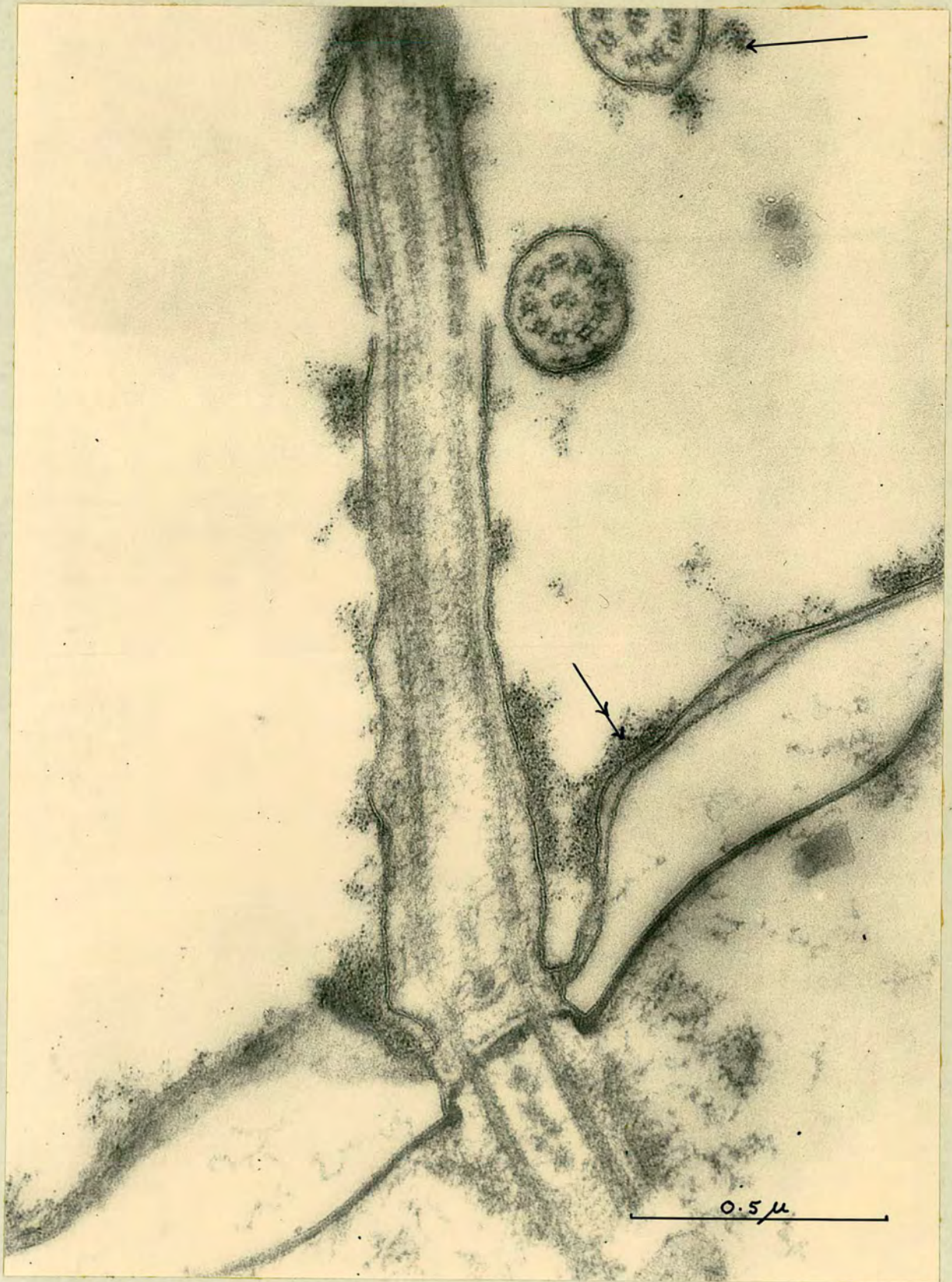


Plate 14.

Plate 15.

Counterstained section of pellicle and cilium of a paramecium treated with ferritin-labelled antibody to the "new" antigen, after 7 hours growth at 35°C. The globulin fuzz and ferritin granules are seen to cover the entire pellicle (double arrow) but are still in isolated sites on the cilium (arrow).



Plate 15.

Plate 16.

Comparison of paramecia:

- a. After 6 hours growth at 35°C
- b. After 7 hours growth at 35°C

The ferritin granules and globulin fuzz are still at isolated sites along the length of both cilia, and on the pellicle of 6 hour animal (16a), but cover entire surface of pellicle of 7 hour animal (16b).



Plate 16a.

plate 16b.

Table 2. Stages during transformation of Paramecium aurelia from antigenic type 168G to type 168D.

by growth at 35°, showing comparisons between the three methods for detecting antigen

Stage	Time of growth at 35° (hr.)	Effect of antisera (dil. 1/50) on live organisms		Fluorescence following treatment with fluorescein-labelled anti-168D serum		Ferritin granules and globulin fuzz following treatment with ferritin-labelled anti-168D γ globulin	
		Anti-168G immobilization time (min.)	Anti-168D immobilization time (min.)	Pellicle.	Cilia	Pellicle	Cilia
0	0	4	Unaffected	Nil	Nil	Nil	Nil
1	1 - 2	4	Unaffected	Nil	Nil	* Granules and fuzz at a few isolated sites	Granules and fuzz at a few isolated sites
2	3 - 5	4	R	* Faint green	Nil	Sites more numerous	Sites becoming more numerous
3	6	5	R	Green	Faint green	Sites very numerous	Sites becoming more numerous
4	7 - 8	* 7	R	Green	Green	Granules and fuzz cover entire surface	Sites becoming more numerous
5	9	15	50	Green	Green	Granules and fuzz cover entire surface	Sites very numerous
6	18 - 20	Unaffected	4	Green	Green	Granules and fuzz cover entire surface	Granules and fuzz cover entire surface

R indicates retarded but not completely immobilized after 2 hr. in serum

* indicates first definite detection of "new" antigen by each method

Plate 17.

Section of pellicle and cilium of a paramecium, treated with ferritin-labelled antibody to the "old" antigen going out, after 9 hours growth at 35°C. The ferritin granules appear to be distributed in a rather thin layer over the entire surface of the cilium (arrow), whereas they are present sparsely scattered in isolated sites on the pellicle (double arrow).

60,000X



Plate 17.

Plate 18.

Unstained section of paramecium, after 2 hours growth at 35°, treated with ferritin labelled antibody to the "new" antigen (and also to all the antigens of the cell).

Heavy ferritin labelling (arrow) is shown on structures in the internal cytoplasm, denoting the location of the internal antigens.

Sparse ferritin labelling (double arrow) on the surface marks the location of the "new" antigen after 2 hours at the raised temperature. Note no ferritin granules in the interior of the cilia, to which labelled antibody probably did not gain access.



Plate 18.

III. Structure of molecules of immobilization antigen as revealed by the electron microscope.

1. Negative staining of antigen molecules.

The antigen molecules were found to be well preserved in phosphotungstic acid to give a mosaic of molecules easily recognised in the electron microscope. This mosaic is shown in a low power photograph (Plate 19) of a preparation of 0.1 mg antigen/ml 1% PTA, applied with a platinum loop to a carbon coated grid from which the formvar had been dissolved.

At higher magnification (Plate 20) the molecules are seen to be four, five or six sided and of a diameter of 150 - 250⁰A. The smaller diameters and irregular shapes may be due to some molecules being more deeply embedded in the negative stain, or may be a different orientation of the molecules. Some internal structure and arrangement of subunits is also indicated as the PTA has penetrated cavities in the molecule. The subunits appear to be arranged in a regular manner generally with an outer ring of possibly five or six subunits, with a central subunit, all in light contrast to the electron dense PTA penetrating the molecule. Plate 21 is a reversed print of Plate 20, so that the protein appears positively stained, to give a more realistic effect for the interpretation of fine structure. Both these plates suggest a symmetrical three dimensional arrangement of the subunits, possibly in the form of a regular polyhedron, an arrangement seen in many particles in negative stained preparations, particularly the viruses (Horne and Wildy, 1961).

The occasional four sided molecule and those which possibly show a central cavity, suggest a prism shape, similar to that seen in the capsomeres

of viruses (Horne and Wildy, 1961) and in molecules such as hemocyanin, (Van Bruggen, Schuiten and Wiebenga, 1963).

Controls of PTA and polystyrene latex particles alone on carbon films with no antigen present, showed no such mosaic. However, too thick carbon films, giving a definite brown colour with the porcelain plate oil drop method of Bradley, resulted in a definite background of carbon particles noticeable in the electron microscope, and obscuring detail in negative stained preparations.

Solutions of antigen in distilled water kept frozen for any length of time, gave a mosaic with blurred, amorphous outlines when later examined with negative stain, indicating possible aggregation of protein molecules. Denaturation could possibly also occur although frozen solutions of antigens retain their antigenic properties fairly well.

2. Platinum shadowing of antigen molecules.

Plates 22 and 23 show reversed prints of photographs of platinum shadowed preparations of 0.1 mg antigen/ml distilled water (applied with a platinum loop to a carbon coated grid from which the formvar had been dissolved). Plate 22 shows a shadow-to-height ratio of 4:1 and plate 23 a ratio of 2.4:1. These ratios were obtained by measuring the shadow-to-height ratios of polystyrene particles present in the same field. The diameter of the molecules obtained from this ratio appeared to be $70 - 100\overset{\circ}{\text{A}}$, much smaller than the diameter obtained with negative stained preparations, and this diameter does not allow for the cap of metal which can be $50\overset{\circ}{\text{A}}$ (Hall, McLean and Tessman, 1959). The molecules are possibly

flattened during drying on the mica (Anderson, 1962) whereas with negative staining the PTA enters the cavities of the molecule and supports it (Anderson, 1962). The molecules measured at right angles to the direction of shadowing, not a very reliable method, gave a molecular diameter of 160 - 180A, which even allowing for a cap of metal, further supports the probability of collapse and flattening of the molecules in drying.

Solutions of antigen made up in the volatile ammonium carbonate - ammonium acetate buffer to preserve a neutral pH on drying and so prevent possible denaturisation of the protein (Hall and Doty, 1957; Hall and Litt, 1958) unfortunately gave an amorphous effect when shadowed. This effect, may have been due to the use of a frozen antigen solution, for ~~when~~ solutions of antigen in distilled water after storage in the deep freeze, gave amorphous lumps on platinum shadowing, suggesting aggregation of the molecules as with negative stained preparations.

Some specimens for shadowing were placed on formvar and carbon films. These were found to be unsatisfactory, due to the fact that the films sometimes tended to drift, though stabilized with the carbon, and also because the surface of the formvar was found to be too rough, which was especially obvious with a small shadowing angle. This resulted in a background with the appearance of a beach at low tide, obscuring the details of objects placed upon it.

Later preparations made by spraying the antigen solution on freshly cleaned mica, gave a smooth background and better dispersion of the antigen molecules as seen in Plate 24. Controls of freshly cleaned mica

shadowed at angles of $22^{\circ} 30'$ (approximately 2:1) and $11^{\circ} 15'$ (5:1) gave a smooth surface of platinum granules.

The external structure of the molecules unfortunately was obscured by the platinum granules (Plate 23), but the general effect does not contradict the structures suggested by negative staining. Although platinum is stated to give a fine grain, and a resolution of better than 50A (Bradley 1961) a carbon-platinum rod has been obtained (from Edwards High Vacuum, Ltd. Manor Royal, Crawley) with which it is claimed a finer grain deposit of metal and carbon can be obtained. It is hoped to obtain further detail of the external structure by shadowing by this method.

3. Negative staining of ferritin-labelled antibody - antigen complex.

Negative staining showed the complex to be a triad of the three molecules, antigen, antibody-globulin, and ferritin, as shown in Plates 25 and 26, which are micrographs of a preparation of 0.05 mg protein/ml phosphotungstic acid applied with a platinum loop to a carbon coated grid. The measured diameters show the antigen molecule to have a diameter of approximately 125A, and the ferritin molecule to have a diameter of approximately 110A with its characteristic electron dense central core, in accordance with the findings with negative stained preparations of Valentine (1959), van Bruggen, Wiebenga and Gruber (1960), Morgan, Rifkind and Rose (1962) and others. The globulin molecule was found to be a spherical molecule of rather less dense appearance and a diameter of approximately 80A. This spherical appearance agrees with that of globulin negatively stained by Valentine (1959), who shows a spherical globulin molecules of 70A diameter attached specifically to the

surface of a rod-shaped virus. However, globulin is frequently shown in negatively stained preparations as a thread-like molecule of approximately $250\text{A} \times 20\text{A}$, attached by active sites at both ends to antigenic sites on virus particles. (Anderson, 1962; Lafferty and Oertelis, 1963).

Only one antibody molecule appears to combine with one antigen molecule, but the presence of unconjugated globulin and ferritin in the preparation tends to obscure the interpretation of the micrographs. Complexes made with pure antigen and pure ferritin - conjugated antibody will be used in future.

4. Platinum shadowing of ferritin labelled antibody - antigen complex.

As with the platinum shadowing of the pure antigen, the shadowing of the complex was not very successful. Plate 27 is a micrograph of a preparation of 0.05 mg protein in ammonium-acetate-carbonate buffer, applied with a platinum loop to a carbon coated grid. Individual ferritin and globulin molecules can be seen and some combinations of molecules, but these are very difficult to resolve. As with the negatively stained preparations, purifying the solutions would remove extraneous, unbound molecules. A finer grain shadowing material and a shadow-to-height ratio of less than 4:1 would lessen the distracting background.

5. Negative staining and platinum shadowing of ferritin-labelled antibody, and of pure ferritin.

Negative staining of a solution of a ferritin labelled globulin of 0.05 mg protein/ml distilled water showed a 1:1 ratio of ferritin molecules to globulin (Plate 28) in accordance with the predictions for the Singer (1959) diisocyanate conjugation method used (Spendlove and Singer, 1961).

Platinum shadowing of the same solution of ferritin labelled globulin (Plate 29a) showed the same combination of the two molecules, but also showed a large amount of unlabelled globulin, which appeared not to be homogeneous, varying from approximately spherical to elongated forms, in accordance with the findings of Hall, Nisonoff and Slayter (1959) with shadowed globulin molecules.

A pure solution of ferritin, however, gave excellent shadowed preparations (Plate 29b). A solution of 0.05 mg of protein/ml distilled water was shadowed on a carbon film on a grid at a shadow-to-height ratio of 2:1. From measurement of the shadow length, the ferritin molecule was found to have a shadowed diameter of approximately 160A, which, when corrected for the cap of metal of from 50 - 60A, gave a molecule diameter of 100 - 110A, the known diameter for ferritin.

6. Negatively stained and shadowed fragments and cilia of Paramecium.

Attempts at using these two techniques to determine fine surface structure on the cilia and pellicle have so far been unsuccessful. A negatively stained preparation of fragments of pellicle is shown in Plate 26. Some contours of the surface can be seen, but it is mainly of interest to show the ciliary - kinetosome junctions of single and paired cilia, the positions of the fibrils through the septum, and the large parasomal sac openings.

Plate 19.

Antigen molecules embedded in phosphotungstic acid at low magnification. A general mosaic of molecules can be seen, with some indication of internal structure.

90,000X

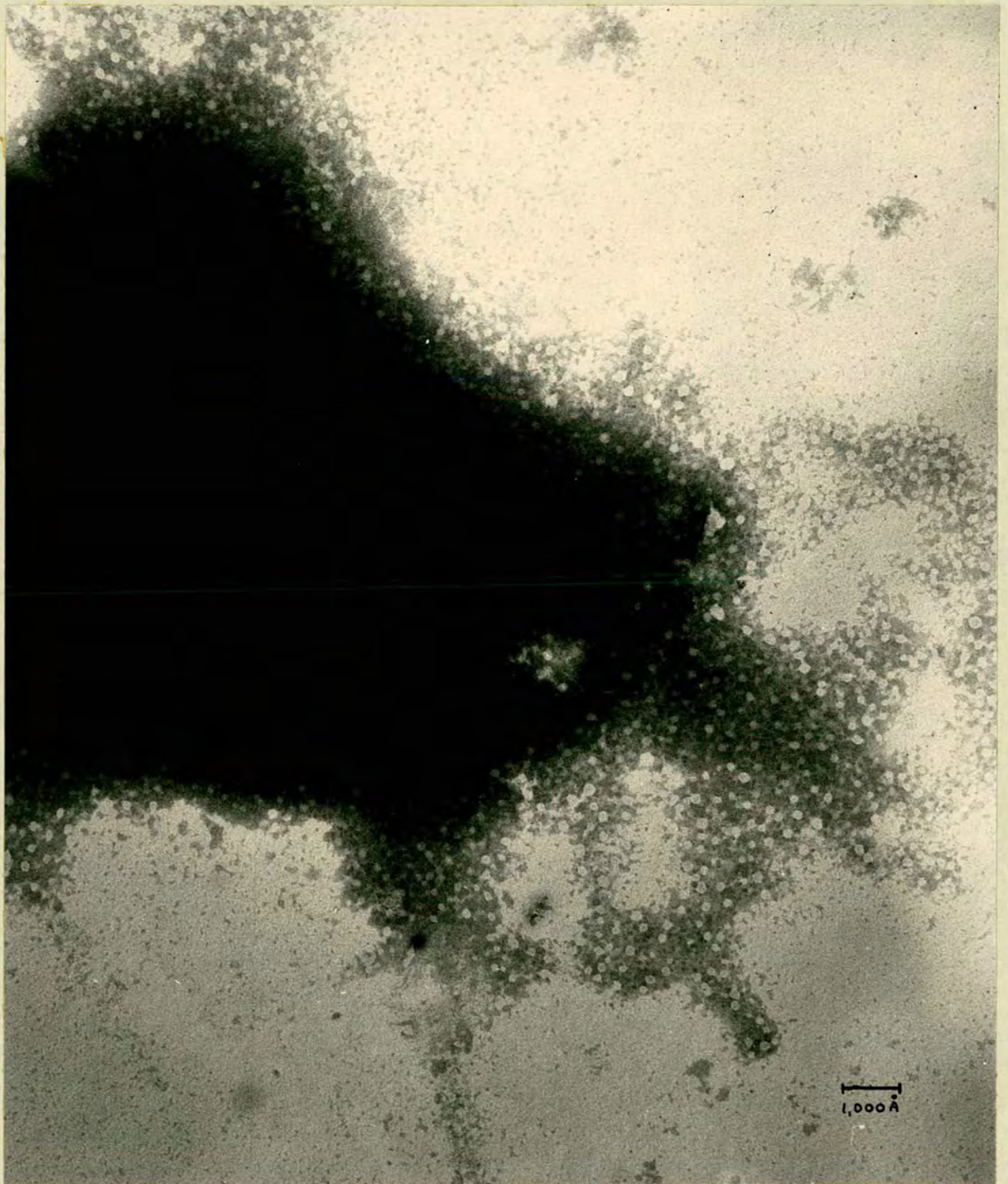


Plate 19.

Plate 20.

Antigen molecules embedded in phosphotungstic acid at higher magnification. The molecules can be seen to vary in size and shape, but many 5 or 6 sided particles can be seen. Some indication of structural subunits can also be noted.

300,000X

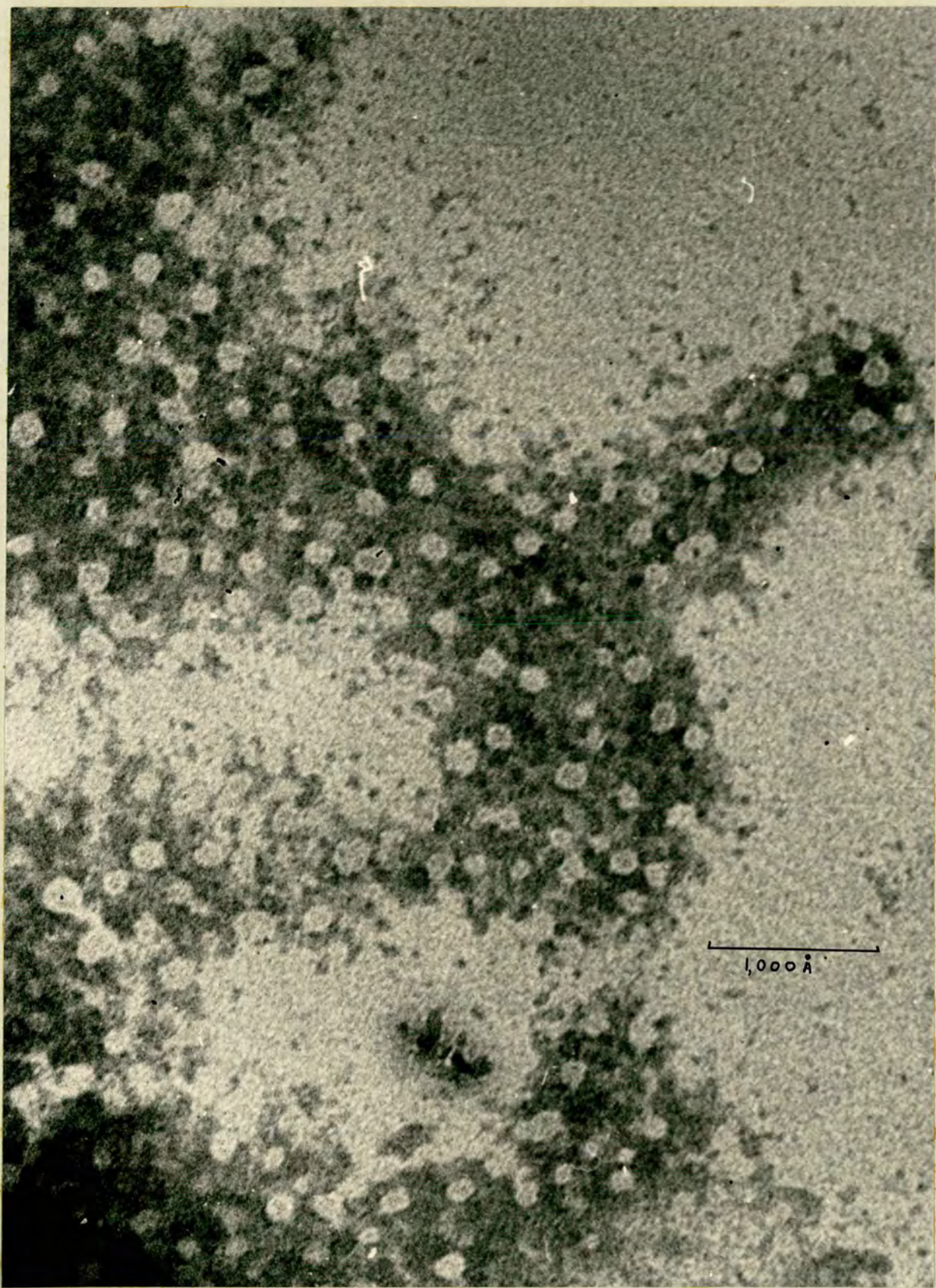


Plate 20.

Plate 21.

Reversed print of Plate 20.

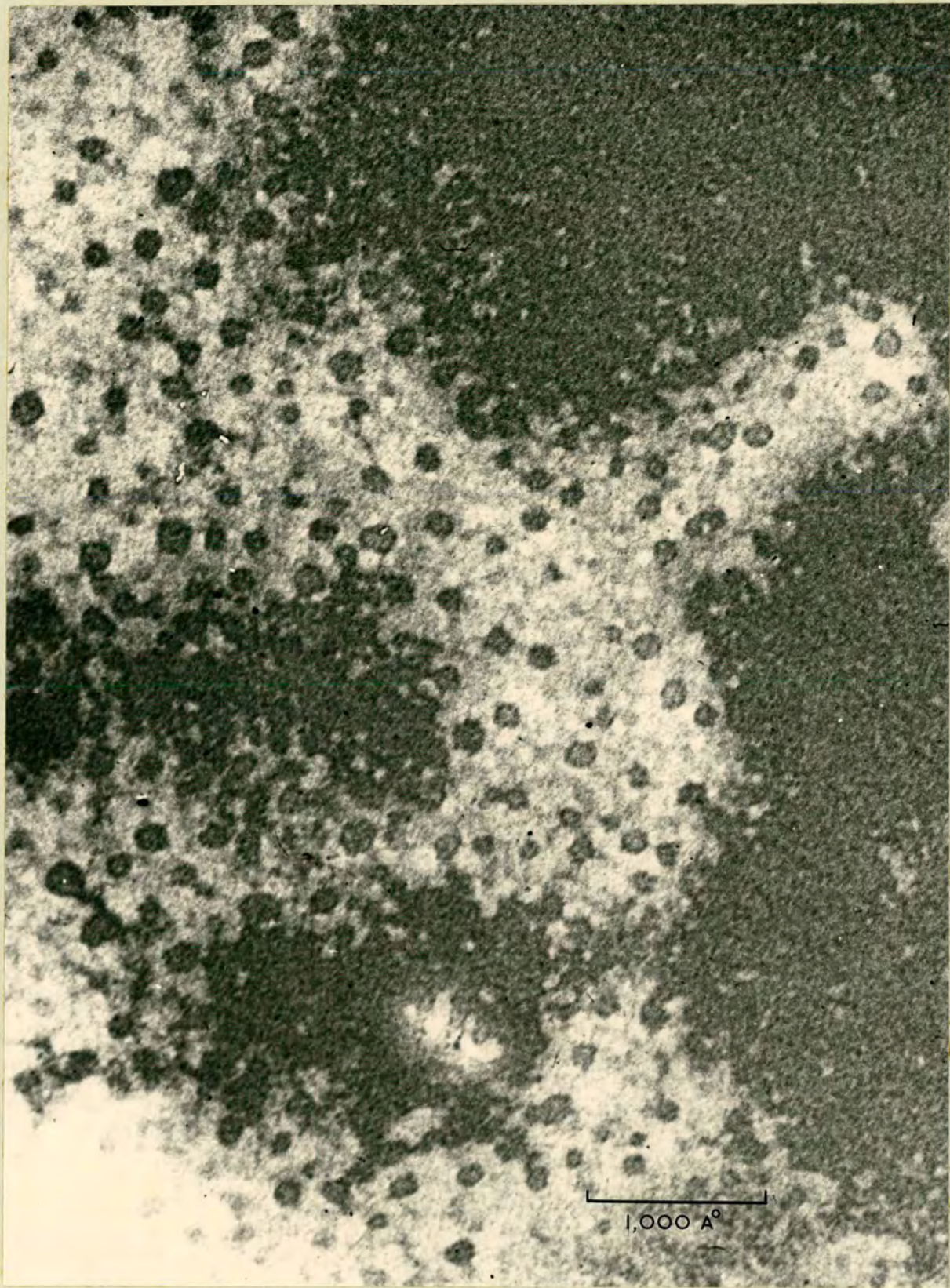


Plate 21.

Plate 22.

Reversed print of antigen molecules shadowed with platinum
at a shadow-to-height ratio of 4:1, with a polystyrene latex particle
of 0.26 μ diameter.

120,000X

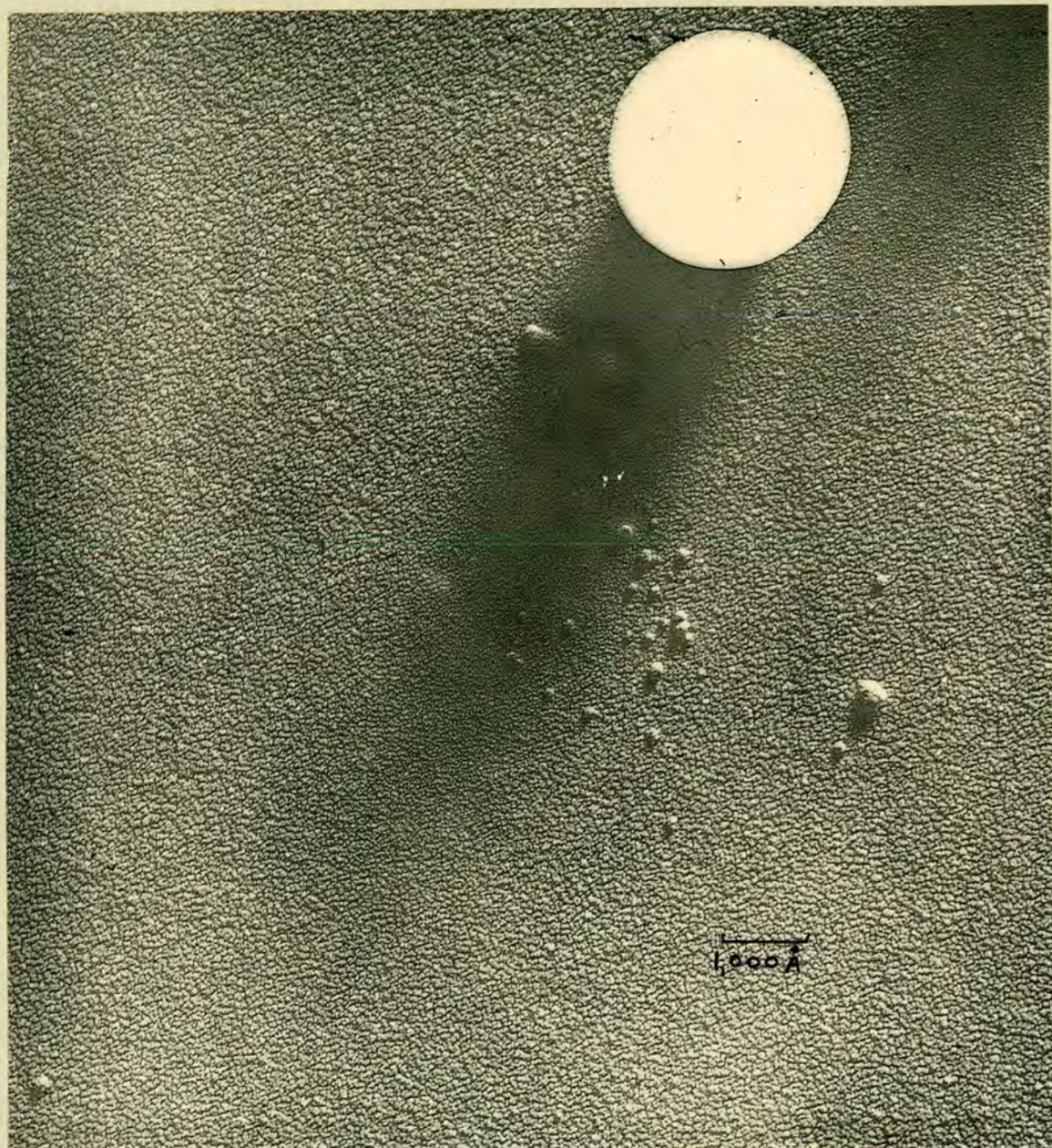


Plate 22.

Plate 23.

Reversed print of antigen molecules shadowed with platinum at a shadow-to-height ratio of approximately 2.4: 1, with a psl particle of 0.264μ diameter.

220,000X

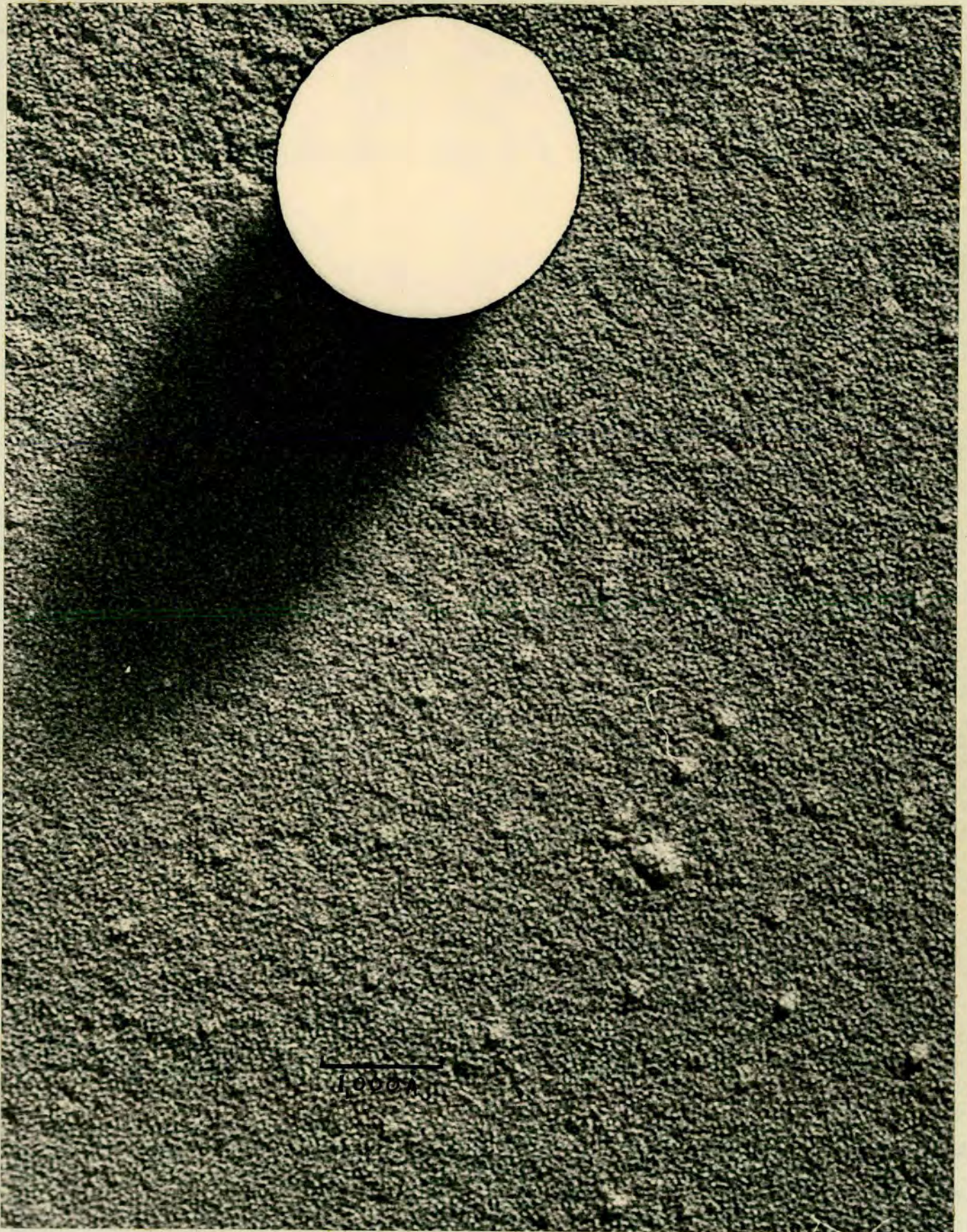


Plate 23

Plate 24.

Reversed print of antigen molecules sprayed on freshly
cleaved mica shadowed with platinum at a shadow-to-height ratio of
approximately 2:1.

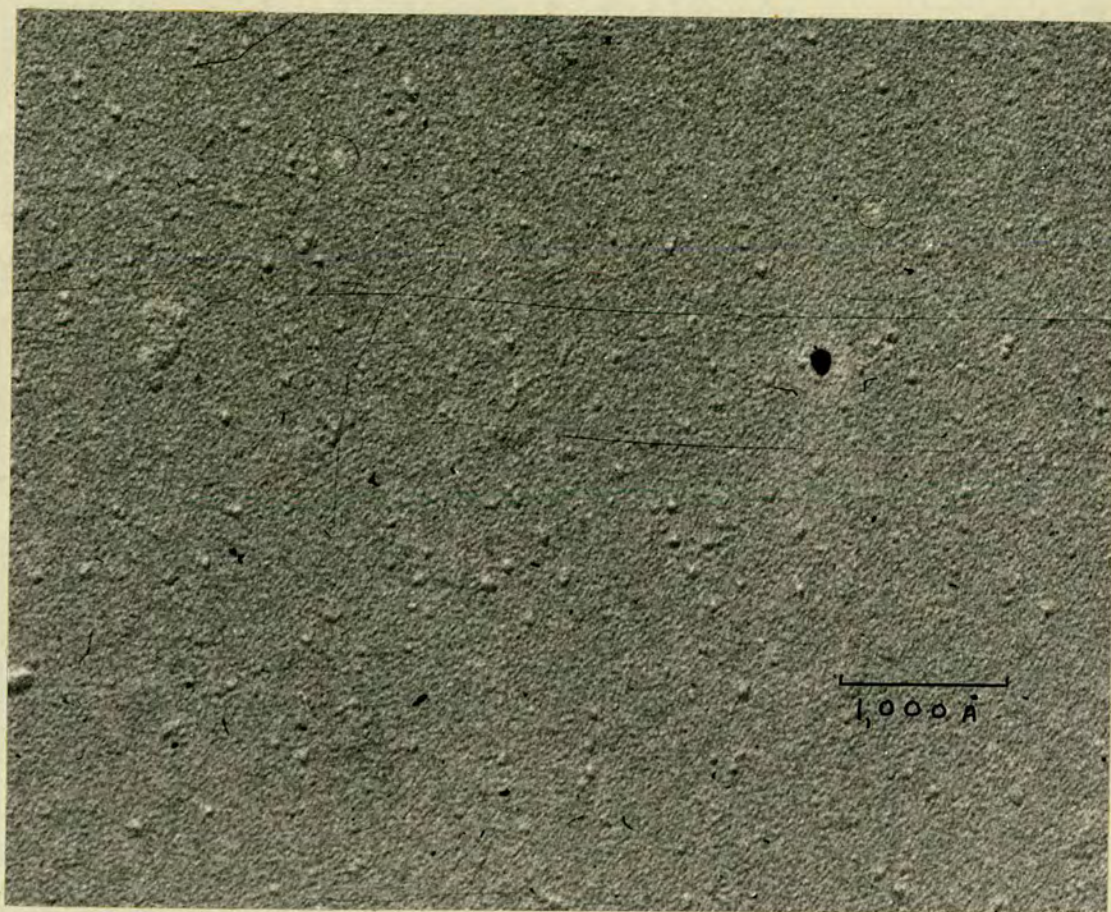


Plate 24

Plate 25.

Low power print of a negatively stained preparation of antigen - ferritin labelled globulin complex. A possible triad of molecules is shown (arrow).

120,000X

Plate 26.

High power print of the same preparation. The triad shows a ferritin molecule, a globulin molecule and an antigen molecule.

400,000X

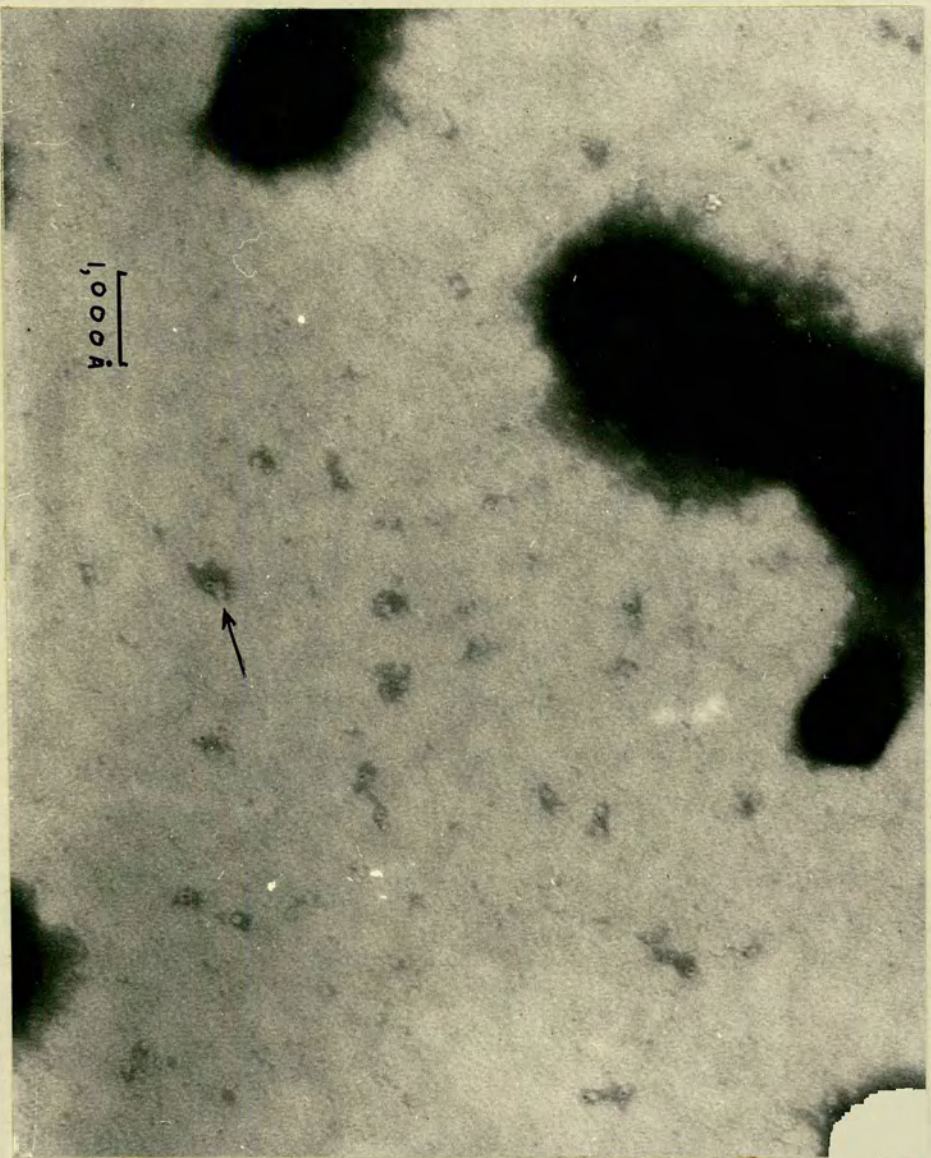


Plate 25

1. 71.

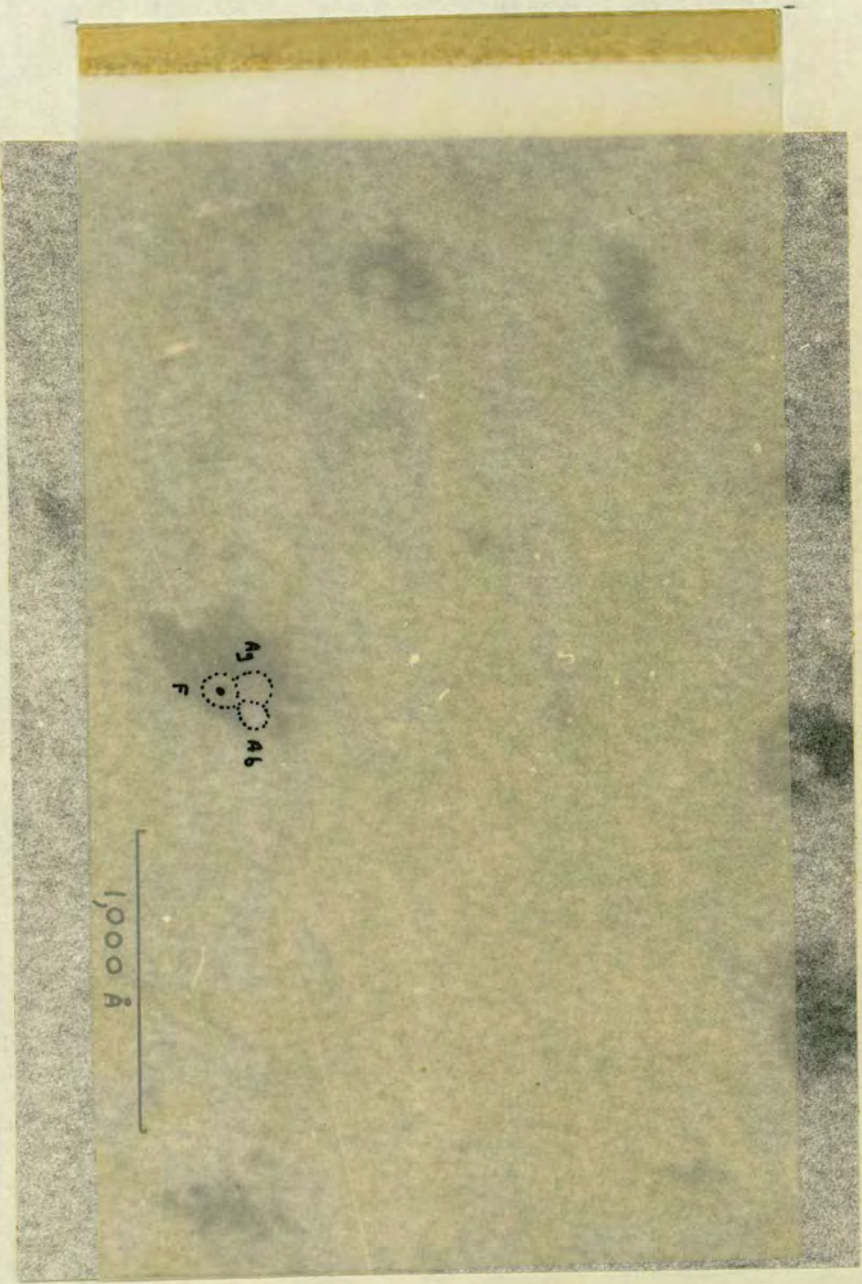


Plate 26

Plate 27

A preparation of antigen - antibody complex (ferritin labelled), shadowed with platinum at a shadow-to-height ratio of 4 : 1.



Plate 27

Plate 28.

Ferritin conjugated antibody negatively stained with phosphotungstic acid, showing one globulin molecule coupled to one ferritin molecule. Polystyrene latex particles = 0.088 μ .

Plate 29a.

Ferritin conjugated antibody platinum shadowed at a shadow-to-height ratio of 3.5:1. Globulin molecules can be seen coupled to ferritin molecules, in addition to a large number of unconjugated globulin molecules. Polystyrene latex particle = 0.088 μ .

Plate 29b.

Pure ferritin platinum shadowed at a shadow-to-height ratio of 2:1. Polystyrene latex particle = 0.088 μ .

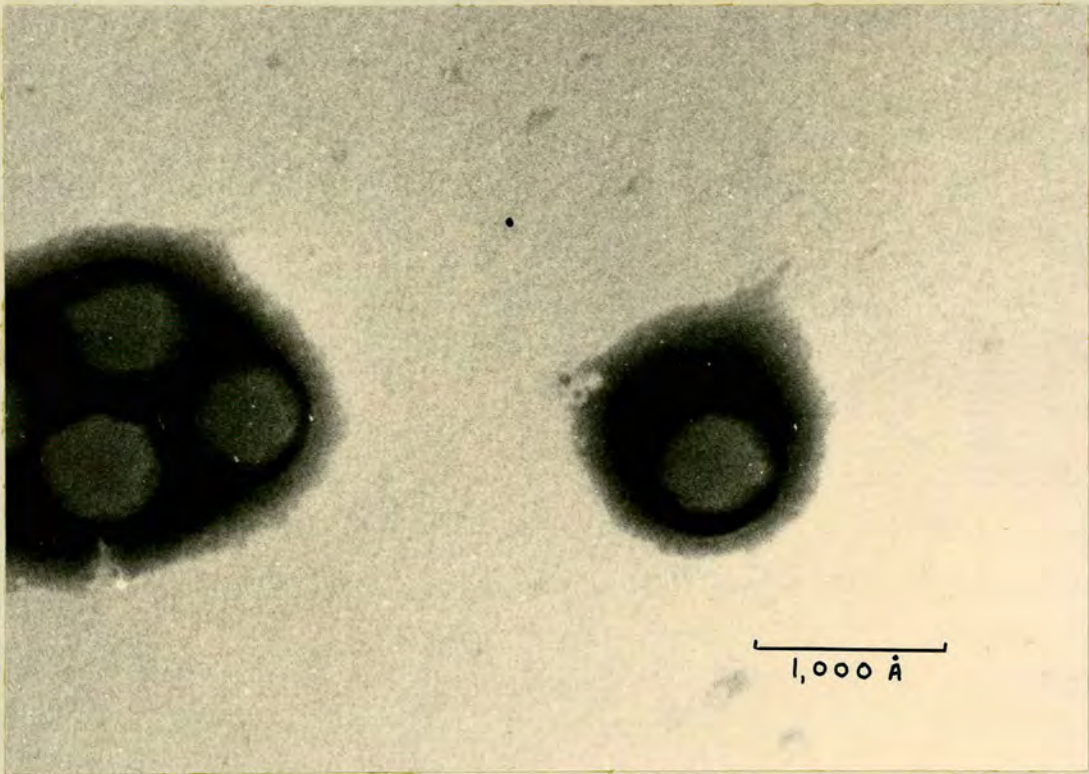
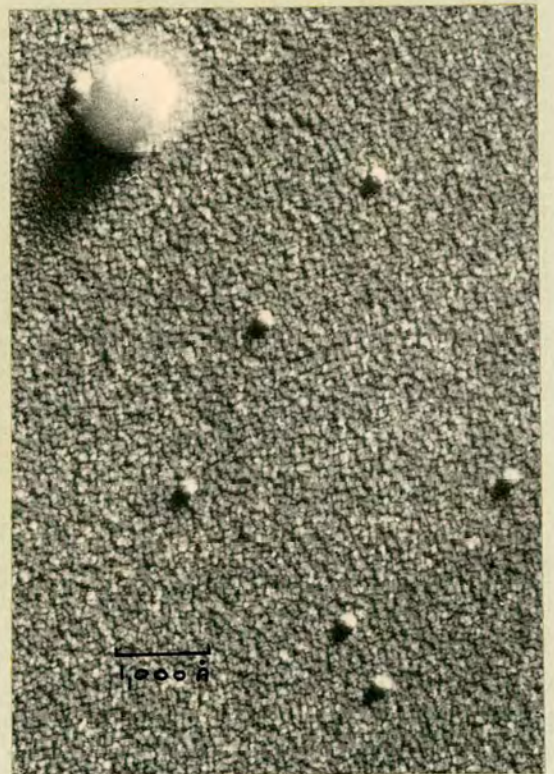


Plate 28



a.



b.

Plate 29

Plate 30.

Fragments of the pellicle of Paramecium, negatively stained with phosphotungstic acid, showing some contours of the surface and also the ciliary-kinetosome junctions of single and paired cilia, and the large parasomal sac opening.

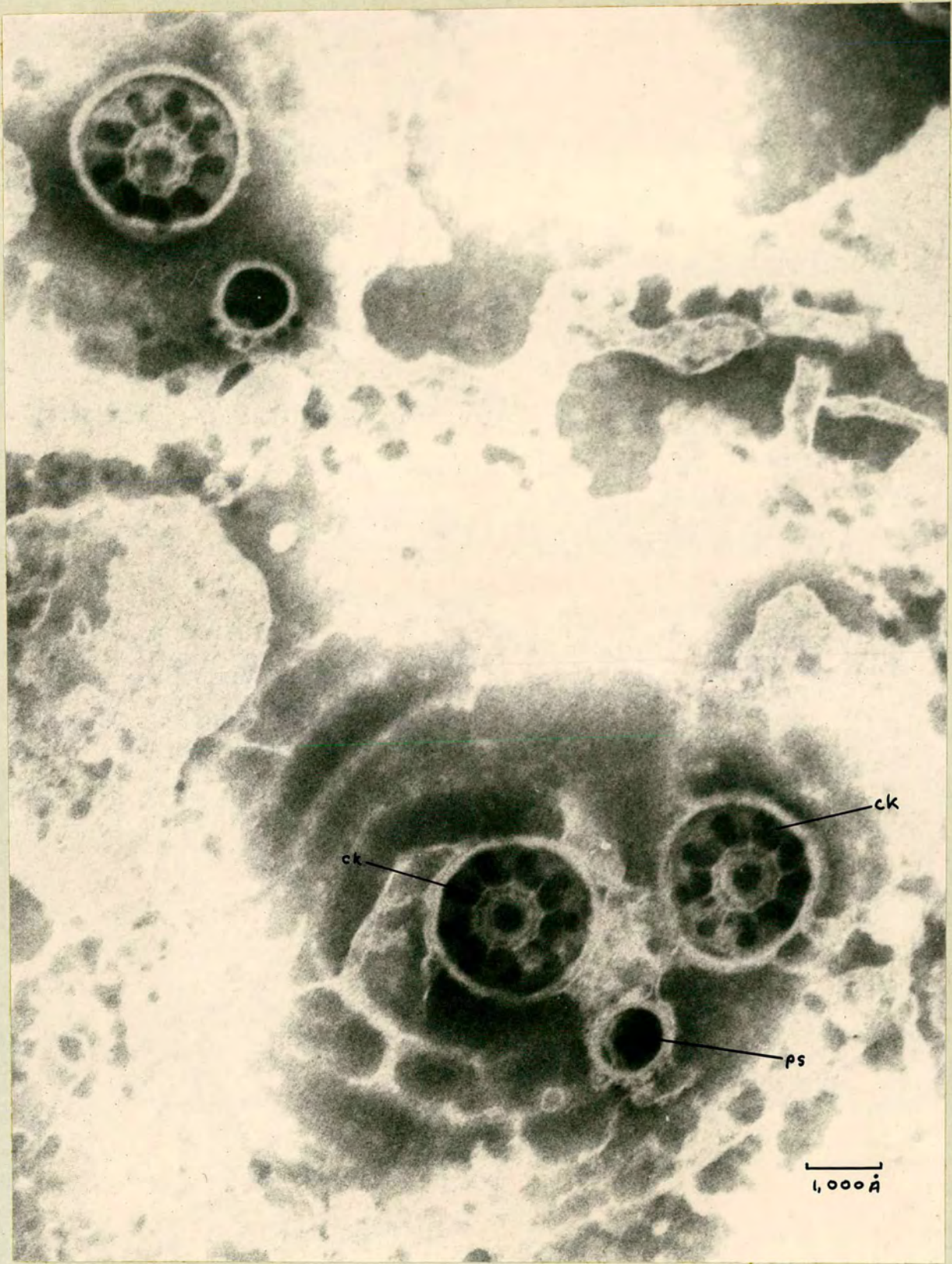


Plate 30

4. DISCUSSION

It has been demonstrated in the studies presented here, by both fluorescent and ferritin antibody-labelling techniques, that the immobilization antigen is situated on the surface of the pellicle and cilia of the paramecium. At the level of sensitivity of the fluorescent labelling method, it has been shown that the immobilization antigen is not present in the interior cytoplasm of the cell. However, as the ferritin labelling method has proved to be a much more sensitive technique, it is possible that extremely small amounts of antigen in the cytoplasm could be undetected by fluorescent labelling.

Unfortunately, the use of this more sensitive ferritin-labelling technique on the interior of the cell has so far been unsuccessful, but it is hoped to continue the investigation as mentioned earlier, perhaps using other water soluble embedding media, such as Aquon, the water soluble component of the epoxy resin Epon (Gibbons 1959) or water soluble glycol methacrylate (Leduc, Marinozzi and Bernhard, 1963) and treating the sections with ferritin-labelled antibody.

However, according to our experience and that of Baxandall, Perlmann and Afzélious (1962) treatment of electron microscope thin sections with ferritin-labelled antibody seems to have little possibility of success (Morgan, Rifkind and Rose, 1962), and also Spendlove and Singer (1961) suggest that Araldite may destroy the antigenic components so that attempts at locating the antigens would be useless.

To study the process of synthesis of the antigen it is therefore hoped to gain access to the interior of the cell by one of several alternative methods which have been used by various workers. The exposed interiors of the fixed cells can then be treated with ferritin-labelled antibody and subsequently embedded and fine sectioned for electron microscopy. Intra-cellular ferritin labelling has been demonstrated by several workers. Morgan, Rifkind, Hsu, Holden, Seegal and Rose (1961) obtained ferritin labelling of vaccinia virus within the host cells, gaining access to the cells by frozen sectioning. Morgan, Rifkind and Rose (1962) demonstrated ferritin labelling of soluble influenza antigen within host cells, fragmenting the cells by freezing and thawing, although this latter method can destroy fine structure. Andres, Morgan, Hsu, Rifkind, and Seegal (1962) showed the location of injected globulin in kidney cells by ferritin antibody treatment of dissected cell fragments.

Other possible methods of gaining access to the interior of fixed paramecia are breakage with ultrasonics or the cutting of frozen sections embedded in agar. According to the method of Dr. A.M. Glauert for embedding bacteria in agar blocks for easy handling for later embedding in Araldite, the agar can be dehydrated and embedded as with a tissue block. This method would possibly be superior to the gelatin frozen sectioning method, used previously in this study for treatment of sectioned animals with fluorescent antibody, as gelatin not removed before Araldite embedding would possibly obscure the picture in the electron microscope.

As the antisera we have so far used for our experiments are produced by injecting whole paramecia, the non-immobilizing antigens, common between paramecia of different serotype would have to be removed, in order that the exposed

interiors of the fixed cells can then be treated with specific ferritin-conjugated immobilization antibody. Absorption of non-specific antibodies from the antisera can be affected with homogenised, heterologous animals at approximately one million animals per 0.1 ml serum (Sonneborn, 1950; Finger, 1956) or boiled, heterologous animals (Balbinder and Preer, 1959). An elegant method to obtain specific antibody, is the use of specific absorbents, such as antigen - polystyrene suspension (Gyenes and Schon, 1960) or an antigen-cellulose suspension (Moudgal and Porter, 1963) from which the specific antibody can later be eluted. Pure antibody can also be obtained by the injection of rabbits with purified immobilization antigen, (Gill and Doty, 1960) plus Freund's adjuvant, but earlier attempts in this department to do this have not resulted in the production of antisera of very high titre .

Apart from the workers mentioned previously, there has been little general success in the study of the interiors of cells with ferritin labelling techniques, although surface labelling has been very successful. Rifkind, Hsu, Morgan, Seegal, Knox and Rose (1960) showed ferritin labelling on the surface of red cells and of virus particles on the surface of chick embryo cells. Baxandall et al. (1962, 1963) used a two layer, or indirect, ferritin staining technique to give increased sensitivity in their study of the surface antigens of sea urchin eggs. The surface antigens of virus particles have been labelled with ferritin antibody by a number of workers, such as Singer (1959) with the original method with Tobacco mosaic virus, and also Morgan, Rifkind and Rose (1962) and Anderson (1962).

Double labelling for the detection of two different antigens in the same preparation could possibly be carried out with one specific antibody conjugated to ferritin, and the other to mercury. (Pepe, 1961; Pepe and Fink, 1961; Pepe, Fink and Holtzen, 1961). The two electron dense labels should be distinct, mercury being a smaller molecule than ferritin and lacking the characteristic atomic orientation, giving only a diffuse increase in electron density in the sections. Mercury coupled antibody has been used to label Araldite sections specifically (Roppel and Melton, 1963), so far not possible with ferritin, but mercury has the disadvantage of vapourising in the electron beam.

Stages of transformation with ferritin labelling were examined to see if the ferritin granules, indicating "new" antigen formation, bore any relationship to particular surface structures. No evidence was found of flow of antigen along the surface from any particular point of emergence, such as possible pores in the membranes. No pores were seen, but frequently a folding was noted (Plates 6 and 10), as mentioned previously, but the appearance of ferritin granules did not particularly coincide with any of these folds. The parasomal sac, connecting as it does, the interior cytoplasm to the surface, would seem a likely exit for internal antigen to flow to the exterior, but ferritin granules, in the transformation studies, were not seen to be particularly associated with the openings of sacs when seen in several sections (Plate 13), nor in the area of the sac generally, nor did there appear to be any flow of antigen from this area.

It was noticed on examination of the micrographs that the density of individual ferritin granules and their distance from the pellicular membrane,

varied greatly. These facts were also noted in the micrographs of ferritin labelling by Morgan, Rifkind and Rose (1962). Both these observations could be due to the thickness of the sections, approximately 600A, and the fact that the membrane was not perpendicular to the plane of section, particularly when there was a protruberance of the surface, which often occur on cilia (Plate 7). The ferritin granules were seldom very close to the membrane and this is possibly due to the fact that the ferritin label could be situated on the globulin at a distance from the active site which combines with the antigen. The width of the globulin fuzz and the position of the ferritin granules could be that of the maximum length of a globulin molecule, 250A (Hall, Nisanoff and Slayter, 1959) and to the linkage of further antibody molecules to those already absorbed specifically on the antigen.

The presence of a small number of antigen molecules in the internal cytoplasm has therefore not been disproved, and thus the immobilization antigen could be formed either in the internal cytoplasm, or on the surface membranes. If the antigen is formed in the cytoplasm, antigen molecules would then have to pass to the exterior of the animal, and if the antigen is formed on the surface, the "message" for its synthesis would in its turn have to reach the exterior. Theories as to how these systems could be operated will be discussed later.

The soluble immobilization antigen when studied by the method of negative staining was found to be made up of molecules of approximately 200A diameter and of roughly hexagonal outline, made up of subunits arranged with the symmetry of a regular solid. The five or six sided outline suggests a regular polyhedron such as an icosahedron or dodecahedron. The diameter fits the physical data, for knowing the antigen to be a protein of M.W. 250,000 and assuming

the molecule to be globular and the density of protein 1.3, a value of approximately 200A is arrived at for the theoretical diameter.

The detail of the subunits was not very clear and future preparations will be made on holey carbon films to eliminate even a carbon background and as an aid to focusing (Huxley and Zubay, 1960). The spherical shape of the globulin molecule seen in the antibody - antigen complex is of interest as many workers with virus particles obtain pictures of antibodies as slender threads 250A X 20A attached to an active site each end (Anderson, 1962; Lafferty and Oertel, 1963; Watson and Wildy, 1963). The dimensions of a proposed model for γ globulin; made by Edelman and Gally based on physical and chemical data and such electron microscope results, are 240A X 57A x 19A giving a cylinder of elliptical cross section with an active combining site each end.

Only one globulin was found to be attached to one antigen, although it would seem possible that the antibody could attach to two antigen molecules as the ferritin label is known not to be conjugated to an active site on the globulin, as double labelling of one globulin molecule does not affect the antibody titre. (Pepe et al, 1961; Hsu, Rifkind and Zabrieski, 1964). Also it is presumed there could be a large number of antigenic sites on the antigen molecule.

The shape of the immobilization antigen is very similar to that found in small viruses using the same general techniques (Smith, 1959).

Crick and Watson (1956) suggest that small spherical viruses must be made up of subunits, related by cubic symmetry elements. Of the possible cubic symmetry patterns the 532 symmetry of the dodecahedron with 12 faces

and 20 vertices and the icosahedron with 20 faces and 12 vertices is the one found in viruses. The icosahedron has been found to be the usual symmetry in small viruses, possibly because the packing of subunits on triangular faces is the easiest way of packing small spheres. For this 532 symmetry the number of subunits must be a multiple of 60. These are symmetry elements and not necessarily shape. The subunits probably are single protein molecules. Viruses studied have all shown this icosahedral symmetrical arrangement of subunits. Hall, Nehean and Tessman (1959) and Sin Sheima (1959) studied bacteriophage ϕ X 174 by both negative staining and platinum shadowing and found a hexagonal outline of 250A, very similar in size to the immobilization antigen molecule. They also obtained good shadowed particles with an apical subunit. Horne, Brenner, Waterson and Wildy (1959) studied adenovirus which was a similar shape, but of larger diameter 700A. A number of workers have studied turnip yellow mosaic virus including Brenner and Horne (1959) for the original negative staining technique, and also Nixon and Gibbs (1960) and Huxley and Zubay (1960), and found hexagons of diameters of 200 - 300A. Horne and Nagington (1959) studied poliomyelitis, of diameter 300A, and Smith and Hills (1960) tipula irridescent virus. Other viruses have been studied and been found to have been made up of subunits arranged with helical symmetry, such as Tobacco mosaic virus (Huxley 1956b), tobacco rattle virus (Nixon and Harrison, 1959) and beet yellow virus (Horne, Russell and Trim (1959).

Viruses, such as the influenza virus of diameter 1,000A, the protein subunits are seen to be elongated projections of hexagonal or pentagonal cross-section, radially arranged over the surface of the particle. These

capsomeres, whose arrangements gives the shape to the virus particle, are not necessarily arranged with cubic symmetry as they are not necessarily packed in multiples of 60. In influenza virus, the capsomeres are seen to be hollow prisms $100\text{\AA} \times 40\text{\AA}$ and are made up of further protein subunits packed to give cubic symmetry. Studies by Wildy, Russell and Horne (1960) on herpes virus, and by Wildy, Stocker, MacPherson and Horne (1960) on poliovirus show similar capsomeres which are however arranged with 532 symmetry.

The capsomeres appear to be the antigenic sites of the virus, for when specific antibody is allowed to react with virus particles, the antibody molecules are seen to be attached by their active sites to the capsomeres (Lafferty and Oertel, 1963). Viruses, can be broken down to give two serologically active components, 'soluble' antigen which contains DNA, and hemagglutinin which is a protein. As shown by the studies of Horne, Waterson, Wildy, and Farnham (1960) and Hoyle, Horne and Waterson, (1961) on the myxoviruses, 'soluble' antigen appeared to be formed from the capsomeres. Studies by Polson (1953) and Hoyle, Reed and Astbury (1953) also showed subunits in the soluble antigen as hexagons of roughly 120\AA size. These had an appearance very similar to the molecule of immobilization antigen. The capsomeres have been seen to retain their shape after disruption of the virus particle and are thought to be proteins of M.W. 500,000. On negative staining of the outer membranes of the paramecium, antigen molecules ^{possibly} may be found to project from the surface in the manner of the capsomeres on virus particles. The antigenic sites on the molecules could be on some of the subunits seen, as the antigen has possibly up to twenty active sites. Gill and Doty (1960) performed an interesting experiment by injecting a synthetic polypeptide

of M.W. 49,000 into a rabbit, with the addition of Freund's adjuvant, in a search for the minimum requirements for antigenicity. They found it to be a structureless coil, not a helix, and it was a straight chain polypeptide of glutamic acid, lysine and tyrosine, the tyrosine probably being the major antigenic factor.

Many examples of molecules have also been studied with negative staining and metal shadowing and they have been seen to be made up of subunits symmetrically arranged. Van Bruggen, Wiebenga and Gruber (1962) and Van Bruggen, Schuiten and Wiebenga (1963) have shown molecules of hemocyanin, which are rather similar in appearance to the molecules of immobilization antigen and very similar to the pentagonal or hexagonal prism of the virus capsomeres (Horne and Wildy, 1961). Other molecules that have been studied with these techniques have shown very varied appearances. Chatterjee, Sadhukhan and Chatterjee (1961) and Levin (1963) studied haemoglobin and myoglobin and found them to be ring shaped molecules of approximately 50Å diameter, which fitted the X ray analysis of Perutz, Rossmann, Cullis, Muirhead and Will (1960) and Kendrew, Dickerson, Strandberg, Hart, Davies, Phillips and Shore (1960). Horne and Greville (1963) studied L-glutamic dehydrogenase and found the subunits arranged to give tetrahedral symmetry. Levin (1962) found cytochrome c to be a compact molecule with very little internal structure. The long strand of DNA has been studied by shadow casting, originally by Horne (1956a, 1956b) and also by Hall and Litt (1957), Hall and Doty (1958), Hall and Cavalieri (1961), Beer (1961) and McLean and Hall (1962). Hayes, Murchio, Lindgren and Nichols (1959) studied lipprotein molecules. It is concluded from these studies that the immobilization antigen is a molecule made up of symmetrically arranged subunits, similar in appearance

to many virus particles and molecules.

The nature of the membranes and the general ultrastructure of the paramecium, as well as the position of the ferritin and globulin labelling of the antigen on the membranes, are of importance in the discussion of the possible nature of the molecular transfer. The structure of both pellicular and peribasal membranes, as mentioned previously, was seen with the staining methods used, to be the "3-ply" unit membrane of Robertson (1957, 1960a, 1960b) consisting of two dark staining outer layers each approximately 25A thick, separated by a clear layer of 25A width, the whole making up a membrane approximately 75A. Knowledge of the actual molecular structure of the cell membrane would be of value in understanding the probable sites of the antigen.

Cell membranes are known to be made up mainly of proteins and lipids, but how these are arranged and the meaning of the three bands, has been the subject of some speculation. (Finean, 1962). Stoeckenius (1962) found with the use of model 'membrane' systems that the dark staining bands in cell membranes were due to the hydrophobic groups of the lipids, plus the protein, on either side of the unstained fatty acid chains in the centre, and therefore cell membranes could be considered a bimolecular leaflet of lipid covered on both sides by layers of protein. (Stoeckenius, 1960).

Interpretation of structures under the electron microscope, however, must be made with some care as yet, due not only to the possibility of artifacts being introduced during dehydration and embedding, but to actual interpretation of structures seen.

The following conclusions are therefore drawn with some reservations.

From the results quoted, it would appear that the outermost layer of the pellicular membrane is probably mainly protein, which would include the immobilization antigen. From a rough correlation of various factors, such as the probably size of the antigen molecule of M.W. 250,000, the amount of antigen, 10^{-7} mg., extracted per single animal, being only 10% of the total surface protein, the relatively vast surface area of a paramecium with from 1,000 - 2,000 cilia (Preer and Preer, 1959), and the thickness, 20A° , of the outer layer of the pellicular membrane, it is probable that the antigen molecules are widely separated or spread over the surface in thin sheets. From the appearance of the globulin fuzz of the antigen-antibody reaction antigen would appear to cover the entire surface, but from the thickness of the sections approximately 600A° , and the fact that the antibody:antigen ratio might be as high as 20 : 1 due to the number of possible active sites on the antigen, this may not be so. It has been found from the present study that no other antigen is present on the surface, and that in the gullet area no antigen, immobilization or otherwise, is present. However, the three layers of the outer pellicular membrane of the gullet area do not seem to vary in width or density compared to elsewhere on the surface of the animal. Antigen may possibly be stimulated to come out onto the surface of a living animal by antibody treatment but this would not be so in a fixed animal, and thus it would appear that the antigen molecules cannot be distinguished from the rest of the protein-lipid layer of the membrane at the magnifications used, approximately X 100,000, and the positive staining methods used.

With the knowledge of the shape of the antigen molecules when present as soluble antigen in solution, it would be of interest to examine the surface membranes of the paramecium at a possibly molecular level in an endeavour to

distinguish antigen molecules on or in the membranes, though these may not necessarily have the same configuration when present in the membranes as they have in solution. Parson (1963) has shown that with negative staining at very high magnifications of from X 700,000 to 1,000,000, flask-shaped subunits of approximately 80A diameter can be seen on the mitochondrial membranes, and he suggests that these may be individual enzyme molecules or clusters of molecules.

Negative staining and high resolution microscopy should certainly show a hexagonal molecule of 200A if the molecules are present on the surface with the same configuration as found in solution. If the membranes could be viewed in section with negative stain, the antigen molecules should be seen projecting from the surface in the manner of the molecules seen by Parsons or of capsomeres on virus particles as mentioned earlier. Previous positively stained preparations of sections of membranes have not been at a sufficiently high power or with enough contrast to indicate this, although sections of membranes fixed in osmic acid and counterstained with potassium permanganate and uranyl acetate, would render electron dense any protein present. (Watson, 1958; Huxley and Zubay, 1961; Sjostrand, 1962). Some micrographs of sections of the external membranes at magnifications of 250,000 X did show some hazy effect on the outermost dark band of the three-ply membrane, although this may have been an artifact and certainly showed no structure. It is also suggested that the surfaces of membranes are more distorted in sections with positive staining, than with negative staining techniques. (Valentine and Horne, 1962).

It is possible that the molecules may appear flattened when on the membranes, but the flattened effect obtained when soluble antigen was platinum shadowed was probably an artifact, as no side-on view of flat

hexagonal molecules was obtained with negative stain, indicating rather a three dimensional solid structure.

That negative staining is suitable for quite large objects such as fragments of paramecia, has been shown by Horne and Whittaker (1962) with studies of the particles resulting from cell disintegration, both with and without osmic acid fixation, frequently in sucrose to preserve the tonicity and hence the preservation of the particles. They found molecular subunits in the membranes, similar to the hexagons later found with artificial membranes. (Bangham and Horne, 1962; Glauert, Dingle and Lucy, 1962). Hexagonal shapes have been seen by Tromans and ~~Hall~~ Horne (1961) on the membranes of cells infected with a virus which shows this configuration in its soluble antigen molecules. Other large particles examined with negative stain have been collagen fibrils examined by Tromans, Horne, Gresham and Bailey (1963) which showed the banded structural units of 15 - 20A of the triple helix. The flagella and fimbriae of bacteria showed subunits when examined in negative stain by Kerridge, Horne and Glauert, (1962) and Thornly and Horne (1962). Metal shadowing of similar large particles on the other hand has not yielded as much information on structure as negative staining, although Metz, Pitelka and Westfall (1953) obtained information of the fibrils of cilia of paramecia and Duguid (1959) used this method ^{to} ~~and~~ study bacterial fimbriae.

Even if the antigen molecules do appear on the membranes as hexagons of approximately 200A ^o diameter, they will be difficult to locate as the immobilization antigen is only 10% of the total surface proteins, although it is the largest single component. A suggestion is that the antigen molecules could be located by treating the membranes, or whole paramecia before fragmenting, with ferritin labelled specific antibody to the

immobilization antigen present. On examination with negative stain the ferritin labelled globulin molecules should be able to be distinguished, absorbed on the antigen on the membrane. This ferritin labelling of molecules in membranes has been suggested by Novikoff, Essner, Goldfischer and Heus (1962). For this high resolution ferritin labelling, it would be desirable to separate the ferritin conjugated γ globulin from unconjugated globulin. Ultra centrifugation at 100,000G precipitates the ferritin conjugated globulin, leaving the unconjugated globulin in the supernatant. (Morgan, Rifkind, Hsu, Holden, Seegal and Rose, 1961; Singer and Schick, 1961; Hsu, Rifkind and Zabrieski, 1964). Continuous flow electrophoresis or column chromatography (Borek, 1961; Borek and Silverstein, 1961) or a sucrose or C_2Cl_2 density gradient (Anderson, 1962) would separate the ferritin conjugated globulin from both unconjugated globulin and ferritin. A new one stage method by Tawd and Ram (1962) for conjugating ferritin to globulin may result in less unconjugated globulin molecules.

As mentioned in the introduction, the antigen system is influenced by the interaction of three factors, the genes, the cytoplasmic states, and the environment. Each gene, eg^d60, in the nucleus in the interior of the cell, controls one antigen in this case 60D, on the exterior surface of the cell, and thus there must be some sort of chain-reaction from the gene, through the cytoplasm, to the cell surface as well as a "feed-back" from the cytoplasm to influence the gene. It is thought the action of the gene could be controlled in one of several ways, either by activation of certain systems always in readiness, or de-repression of some agent keeping these systems in check (Waddington, 1962) such as the removal of an inhibitor from an enzyme, thereby allowing the

enzyme to act. When a certain chain of reactions, resulting in the formation of a certain antigen, is complete it could then inhibit other reactions, at any point in the cell, in the manner of a 'steady-state' system (Beale 1958) by de-activation or repression, as above.

It would be interesting to use the sensitive ferritin-labelling technique on studies of transformation of antigen by gene change, to determine whether the long delay and abrupt change of antigen after five fissions noted with the immobilization technique, was confirmed. An abrupt change of antigen could denote the sudden removal of an inhibitor, as in the Killer system (Gibson and Beale, 1963) or the disappearance of an unstable messenger, following the change of a gene.

A great deal of work has been done on the mechanism of the synthesis of proteins in a cell (Crick, Barnett, Brenner and Watts-Tobin, 1961:) and it is generally accepted that the deoxyribonucleic acid (DNA) of the gene controls the structure of the protein synthesised. Both the DNA of the gene, and the protein, are similar in being linear sequences, of nucleotides in the case of the DNA, and of amino acids in the case of protein, but the gene is thought not to act directly to form the protein but through intermediate substances, the ribonucleic acids (RNA). Genetic information for protein structure is thought to be coded in the DNA and the information carried by unstable intermediate messenger RNA, (Brenner, Jacobson and Meselson, 1961; Jacob and Monod, 1961) of MW = 170,000, to the ribosomes. These are non-specialized structures of approximately 200A diameter (Rich, Warner and Goodman, 1963). The messenger RNA varies in length as it can code for long polypeptide chains, as the nucleotide base sequences of the

messenger RNA correspond to that of the DNA and control the amino acid sequence in the protein molecule. (Nirenberg and Matthai, 1961; Matthai and Nirenberg, 1961). Ribosomes consist of a protein shell containing a core of ribosomal or template RNA (Nirenberg and Matthai, 1961) of M.W. 170,000, similar to that of messenger RNA. The amino acids are brought to the ribosomes by soluble or transport RNA of low MW = 25,000 (Gros, Hiatt, Gilbert, Kurland, Risebrough and Watson, 1961). The ribosomes work their way along the messenger RNA thread in ratchet-like steps, adding amino acids as they go so that the polypeptide chain increases in length until eventually the final proteins and the ribosomes detach. (Warner, Rich and Hall, 1962). The three nucleotide bases on this messenger RNA strand are probably responsible for the coding of a single amino acid (Crick, Barnett, Brenner and Watts-Tobin, 1961). A great deal of work has been done on the nature of the code, the replication of the DNA and RNA and the helical structure of the molecules. (Watson and Crick, 1953; Griffith and Orgel, 1957; Lengyel et al, 1961; Speyer et al, 1962; Lengyel et al, 1962; Basilio et al, 1962; Gardner et al, 1962; Wahba et al, 1963, as well as Watson and Kendrew, 1961; and Goldstein, 1962).

In the light of these theories, it would appear that the immobilization antigens, which have been found to be proteins, could be synthesised on the ribosomes in the cytoplasm, where they could exist as formed antigen molecules, not necessarily very many at any one time, and as such, pass to the exterior of the cell. These ribosomes could be associated with the membranes of the endoplasmic reticulum (Palade 1956) which can have many ribosomes on their surfaces and are known to be sites of active synthesis.

Ribosomes are also known to be present on the cytoplasmic side of external cell membranes (Palade, 1955; Palade and Siekevitz, 1956a) and the antigen could thus be formed close to the cell surface.

If the antigen itself is formed on the ribosomes, internal ferritin labelling may show antigen associated with polyribosomes made up of a definite number of ribosomes, as various classes of polysomes appear to synthesise similar polypeptide chains. In specialized cells, such as reticulocytes, making a single protein, haemoglobin, the polyribosomes are seen to be in similar clusters i.e. pentameters, attached to a strand of messenger RNA (Warner, Rich and Hall, 1962; Marks, Burka Rifkind and Danon, 1963; Rich, Warner and Goodman, 1963). On the other hand, cells which are not specialized, but are manufacturing the entire complement of proteins necessary for the cell, show polyribosomes made up of different numbers of ribosomes.

The alternative to the antigen being synthesised in the cell and moving to the exterior, is that the information only, as messenger RNA, could pass from the gene to the external membranes and the antigen by synthesised there. There is the possibility that this RNA could take the form of an intermediate particle located in the cytoplasm similar to the metagons in the Killer system in paramecium (Gibson and Beale, 1963). The fibrils, both in the cilia and in the cytoplasmic network, are also known to have enzymatic activity (Nelson, 1959), and Pitelka (1963) suggests that they could transmit information or messenger substances.

These systems would necessitate the movement of either the antigen protein or the RNA, molecules of approximately 200,000 molecular weight to the exterior of the cell. From the study of the ultrastructure, it appears this passage is possibly not through openings such as pores or the

parasomal sac, but through the membranes themselves. If the membranes were static, structural entities, there would seem to be several mechanical barriers to a large molecule passing to the exterior. Three 'three-ply' membranes and the large peribasal space separate most of the cytoplasm from the exterior, as well as the dense homogenous layer or membrane mentioned earlier. This dense layer also appears to be continuous with the septum across the base of the cilium, which would appear to be a barrier from the general cytoplasm to the interior of the cilia.

It is well known, however, that membranes contain enzymes and that there is a flow of molecules through cell surfaces in metabolism. It is thought that cell membranes are impermeable to a great many substances, unless metabolized by the enzymes in and behind the membranes (Danielli, 1954) Mitchell and Moyle, (1959). This membrane-selective chemical mechanism of Davson and Danielli (1952) could account for the fact that large molecules, such as the antigen or the RNA, both molecules of M.W. approximately 200,000 and diameter approximately 200A, could pass through the membranes to the exterior, whereas others, such as the globulin of the antibody-labelling, of molecular weight 156,000 and possible dimensions of 240A X 57A X 19A (Edelman and Gally, 1964), were unable to penetrate the cell wall, possibly due to lack of the necessary enzymes in the membranes. A mechanical transport, such as pores in the membranes, would not give this specificity (Danielli, 1952). Several workers (Novikoff, Essner, Goldfischer and Heus, 1962) hope to use the sensitive ferritin antibody-labelling described in this study, to detect enzymatic activity in the cell which may be too low to detect by present methods, particularly on the cytomembranes. These workers believe that enzymes take part in the transport of special molecules by the formation and dissolution of the cytomembranes which are in a constant state of flux and transformation

(Bennett, 1956) having functional rather than structural continuity, and are thought to be continuous with the nuclear membrane, and possibly the cell surface (Watson, 1955b; Palade, 1956; Palade and Siekevitz, 1956b; Gray, 1964). This may also be true of the external cell membranes, for these also must be capable of active transformation, such as the instantaneous production of pseudopodia (Pitelka, 1963) and of the ability to flow under certain conditions, in the manner of a plastic-solid (Waddington, 1962). It is hoped that the use of the sensitive ferritin-labelling in the interior of the cell and the technique of negative staining at the molecular level, to finally elucidate the problem as to the possible sites and mechanisms of the production of the immobilization antigens. Elucidation of the complicated interaction of the factors influencing the antigen system, could produce a model for cell differentiation in multicellular animals, where all the cells of one animal have the same set of genes, yet produce very different phenotypes.

5. SUMMARY

1. The immobilization antigens of Paramecium aurelia were located by treatment of the fixed animals with labelled antibody. Two different methods of labelling were studied, first the use of fluorescent dyes for examination of preparations under ultra-violet light, and secondly, the use of electron-dense ferritin for examination in the electron microscope.

2. With the method of fluorescent labelling, antibodies were conjugated with either fluorescein or rhodamine, giving double labelling for two different serotypes. On treatment of sections of fixed animals with fluorescent antibody, fluorescence was observed on the pellicle and cilia and in the internal cytoplasm. Fluorescence of the cytoplasm was prevented by pretreating the sections with heterologous, non-fluorescent antiserum. Following this treatment, fluorescence occurred on the pellicle and cilia only of homologous animals. It was concluded that the immobilization antigens are located on the pellicle and cilia, and that the internal cytoplasmic antigens are relatively invariant in paramecia of different immobilization antigenic type.

3. On treatment of fixed whole animals with ferritin-labelled antibody, followed by sectioning, ferritin granules were seen on the pellicle and cilia of homologous animals only. By counterstaining the sections with an electron-dense stain of potassium permanganate and uranyl acetate, the absorbed globulin of the anti-body was revealed as

a thick fuzz on pellicle and cilia. The internal antigens could not be studied by this method, as labelling of sections with ferritin was not successful.

4. Transformation from one antigenic type to another by change of temperature, was studied, using both labelling techniques, both of which showed the 'new' antigen appearing initially on the pellicle and subsequently on the cilia. The ferritin labelling method, however, proved to be the more sensitive, ferritin granules and globulin fuzz denoting 'new' antigen appearance being detected at isolated sites on the pellicle and cilia after only one hour of growth at the higher temperature. The number of these sites then increased, until the granules and fuzz were seen to cover the entire surface of first the pellicle and subsequently the cilia.

5. From the observations made using both labelling techniques, it is concluded that the immobilization antigens are present on the pellicle and cilia of the paramecium, and at the level of sensitivity of the fluorescent labelling method, immobilizing antigens have not been found to be present in the internal cytoplasm. In the light of these observations, therefore, several theories as to the possible sites and mechanisms of the production of immobilization antigen are proposed and discussed.

6. Molecules of purified immobilization antigen were studied in the electron microscope, using the methods of negative staining with phosphotungstic acid, and shadow casting with platinum, to give increased contrast to small particles. Both single antigen molecules, and the antigen-antibody (ferritin-labelled) complex, were studied by these

methods, and an attempt was made to study the surface of the external membranes of the paramecium.

7. From observations made by these methods shapes of regular solids are discussed as possibilities for the structure of the antigen molecule. The antigen-antibody (ferritin-labelled) complex was seen as a triad made up of individual molecules of antigen, antibody and ferritin. No obvious molecular structures were demonstrated on the surface of the external cell membranes of the paramecium, with the magnifications and techniques so far employed.

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CYTOCHEMICAL LOCALIZATION OF ANTIGENS OF *PARAMECIUM* BY
FERRITIN-CONJUGATED ANTIBODY AND BY COUNTERSTAINING THE
RESULTANT ABSORBED GLOBULIN

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PLATES 61-63

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SYNOPSIS

The localization and transformation of the immobilization antigens of *Paramecium aurelia* were studied with the use of ferritin-conjugated antibody. The reaction was extremely specific, ferritin granules being found only on the pellicle and cilia of homologous animals. In the transformation from one antigenic type to another, ferritin granules, conjugated with antibody specific for the "new" antigen, appeared initially on the pellicle and subsequently on the cilia.

An electron-dense counterstain, of potassium permanganate and uranyl acetate, not only increased the detail of the structure of the organism, but also stained the absorbed globulin of the specific antibody as a thick fuzz on pellicle and cilia. This fuzz, unlike the ferritin granules, was clearly visible at very low magnifications of the electron microscope. This gave a quick scanning method, especially useful in transformation experiments, when various stages could be quickly scanned for the first appearance of globulin fuzz.

INTRODUCTION

THE ciliate, *Paramecium aurelia*, contains excellent antigens so that, when suspensions of the broken animals are injected into a rabbit, immune antibodies quickly develop.

When live animals of the same type as those injected are placed in diluted antiserum, a characteristic clumping of the cilia takes place, causing immobilization of the organisms, and with stronger treatment, eventual death of the animals. This antigen-antibody reaction is highly specific and therefore much used in the genetical work of the Institute by Dr. G. H. Beale, under whom this work has been done (Beale, 1954).

METHOD

To localize the immobilization antigens on the paramecium in earlier work, preparations were treated with antibody conjugated with fluorescent dyes, fluorescein and rhodamine, and examined by ultraviolet (U.V.) fluorescence microscopy. Later, when the greater resolution of the electron microscope was required, the antibody was conjugated with ferritin. Both these methods were found to be extremely specific, but that involving ferritin was the more sensitive, in that it revealed the presence of smaller amounts of antigen.

The animals were fixed in buffered osmium sucrose solution at pH 7.4, causing no loss

of antigenic activity, and were subsequently treated with the ferritin-conjugated antibody. Between all treatments the animals were well washed with isotonic buffered salt solution.

The fixed animals were first pre-treated with a non-conjugated, heterologous antiserum in order to block any non-specific antigens which might be present, as all antisera were made against whole paramecia.

The fixed animals were then exposed to the ferritin-conjugated globulin, specific to a given antigenic type. The globulin fractions of the antisera used were precipitated in half saturated ammonium sulphate solution and the ferritin-globulin conjugate prepared by the diisocyanate method of Singer (1961).

After dehydration in graded alcohols, the treated animals were embedded in Araldite, which was found to give better preservation of fine structure, particularly membranes, than other embedding media tried, such as methyl and butyl methacrylate. Sections were cut at about 600 Å and mounted on copper grids, previously coated with formvar and carbon.

Excellent specificity was obtained. When sections were viewed under the electron microscope, ferritin granules were seen on the pellicle and cilia of homologous animals only, none at all being present on non-homologous animals.

In Pl. 61, fig. 1, are shown ferritin granules on pellicle and cilia of an animal treated with ferritin-conjugated homologous antibody.

In experiments on the transformation from one antigenic type to another, live animals were exposed, by raising the temperature, to an environment favouring the formation of an antigen different from that initially present. Samples of animals were taken at intervals, fixed, and treated as before, exposing them to the ferritin-conjugated globulin specific for the new antigen. Ferritin granules, conjugated with antibody specific for the "new" antigen, were seen to appear initially on the pellicle and subsequently on the cilia.

As the contrast of the cells with Araldite is low under the electron microscope and results in poor structural detail of the sections, especially of the membranes, some grids were treated with an electron-dense counterstain. This was a combination of potassium permanganate and uranyl acetate, a modification of methods by Lawn (1960) and by Watson (1958), suggested by Mr. A. E. G. Dunn of Aberdeen University.

This stain gave excellent fine detail of the structure of the animal, especially the outer membranes, which were seen to consist of two distinct double layers, as previously noted by Stewart and Muir (1962). But it was of special interest to find that the counterstain revealed the absorbed globulin of the specific antibody as a thick fuzz, in which could be seen the conjugated ferritin granules, covering the pellicle and cilia of homologous animals. No such fuzz was present on non-homologous animals.

All the animals in the figures have been treated with the ferritin-conjugated antibody.

Pl. 61, fig. 2, shows the pellicle of a non-homologous animal. It exhibits two double membranes and *no* fuzz or ferritin granules.

Pl. 62, fig. 3, shows the pellicle of an homologous animal, with two double membranes, fuzz, and ferritin granules.

Pl. 62, fig. 4, shows the cilium of a non-homologous animal, with one double membrane, *no* fuzz or ferritin granules.

Pl. 63, fig. 5, shows the cilium of an homologous animal, showing one double membrane, fuzz, and ferritin granules.

The entire lack of globulin fuzz on the non-homologous animals not only showed that there was no specific antigen-antibody reaction but also that pretreatment with heterologous antibody was unnecessary. Any non-specific antigen-antibody reaction resulting

from this pretreatment would have resulted in the staining of any globulin absorbed. Therefore the surface antigens of paramecia appear to be immobilization antigens only.

The counterstaining of any absorbed globulin proved very useful as a quick scanning technique, for, unlike ferritin, the globulin fuzz was clearly visible at very low magnifications of the electron microscope. Therefore results could be obtained using the E.M. 75 in our department, at the maximum magnification of which ferritin granules are practically impossible to determine by eye, though they come up quite well in a micrograph. After preliminary scanning, interesting sections could then be studied and photographed with a Siemens microscope also at our disposal.

This was particularly useful in following transformation of the antigens, when successive stages could be quickly scanned for the first appearance of globulin fuzz. This was especially so in the early stages, when little specific antigen had then been formed, for the faint globulin fuzz of the antigen-antibody reaction was easier to pick up than a few sparsely separated ferritin granules.

There was no trouble in conjugating the globulin with ferritin and obtaining specific results, but unconjugated specific antibody, and counterstaining, could be of use, to save time and effort, both in preparation and in obtaining results.

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DESCRIPTION OF PLATES 61-63 (see after p. 158)

PLATE 61

- Fig. 1.—Section through paramecium treated with ferritin-conjugated homologous antibody, showing ferritin granules around pellicle and cilia.
 Fig. 2.—Section of pellicle of non-homologous animal, treated with ferritin-conjugated antibody, and counterstained, showing two double membranes, and no fuzz or ferritin granules.

PLATE 62

- Fig. 3.—Section of pellicle of homologous animal, treated with ferritin-conjugated antibody and counterstained, showing two double membranes and fuzz and ferritin granules.
 Fig. 4.—Section of cilium of non-homologous animal, treated with ferritin-conjugated antibody and counterstained, showing double membrane, and no fuzz or ferritin granules.

PLATE 63

- Fig. 5.—Section of cilium of homologous animal, treated with ferritin-conjugated antibody and counterstained, showing double membrane, and fuzz and ferritin granules.

DISCUSSION

Baxandall.—Were there any isolated ferritin particles on the surface?

Mott.—No, except that at early stages in the transformation experiments only a small amount of ferritin is seen, but this is conjugated with antibody and indicates specific uptake by the small amount of homologous antigen present.

Baxandall.—If there are only a few isolated ferritin molecules on the cell surface, although the gamma globulin stains with the permanganate-uranyl acetate solution all

along the surface, it looks as if the ferritin label is only revealing a small proportion of the antigenic sites present.

Mott.—This is not so, both ferritin labelling and globulin fuzz are quite specific. When the ferritin granules are sparse, so is the globulin fuzz. Both are completely absent in an animal treated with a non-homologous antibody conjugate, and both heavy in the homologous animal. However, the ferritin labelling could be increased by purification to remove unconjugated globulin.

