THE CONTROL OF ANTIGEN FORMATION IN CILIATES.

John Sommerville, B.Sc.

Submitted to the University of Edinburgh as a thesis in fulfilment of the requirement for the degree of Doctor of Philosophy.

Institute of Animal Genetics,
University of Edinburgh.

July, 1967.

TABLE OF CONTENTS

SUMMARY

1. The protozoan ciliate, Paramecium aurelia, has been found to be a convenient organism for the study of the synthesis of the cell-surface immobilization antigens (i-antigens).

2. Cell fractionation procedures have been employed. On sucrose density gradient centrifugation of paramecium post-mitochondrial supernatants, four major fractions have been distinguished: a) membrane fraction containing bound ribosomes b) membrane-free polysomes c) free monosomes d) soluble fraction. These fractions were characterized by chemical analysis and electron microscopy.

3. Methods have been examined for the detection of newly synthesised i-antigen. Both living cells and cell-free incorporating systems have been investigated for their ability to incorporate radioactively-labelled amino acids into protein immunologically identifiable as i-antigen. Labelled i-antigen has been separated by the techniques of column chromatography, immunoelectrophoresis and direct precipitation by specific antisera, and radioactivity in these preparations has been assayed by liquid scintillation counting and by autoradiography.

4. The most efficient method of labelling living paramecia was to introduce the radioactively-labelled amino acid by way of the bacterial food source. During the early stages of labelling (30-45 min.), the highest i-antigen specific activity was found in the membrane fraction. Labelled i-antigen was detectable on the pellicle and cilia after 1 hr. and thereafter accumulated at these

sites, whereas the labelled i-antigen associated with the membrane fraction increased little after 1 hr..

5. Paramecium post-mitochondrial supernatants have been shown to be capable of protein synthesis and the properties of this system have been characterized. Both the free ribosomes and the membrane fraction have been shown to be active components in the cell-free incorporating system. Labelled i-antigen has been detected after short periods of labelling (5 - 15 min.) and its synthesis was found to be mainly associated with the membrane fraction.

6. There was little release of labelled soluble protein in the cell-free system. Puromycin treatment has enabled the artificial release of up to 60% of the labelled protein. Some of this released labelled protein behaved immunologically and physically as native i-antigen.

7. The relationship between ribosomes with associated i-antigen activity and the membrane material has been studied. Free ribosomes derived from both in vivo and in vitro labelling experiments, have been detected with associated newly synthesised i-antigen activity. These ribosomes have been found to readily adsorb on to washed membrane fraction: yet the membrane-bound ribosomes appear to be firmly attached, since they were not separated from the membranous elements on prolonged centrifugation through dense sucrose. These membrane-bound ribosomes were, however, released on treatment with deoxycholate and some of the labelled i-antigen remained in

association with the released polysomes.

8. Radioactivity has been specifically precipitated from free ribosome fractions, as well as from the membrane fraction, by treating with antiserum against reduced-carboxymethylated i-antigen. In general, this antiserum reacted with smaller polysomes than did antiserum against native i-antigen. Also, the puromycin released protein reacting with this antiserum sedimented at a slower rate than did native i-antigen.

9. The bearing of these results on the mechanism of i-antigen synthesis has been discussed. The main site of i-antigen synthesis is on membrane-bound polysomes, although an earlier stage in the synthesis may occur on free polysomes which later adsorb on to the membranes. The completed i-antigen is then transported to the cell surface. This system is considered in relation to other protein synthesising systems.

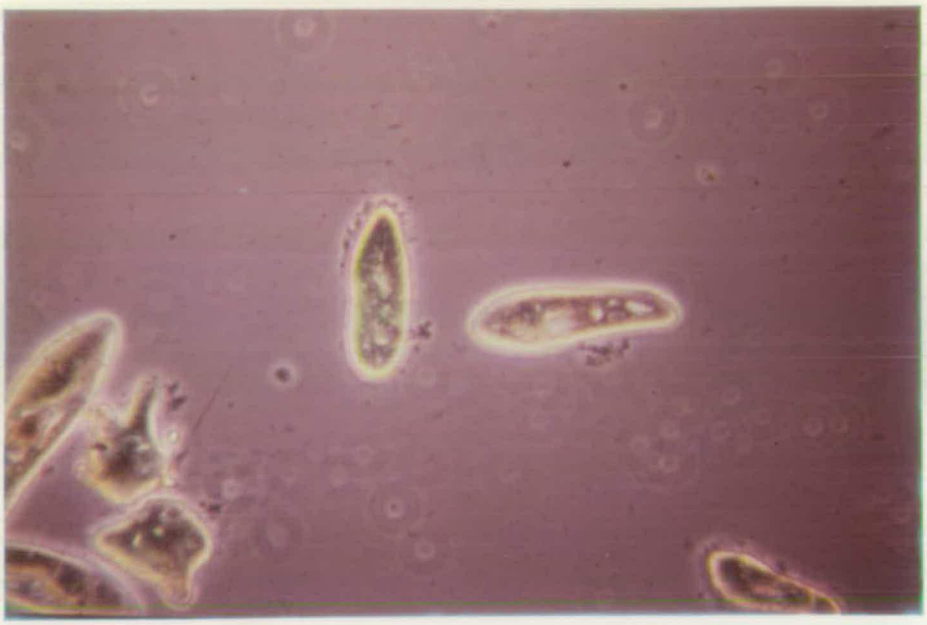


FIGURE 1 Immobilized and unimmobilized cells of Paramecium aurelia, variety 1. A mixed culture of equal numbers of 90D serotype and 90G serotype cells were treated with anti-90D serum stained with Congo red. After 10 min. half of the cells were immobilized. A sample from the culture was then stained with aceto-carmine/fast green and photographed in a phase contrast microscope. x 150. Both immobilized (left centre and bottom right) and unimmobilized (right centre and extreme left) paramecia can be seen. The immobilized paramecia show clumped cilia and a red-stained pellicle. The cilia of the unimmobilized paramecia are not visible and the pellicle is less stained. Two lysed cells also appear in this figure.

INTRODUCTION

Protozoan ciliates, particularly Paramecium aurelia, afford a convenient system for the study of two interesting and little understood phenomena; the mechanism of synthesis of a specific cell-surface protein, the immobilization antigen (i-antigen) and the control mechanism which governs which of a range of possible i-antigens is expressed.

It has been the purpose of this study to investigate the process of i-antigen synthesis and to consider this process in relation to other protein synthesising systems. Such a study would also provide an experimental approach to the control mechanism.

Diluted rabbit antiserum prepared against Paramecium aurelia immobilizes living organisms of the same type by causing agglutination of the cilia (Figure 1). However, clones grown from other stocks are not immobilized by this antiserum but produce their own specific antisera. Variation in serotype expression is also found within stocks, in clones grown under different environmental conditions.

In preliminary studies the ciliate Didinium nasutum was investigated for the presence of i-antigens. Here, different environmentally influenced serotypes were discovered which were specifically immobilized by antisera prepared

against cells of the same type. However, this organism was found to be less suitable than Paramecium aurelia in that the stocks available did not conjugate, making genetical analysis impossible. Genetically controlled i-antigen formation is known in Tetrahymena pyriformis (Nanney and Dubert, 1960; Nanney et al., 1963, 1964) but this organism is less efficient than Paramecium aurelia in stimulating antibody formation and, as yet, the i-antigens have not been recovered in a soluble form. It was decided to use Paramecium aurelia exclusively as the experimental organism.

Studies on whole paramecia treated with fluorescein-labelled antibody (Beale and Kacser, 1957) and with ferritin-conjugated antibody (Mott, 1963, 1965) have shown the surface coating of the pellicle and cilia to be antigenic in nature. That the i-antigen is mainly associated with, if not restricted to, these sites was demonstrated by extracting antigen from isolated cell fractions (Preer and Preer, 1959) and, more conclusively, by treating sections of paramecia with fluorescein-labelled antibody (Beale and Mott, 1962). There has been no conclusive evidence that immobilization antigen exists internally in the cell. In fact, Mott has shown that in paramecia in the process of changing serotype (transforming), the new i-antigen first appears on the pellicle. Later, however, it spreads to the cilia.

Immobilization antigens are soluble and extracts form precipitates with homologous antiserum in agar diffusion tests (Finger, 1956; Preer, 1956, 1959 a-d) but tend to give greater cross-reaction with heterologous antiserum by this method than is detected in immobilization reactions (Bishop, 1963). These antigens can be purified by fractional precipitation with ammonium sulphate (Preer, 1959d), by starch-gel electrophoresis (Bishop, 1961), or by column chromatography using cation exchangers (Bishop,1961; Bishop and Beale, 1960; Jones, 1965a,b; Jones and Beale, 1963) and have been shown to be proteins of high molecular weight (240,000 - 260,000). However, the best value of molecular weight is probably 310,000 (Steers, 1965). Reduction and alkylation of the many disulphide linkages (252 - 272 half-cystine residues) found in purified immobilization antigen yields fragments of about 35,000 molecular weight (Steers, 1965) or of more variable value, 16,000 - 87,000 (Jones, 1965a). Evidence from peptide mapping has indicated that the molecule is either a dimer or a trimer, each unit consisting of three non-identical chains giving six or nine chains in all.

Different i-antigens have different physical and chemical properties, similarities reflecting the extent of their immunological cross-reactions. Cross-reacting i-antigens are often closely related in solubility (Preer, 1959d), in

electrophoretic migration (Bishop and Beale, 1960; Steers, 1961) and in the fingerprints of their tryptic peptides (Steers, 1962; Jones and Beale, 1963). In general, allelic i-antigens have fewer differences in the above properties than do non-allelic i-antigens. Experiments involving the analysis of the type of i-antigen formed in heterozygotes (Finger and Heller, 1963, 1964a; Finger et al., 1965; Jones, 1965a) are consistent with the view of a random assortment of two or three non-identical chains.

As already mentioned, a series of i-antigens may be expressed in any one stock of paramecia. These antigens, only one of which can normally be detected in a single animal at a given time, are controlled by a series of unlinked genes. Transformations from the expression of one serotype to the expression of another can be facilitated by such changes in environment as temperature change, alteration of the food supply or change in ionic concentration. However, certain "cytoplasmic" factors tend to perpetuate the expression of the pre-existing type. The "cytoplasmic state" is itself influenced by the nuclear genes present and the past history of the clone. The genetics of this system have been extensively reviewed (Beale, 1957).

Thus, Paramecium aurelia provides, in the synthesis of its surface antigens, a system suitable for the study of the

biosynthesis of a family of large and complex proteins. Although both the genetic basis of i-antigen determination and the nature of the i-antigen molecule have been extensively studied, little is known of the biochemical nature of the intermediates, the cellular components involved, and how they interact to give rise to a precisely controlled synthetic process. In other words, the vague terms mentioned above, describing i-antigen expression, need to be translated into a biochemical description of the system.

It is the purpose of this study to examine methods for the detection of the small amounts of newly synthesised i-antigen and to use these methods in experiments designed to be informative about the sites and kinetics of i-antigen synthesis. The general approach decided upon was to measure the incorporation of C^{14}-labelled amino acids into protein immunologically identified as i-antigen. Both living cells and "in vitro" systems have been examined for their synthetic potential. Since the nature of the study involves subcellular fractionation, a section is devoted to the identification and characterization of these fractions.

MATERIALS AND METHODS

1. Materials.

'SE-Sephadex C-50' is a product of Pharmacia, Uppsala; 'Ionagar' and 'Membrane Filters' are products of Oxoid, London. Sheep precipitating serum was purchased from Wellcome Research Laboratories, Beckenham. 'Drakeol 6VR' and 'Arlacel' were gifts from Dr W.J.Herbert. ATP, GTP, yeast soluble RNA, creatine phosphate, creatine phosphokinase (EC 2.7.3.2.), L-amino acids, 2-mercaptoethanol, bovine serum albumin, DLα-lecithin, haemoglobin and catalase were purchased from Sigma Chemical Company, London, and Tris ('Sigma 7-9') was used for the pH7.6 Tris-HCl buffer. Ribonuclease, 5 x crystallized and 'Tween-80' were products of Koch-Light Laboratories Ltd., Colnbrook. Actinomycin D and Chloramphenicol were gifts from Merck, Sharp and Dohme Inc., New Jersey, and Parke, Davis and Company, London, respectively. Puromycin was obtained from Lederle, New York. Uniformly labelled L-leucine-C^{14}(165mC/m.mole), L-phenylalanine-C^{14} (252mC/m.mole) and L-cystine-S^{35}(58mC/m.mole), and protein hydrolysate-C^{14}(640μC/mg.) were obtained from the Radiochemical Centre, Amersham. The scintillators, 2,5-diphenyloxazole - PPO - and 2-p-phenylbis(4-methyl-5-phenyloxazole) - dimethyl POPOP - were purchased from Nuclear Enterprises (GB) Ltd., Edinburgh. 'Araldite' is a product of Ciba (ARI) Ltd.,

Cambridge; 'Royal Blue' film and 'DX-80' developer are products of Kodak Ltd., London.

All other chemicals used were of analytical reagent grade.

2. Solutions.

Wash buffer:	0.013M - NaCl
	0.003M - KCl
	0.003M - $CaCl_2$
	0.004M - phosphate (pH 6.8)
Homogenization buffer:	0.025M - sucrose
	0.100M - Tris-HCl (pH 7.6)
	0.050M - KCl
	0.010M - $MgCl_2$
	0.010M - 2-mercaptoethanol
Gradient and Dialysis buffer:	0.050M-- Tris-HCl (pH 7.6)
	0.025M - KCl
	0.005M - $MgCl_2$
	0.005M - 2-mercaptoethanol

3. Culture Methods.

Paramecium aurelia, variety 1, stock 90 was grown at either 31°C (stable for the D serotype) or 18-24°C (stable for

the G serotype) in either lettuce or grass infusion bacterized with Aerobacter aerogenes and buffered with Na_2HPO_4 at pH 6.8. Normally, cultures of the required serotype were maintained in lettuce medium. When growing and dividing cells were required for an experiment, one volume of lettuce medium containing stationary-phase paramecia was diluted with one volume of fresh grass medium and dispensed in 1 litre portions into Thomson bottles. Exponentially growing cells were obtained after 18-24 hr.. Batch cultures (50-100 l.) were grown according to Jones (1965).

4. Harvesting.

Batch cultures were collected in an Alfa-Laval cream separator after filtering through muslin. Smaller (2-5l.) growing and dividing cultures were filtered through absorbent cotton wool and concentrated by centrifugation at 1000g for 1 min. on an M.S.E. oil-testing centrifuge. All concentrates were pooled, washed at least twice with 100 ml. of wash buffer and once with 100 ml. of the buffer the cells were being transferred to. Generally, 2-3 l. of culture yield 1 ml. of packed cells.

5. Extraction and purification of i-antigens (Jones, 1965).

Concentrated living paramecia were treated with 4 volumes of 15% ethanol in 0.045% NaCl for 1 hr. at 2-3°C (Preer, 1959b),

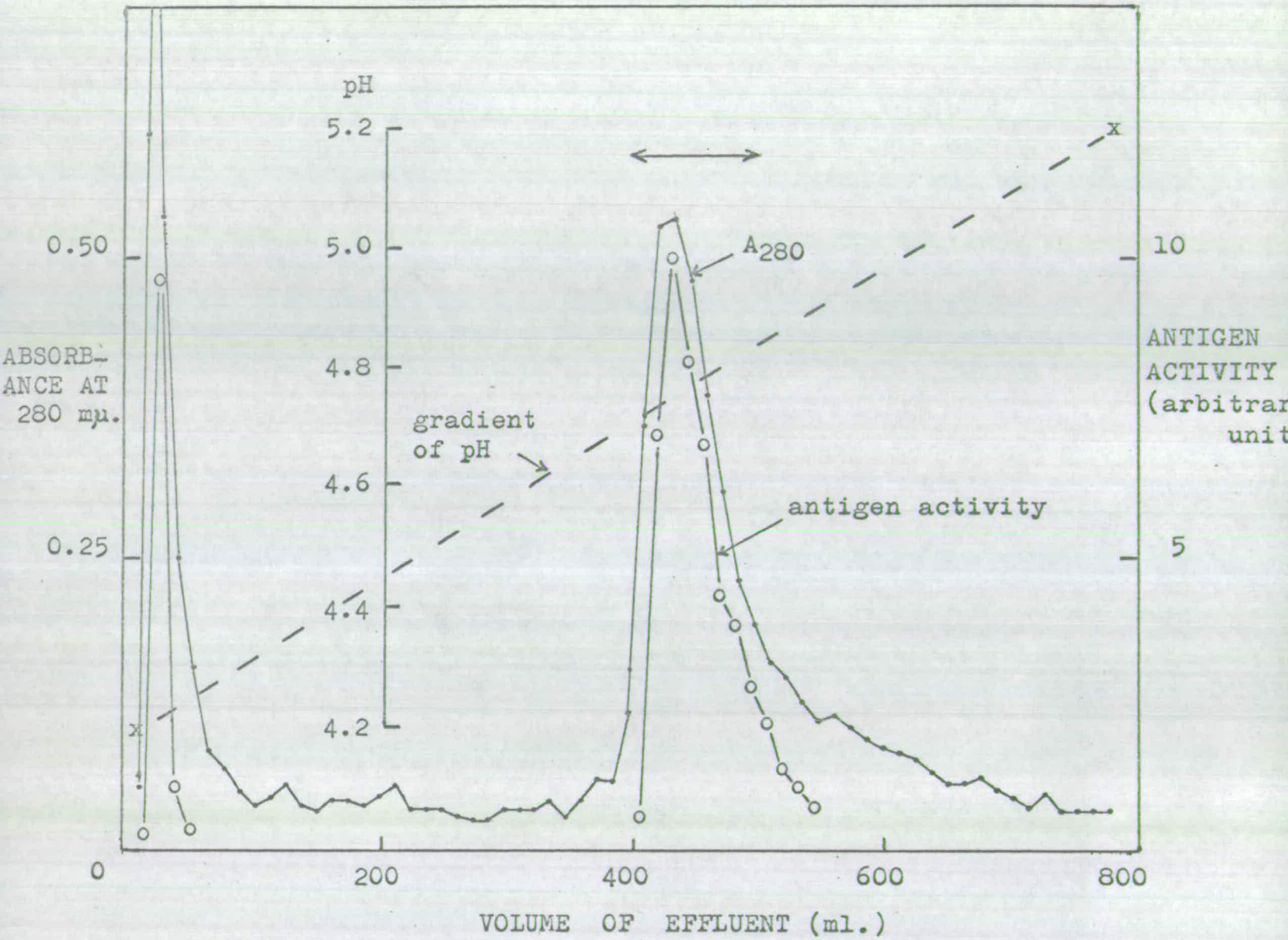


FIGURE 2. Elution diagram of column chromatography purification of i-antigen (90G serotype)
Column: SE-Sephadex
Gradient: 800 ml. of 0.05M - sodium acetate between pH 4.2 and 5.2.
The material within the arrows was pooled, dialysed and lyophilized.

then centrifuged at 20,000g for 10 min.. The clarified supernatant was adjusted to 75% saturation by adding solid $(NH_4)_2SO_4$ and stirred overnight at 2-3°C. The precipitate was collected by centrifugation at 10,000g for 15 min., resuspended in a minimal volume of water, dialysed for 48 hr. against several changes of distilled water and finally lyophilized. SE-Sephadex C-50, after washing and swelling, was suspended in 0.05M sodium acetate buffer, pH 4.2, and poured on to a column to give a packed bed 1.5 x 15 cm.. After equilibrating the column by washing overnight with the starting buffer, the protein was applied as a 3% solution and elution was performed with 500 or 800 ml. of 0.05M sodium acetate buffer, giving a linear gradient of pH 4.2 to 5.2. The eluted material was monitored by measuring absorbance at 254 mμ using an LKB Uvicord 4701A Control and Optical Unit coupled to a Beckman DB Spectrophotometer with 24 hr. chart recorder. A typical elution is shown in Figure 2. Peak fractions were re-read for absorbance at 280 mμ and antigen activity was determined (Bishop, 1963). Fractions containing i-antigen activity were pooled, dialysed against distilled water and lyophilized.

6. Assay of i-antigen activity (Bishop, 1963).

Equal volumes (normally 0.1 ml.) of the solution to be assayed and diluted antiserum and homologous paramecium culture

were mixed in a depression slide and the time taken for the paramecia to be completely immobilized (t) was measured. The immobilization time with the antigen solution replaced by wash buffer (t_o) was also measured. Then the activity of the antigen solution, in arbitrary units, (g), is given by:

$$g = 3(1/t_o - 1/t)$$

This method is only valid if:

$$3 \text{ min.} \leqslant t \geqslant 10 \text{ min. and } t \leqslant 2t_o.$$

These conditions are satisfied by adjusting the concentration of the antigen solution.

7. Preparation of antisera.

Multiple emulsions were prepared by the method of Herbert (1965). A solution of purified i-antigen in 0.9% saline was incorporated into a water-in-oil emulsion. Nine parts 'Drakeol 6VR' were mixed with one part of the emulsifier 'Arlacel A' as the oil phase. Equal volumes of oil and i-antigen solution were thoroughly mixed by cycling them through a hypodermic syringe fitted with a 0.6 mm. bore needle until a thick viscous emulsion was formed. The water-in-oil emulsion was then re-emulsified in an equal volume of 2% 'Tween-80' in 0.9% saline and recycling through the syringe was continued until a free-flowing liquid was obtained. Production of the required type of multiple emulsion was confirmed by microscopic

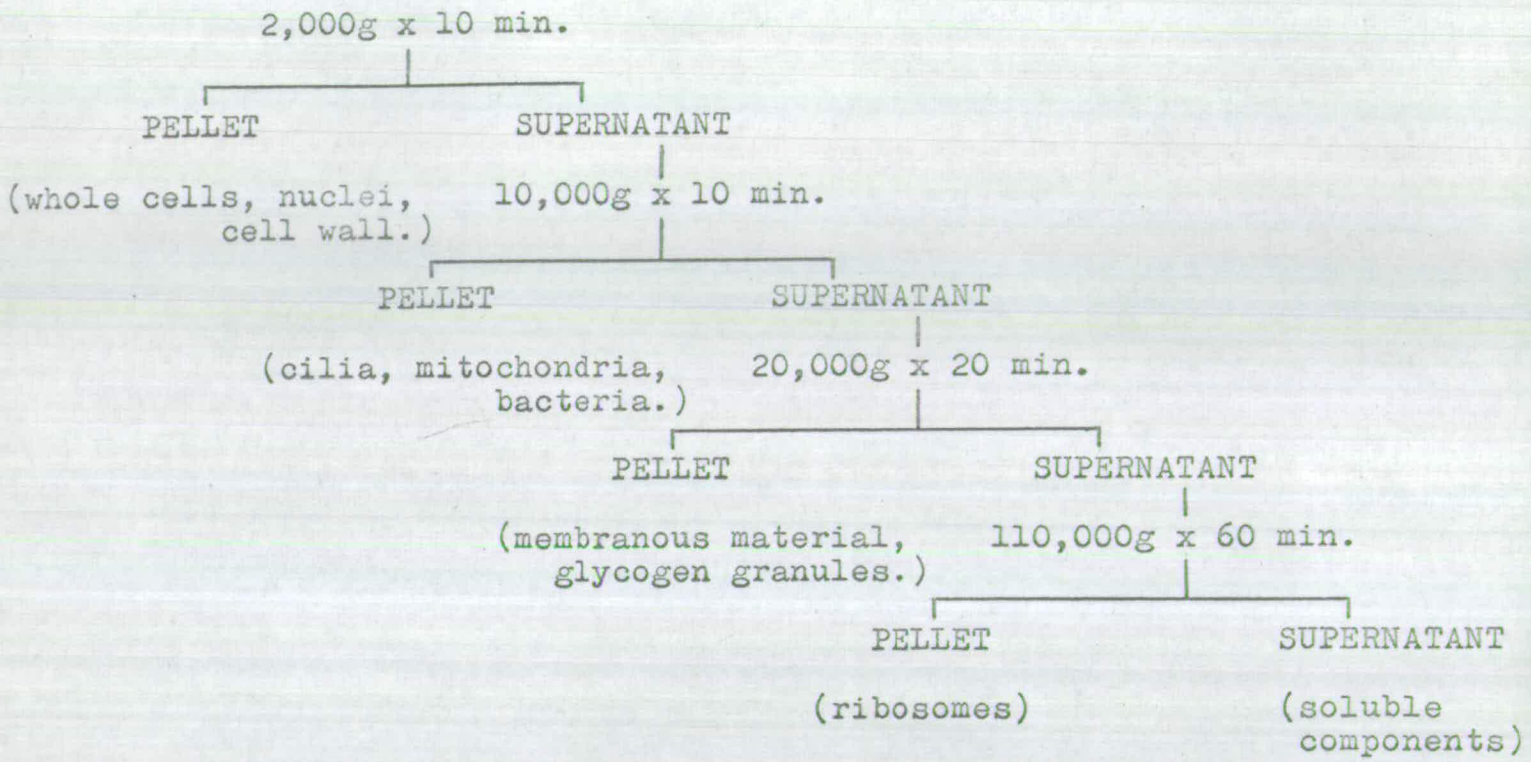


FIGURE 3. Flow-diagram of fractionation of paramecium homogenates.

examination.

Rabbits of 9-12 months were injected subcutaneously with between 2 and 5 mg. purified i-antigen contained in a total volume of 2 ml. of multiple emulsion. Six weeks after the injection, a blood sample was taken from the marginal ear vein. The blood was allowed to clot, the serum drawn off, heated at 56°C for 30 min. to inactivate the complement and dialysed overnight at 2-3°C against 0.9% saline. If a reasonable titre was obtained (over 1:800), bleedings were continued weekly.

8. Homogenization and fractionation.

One volume of washed cells was added to 4 volumes of ice-cold homogenization buffer. All subsequent procedures were carried out at 2-3°C. Homogenization was performed with a 'Tri-R' Teflon Homogenizer until over 95% of the cells were broken (10-20 strokes at speed 8). The homogenate was then fractionated by centrifugation according to the flow-diagram, Figure 3. The composition of the fractions, particularly the low-speed pellets, was checked by microscopic examination and by reference to other work (Preer and Preer, 1959). However, the principal fractions used in this study were contained in the supernatant after centrifugation at 10,000g for 10 min.. This supernatant did not contain visible particles on examination with the phase-contrast microscope and is sometimes referred

to as the 'post-mitochondrial' fraction. The characterization and composition of this material is reported later in Results Section I.

9. Sucrose gradients.

'Post-mitochondrial' supernatants were often further separated by centrifugation through sucrose density gradients. Gradient tubes were prepared by introducing a 5 ml. cushion of 60%(w/w) sucrose and overlaying this with a 10 to 25% (w/w) linear sucrose gradient of 25 ml. volume using a technique similar to that described by Britten and Roberts (1960). The sucrose solutions were made up to give the final concentration of components in gradient buffer (see Solutions). Gradients were allowed to stand at 2-3°C for 2 to 4 hr. before centrifugation. One or 1.5 ml. samples were layered on the top of the gradients. Centrifugation, in the M.S.E. 3 x 40 ml. rotor, was for 2 to 2½ hr. at 20,000 r.p.m. at 2°C. The tubes were punctured at the bottom and 2 ml. fractions were collected after monitoring the absorbance at 254 mμ of the effluent. The flow rate was controlled with a Sigma Motor Pump at between 0.5 and 1 ml./min.. Absorbancy of the fractions was remeasured at 260 mμ.

Density gradient analysis was also used in the study of the sedimentation of proteins. In this case, sucrose gradients

were prepared as above but with 5 to 20%(w/w) gradients and a total volume of 20 ml.. The sample volume was 1 ml.. Centrifugation in the M.S.E. 3 x 20 ml. swing-out rotor was for 24-30 hr. at 30,000 r.p.m. and 1 ml. fractions were collected.

10. Electron microscopy. (Kuff et al., 1966).

Fractions derived from sucrose gradient analysis were pooled, mixed and centrifuged for 3 hr. at 42,000 r.p.m. in the M.S.E. 10 x 10 ml. rotor. The pellets were fixed for 1 hr. in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, containing 15%(w/w) sucrose and 2.5mM $MgCl_2$. The pellets were then washed in changes of cacodylate buffer for 15 min., 1 hr., then overnight. Postfixation was carried out for 1 hr. in 1% OsO_4 in 0.1M phosphate buffer at pH 7.4. After dehydration in ethanol for 1½ hr., the pellets were embedded in 'Araldite', polymerized by heating at 48°C for 48 hr. and cut in 200-400A° sections. The sections were stained with 1% $KMnO_4$ in 2.5% uranyl acetate and photographed using a Phillips '75' or an A.E.I. 'E.M.6' electron microscope.

11. Chemical analysis.

Ultraviolet absorption measurements were made at 260 mμ and 280 mμ in a Beckman DB spectrophotometer to assay RNA and

protein respectively. Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Lipids were extracted by the method of Folch et al. (1957) in chloroform-methanol (2:1). The chloroform phase was concentrated to dryness and total lipid was determined by the method of Bloor (1947) using DLαlecithin as a standard.

12. Incorporation of labelled amino acids.

a) Exogenous labelling of living cells.

Preliminary experiments in labelling paramecia were conducted by simply adding C^{14}-labelled amino acids to paramecia suspended in a non-nutrient solution. Since the presence of bacteria would confuse the incorporation kinetics and washed paramecia were never completely freed from bacteria, it was necessary to add a bacteriostatic drug. Washed paramecia were suspended in 20 volumes of a salts solution adapted from van Wagtendonk (1955).

	Final concentration (µg./ml.)
$CaCl_2.2H_2O$	20
$MgSO_4.7H_2O$	40
$Fe(NH_4)_2(SO_4)_2.6H_2O$	10
Na_2EDTA	20
KH_2PO_4	570
K_2HPO_4	570

Chloramphenicol was added to a final concentration of 100 µg./ml. and C^{14}-labelled phenylalanine (10 mC./m.mole) was added to give 0.25 µC./ml..

b) Labelling paramecia by feeding them labelled bacteria.

The general procedure for the labelling of bacteria was that suggested by Roberts et al. (1955). Aerobacter aerogenes were cultured in a glucose/salts solution (Davis, 1950):

	Final concentration (mg./ml.)
sodium citrate.$5H_2O$	0.5
$MgSO_4.7H_2O$	0.1
$(NH_4)_2SO_4$	1.0
K_2HPO_4	7.0
KH_2PO_4	2.0
glucose	1.0

This amount of glucose is sufficient for the growth of only 1 mg./ml. wet weight bacteria, and exponential growth stops without entering stationary phase when the glucose is exhausted. After incubating overnight at 37°C, the culture was centrifuged at 12,000g for 10 min. and the pellet was rinsed with 0.9% NaCl. The bacterial pellet was then resuspended in sufficient glucose/salts solution to allow 2 generations of growth and C^{14}-leucine (155 mC./m.mole) was added to give a concentration of 0.6 µC./ml. of culture. The labelled s culture was incubated at 37°C for 1 hr., then chilled and

centrifuged at 12,000g for 5 min.. The pellet was rinsed, resuspended in saline and re-centrifuged. The labelled bacteria were finally suspended at a concentration of 10 mg./ml. in wash buffer. Radioactivity of the bacterial protein was generally about 3×10^5 c.p.m./mg. wet weight bacteria.

Washed paramecia were suspended in 20 volumes of wash buffer and the labelling period was started by the addition of C^{14}-leucine labelled bacteria, sufficient to maintain optimal feeding during the course of the experiment. The conditions of labelling paramecia were usually 1 mg. bacteria/ml. suspension, incubated at 30°C for 15 or 30 min.. The paramecia were then washed free from the radioactive bacteria and resuspended in 200 volumes of "cold" bacterized grass medium to give "chase" conditions. Samples of labelled paramecia were then removed at the required times.

c) Amino acid incorporation by cell-free systems.

'Post-mitochondrial' supernatants (see Fractionation) were dialysed against dialysis buffer for 3 hr. at 2-3°C then tested for their ability to incorporate C^{14}-labelled amino acids into acid-insoluble material. The complete reaction mixture, unless otherwise stated, contained per ml. :
50 µ.moles Tris-HCl buffer (pH 7.6), 25 µ.moles KCl, 5 µ.moles $MgCl_2$, 5 µ.moles 2-mercaptoethanol, 0.3 µ.mole ATP, 10 µg. creatine phosphokinase, 5 µ.moles creatine phosphate,

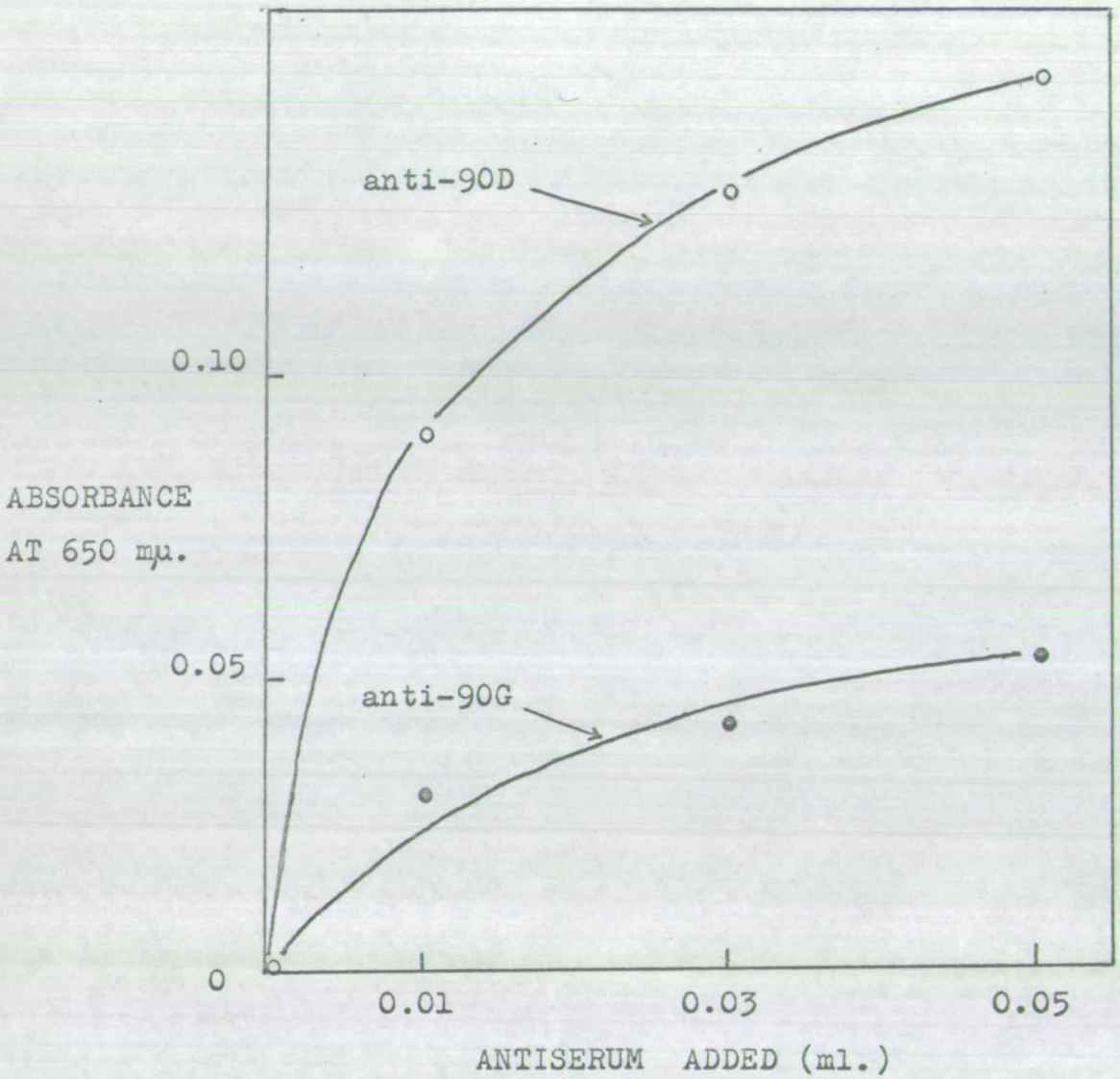


FIGURE 4. Specificity in precipitate formation by homologous antiserum. Increasing amounts of homologous (anti-90D) and heterologous (anti-90G) antisera, of the same titre, were added to 0.05 mg. 90D i-antigen in a volume of 2 ml.. After incubation at 37°C for 1 hr. the absorbance at 650 mμ. was measured.

0.2 μ.mole GTP, 6 mμ.moles (1 μC) C^{14}-leucine, 10 mμ.moles each of 17 amino acids. Each ml. of reaction mixture contained, in addition, 0.2 or 0.4 ml. of dialysed supernatant. After incubation at 30°C, 0.6 μ.mole of C^{12}-leucine was added and the reaction mixture was chilled.

13. Separation of labelled i-antigen from other labelled proteins.

a) Precipitin reaction in solution.

The method used was basically that of Glick et al. (1958). Figure 4 shows the effect of precipitation by adding increasing amounts of homologous and heterologous antiserum to a standard amount of i-antigen solution. The antigen standard was 0.05 mg. in 1 ml. of 0.9%(w/v) saline. The antiserum added was ranged from 0.01 to 0.05 ml. and had a titre of 1/800. The mixture was agitated using a vortex mixer ('buzzed'), incubated at 37°C for 1 hr., left overnight at 2-3°C and 'buzzed' again before measuring the absorbance at 650 mμ. Specificity in such precipitation experiments was always observed.

For the purpose of demonstrating radioactive i-antigen by specific precipitation, it was necessary to examine the possibilities of an adequate control to ensure a reliable estimate of non-specific precipitation. This problem has been encountered by other workers (e.g. Scharff et al., 1963; Scharff and Uhr, 1965; Williamson and Askonas, 1967) and the

controls used here are similar to theirs.

The radioactive samples, to be assayed for labelled i-antigen, were treated by the method described above, using 0.05 ml. homologous antiserum in the experimental tubes and 0.05 ml. heterologous antiserum (or normal rabbit serum) in the control tubes. After incubation at 37°C for 1 hr. the tubes were chilled. In order to produce an equal amount of precipitate in both experimental and control tubes, and also to detect any soluble antigen-antibody complexes, an excess of sheep anti-rabbit serum was then added to all tubes. This technique yielded precipitates sedimenting equal amounts of non-specific radioactivity. After incubating for 1 hr. at 37°C, the precipitates were sedimented by centrifugation at 4,000g for 30 min.. The precipitates were washed twice by centrifugation at 2-3°C through 0.9% saline and the pellet was finally suspended in 0.1 ml. saline and protein was precipitated, washed and counted.

All values for radioactivity precipitated with homologous antiserum were corrected for the non-specific precipitation found in the corresponding controls. The degree of non-specific precipitation of radioactivity varied with different types of sub-cellular preparation being assayed for radioactive i-antigen (see Table 1). This effect was presumably due to the differing stabilities of the preparations, as well as to the different

FRACTION	RADIOACTIVE PROTEIN (counts/min.)		NON-SPECIFIC
	ANTISERUM PRECIPITATE	CONTROL SERUM PRECIPITATE	PRECIPITATION (%)
DEOXYCHOLATE(0.5%)	545	176	32
TREATED MEMBRANE	699	203	29
	971	343	35
RIBOSOME	173	78	45
	239	92	39
	365	175	46
SOLUBLE	434	129	30
	631	207	33
	1009	308	31
SALT/ALCOHOL	325	72	22
EXTRACT	532	132	25

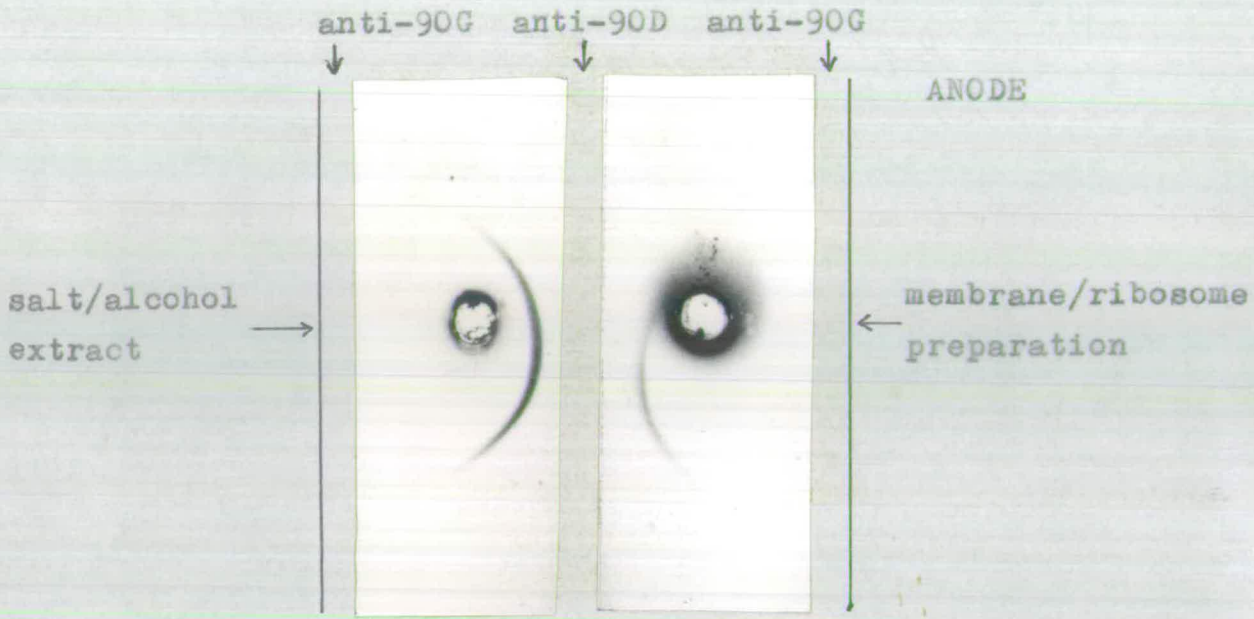
TABLE 1. Specificity in precipitating labelled i-antigen from various types of paramecium sub-cellular extracts. All preparations were derived from paramecia labelled by way of bacteria as for Figure 17. The serotype of the paramecia was 90D, and the antiserum was anti-90D and the control serum was anti-90G.

amounts of cross-reacting materials present. For instance, when ribosome preparations were being treated with antiserum, it was necessary to maintain them in dialysis buffer since spontaneous precipitation occurred in saline. Also, the time of treatment was kept to a minimum i.e. all reactions up to the stage of protein precipitation were performed on the same day. Apart from the relatively high degree of non-specific precipitation (2% to 4%), the precipitation technique was found to be suitable for assaying radioactive i-antigen activity in sub-cellular fractions.

b) Microimmunoelectrophoresis (Grabar and Williams, 1955)

Microscope slides, 3" x 1" were cleaned and dipped into hot 0.2% agar in distilled water, drained and air dried. They were then placed, eight at a time, in a perspex tray and covered with 1% agar in 0.0125M borate buffer pH 8.2 to form a 2 mm. layer. Two holes 1 cm. apart were pierced through the centre of the agar slab and the antigen preparation was applied. Electrophoresis was performed with 0.025M borate buffer pH 8.2, 20 volts/cm. being applied for between 1½ and 3 hr.. For the immunological reaction, filter paper strips, 2 mm. wide, were soaked in antiserum and layed lengthwise on the agar, 2-3 mm. on either side of the wells. By this procedure, one sample could be tested for reaction with both homologous and heterologous antisera. The slides were then

(a) IMMUNOELECTROPHORESIS: stained gels with i-antigen precipitin arcs.



(b) AUTORADIOGRAPHY: assay of radioactivity in i-antigen precipitin arcs.

exposed for 4 weeks in contact with X-ray film.

FIGURE 5. Immunoelectrophoresis and autoradiography of preparations derived from labelled 90D cells. The paramecia were labelled by way of bacteria as for Table 3 (90 min. chase). Membrane/ribosome preparations always gave more precipitation of labelled material in the region of the well than did salt/alcohol extracts or soluble fractions.

left in a humidity chamber at room temperature for 24 hr. for precipitin arcs to form by double-diffusion. Non-precipitated material was washed out of the agar by treating with isotonic veronal, pH 7.4, for 24 hr.. The precipitin arcs were then stained with Naphthalene Black 12B200 and destained with methanol-acetic acid-H_2O (5:1:5). The slides were dried at room temperature. A typical stained preparation is shown in Figure 5a.

14. Assay of radioactivity.

a) Liquid scintillation counting.

If the protein concentration of the radioactive sample was low, 0.1 ml. of 1% serum albumin was added as "carrier". To the chilled sample, NaOH was added to a final concentration of 2N and, after 2 min. at 37°C, the mixture was again chilled and treated with trichloroacetic acid to yield a final concentration of 5% of the acid. After 30 min. at room temperature, the precipitates were collected on 2 cm. diameter membrane filters, washed eight times with 2 ml. of 5% TCA, twice with 2 ml. ethanol and ether (1:1) and once with 2 ml. ether. The filters were then dried in a stream of hot air and placed in 20 ml. vials each containing 15 ml. of scintillation fluid : 0.5% (w/v) PPO, 0.03% (w/v) dimethyl)-POPOP in toluene. Radioactivity was measured in a Nuclear

Chicago 'Unilux' Bench-top Liquid Scintillation Counter. Efficiency in counting quenched C^{14}-protein samples on filters was 45% and the background was less than 30 counts/min..

b) Autoradiography.

After immunoelectrophoresis of radioactively labelled preparations, autoradiographs of precipitin arcs were made by exposing the slides supporting the dried agar film to 'Royal Blue' X-ray film. The surface of the agar and the film were held firmly in contact between two thick glass plates. Exposure time was for 4-8 weeks. Manipulations and development were carried out in total darkness. All exposed films were developed for 4 min. at 20°C in DX-80 developer. An autoradiograph of precipitin arcs is shown in Figure 5b.

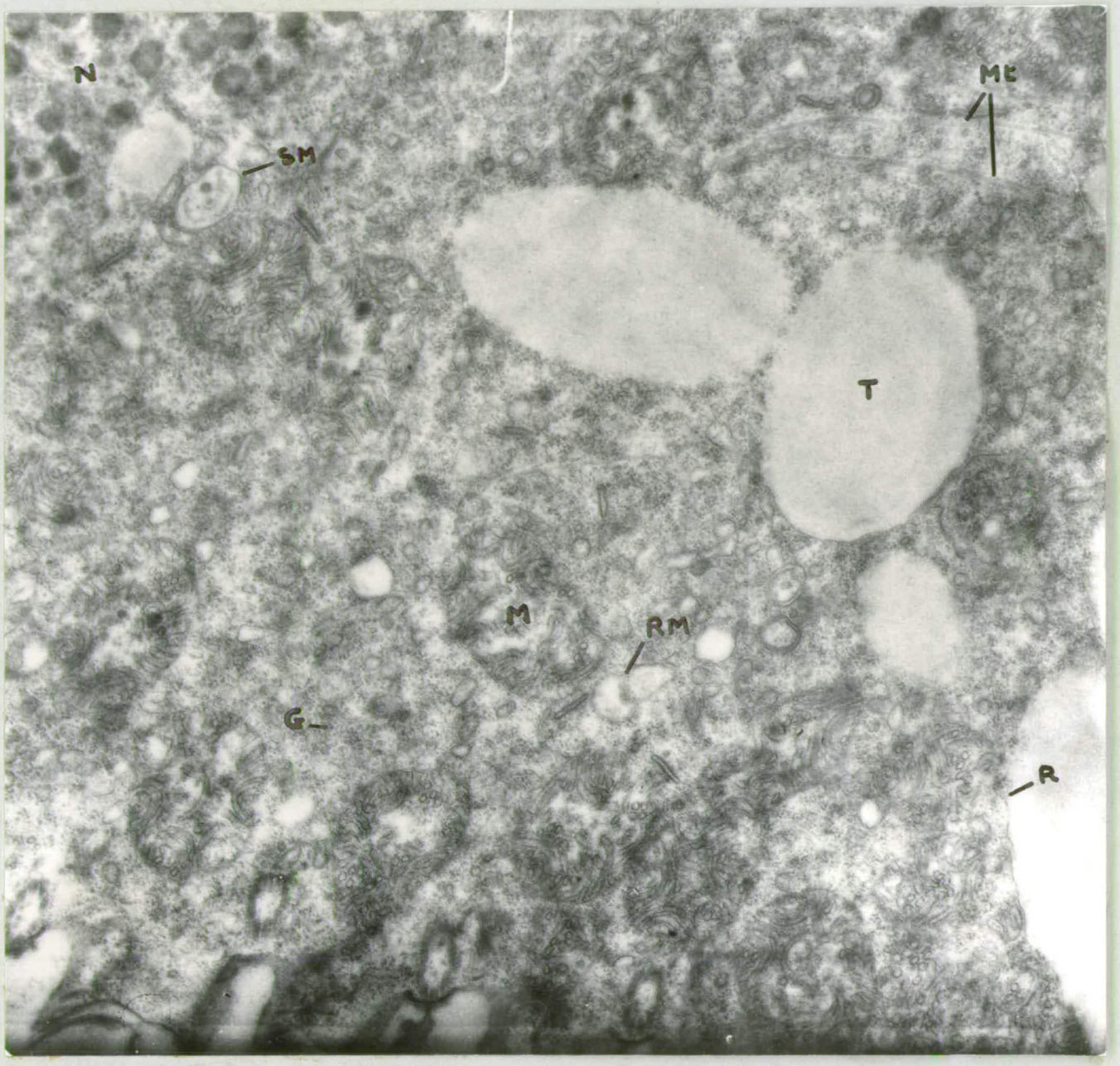


FIGURE 6. Electron micrograph of the cytoplasm of Paramecium aurelia. x 30,000.

KEY:
G - glycogen granules
N - nucleus
M - mitochondrion
Mt - microtubule
R - ribosomes
RM - 'rough'membrane
SM - 'smooth'membrane
T - trichocyst

RESULTS

The experimental results have been divided into three parts. Part I deals with the characterization of the components present in homogenates of Paramecium aurelia. This material is later investigated for newly synthesised immobilization antigen formed by living paramecia (Part II) and by cell-free protein synthesising systems (Part III).

Part I : Characterization of Paramecium Homogenates.

In a study involving the mechanism of protein synthesis, it is essential, first of all, to establish the properties and nature of the extracted cytoplasmic material and to ascertain the purity of the preparations. Since proteins appear to be synthesised in association with either free aggregates of ribosomes, as in cells lacking a definite endoplasmic reticulum such as mammalian reticulocytes, or ribosomes bound to membranes of the endoplasmic reticulum, as in liver cells, it is of particular interest to locate, separate and characterize these, or similar structures in Paramecium aurelia.

The ultrastructural organization of paramecium cytoplasm has been examined by electron microscopy (Sinden, R.E., unpublished). Both free particles and membrane-bound particles resembling

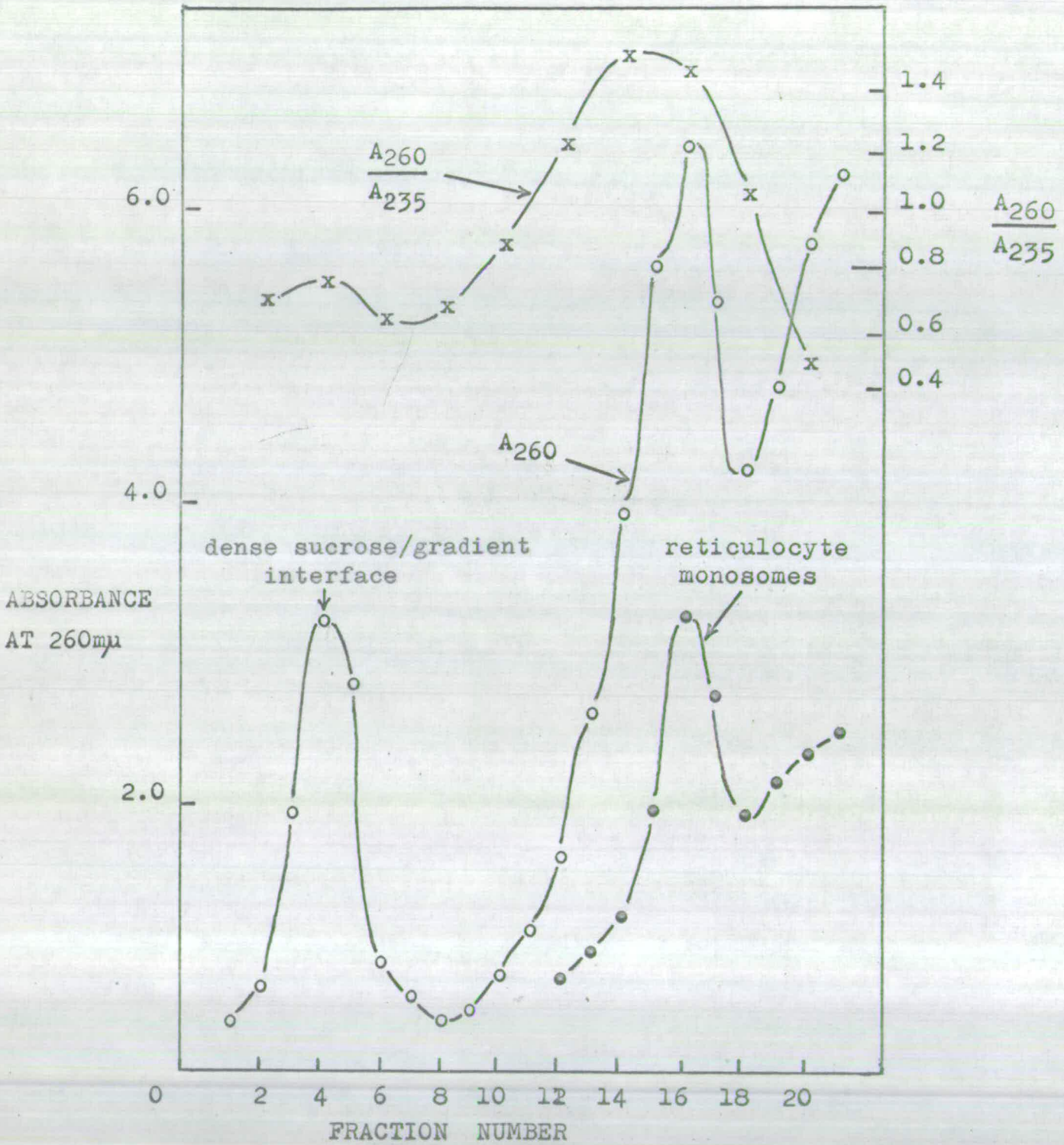


FIGURE 7 Absorbancy profile of paramecium 10,000g supernatant fractionated by centrifugation on a sucrose density gradient. Reticulocyte monosomes were used as a 78s marker.

ribosomes have been observed, but quantitative estimates of the proportion of the two forms has been difficult due to the density of the cytoplasm which contains numerous glycogen granules. As a reference for the later plates, an electron micrograph of the cytoplasm of Paramecium aurelia is shown (Figure 6).

1. Sucrose gradient analysis.

Paramecium homogenates, partially clarified by centrifugation at 10,000g for 10 min., were layered on linear sucrose gradients. After centrifugation, sedimentation patterns were obtained (Figure 7). The plot of absorbance at 260 mμ was based on the profile obtained by automatically monitoring the passage through a flow cell during sampling. A roughly bimodal distribution of absorbing material was observed, one peak forming near the bottom of the tube, another near the top. In all, three regions could be distinguished : (a) fractions 2 to 7 including the dense sucrose cushion and the cushion-gradient interface, were markedly white and turbid in appearance and gave a peak of absorbance at 260 mμ. (b) fractions 8 to 17 contained a series of ascending shoulders of absorbing material, reaching a peak in fraction 16 which dropped sharply on the side near the top of the tube. These fractions, representing practically the whole of the gradient, contained colourless material. (c) fractions 18 to 21 including the original sample

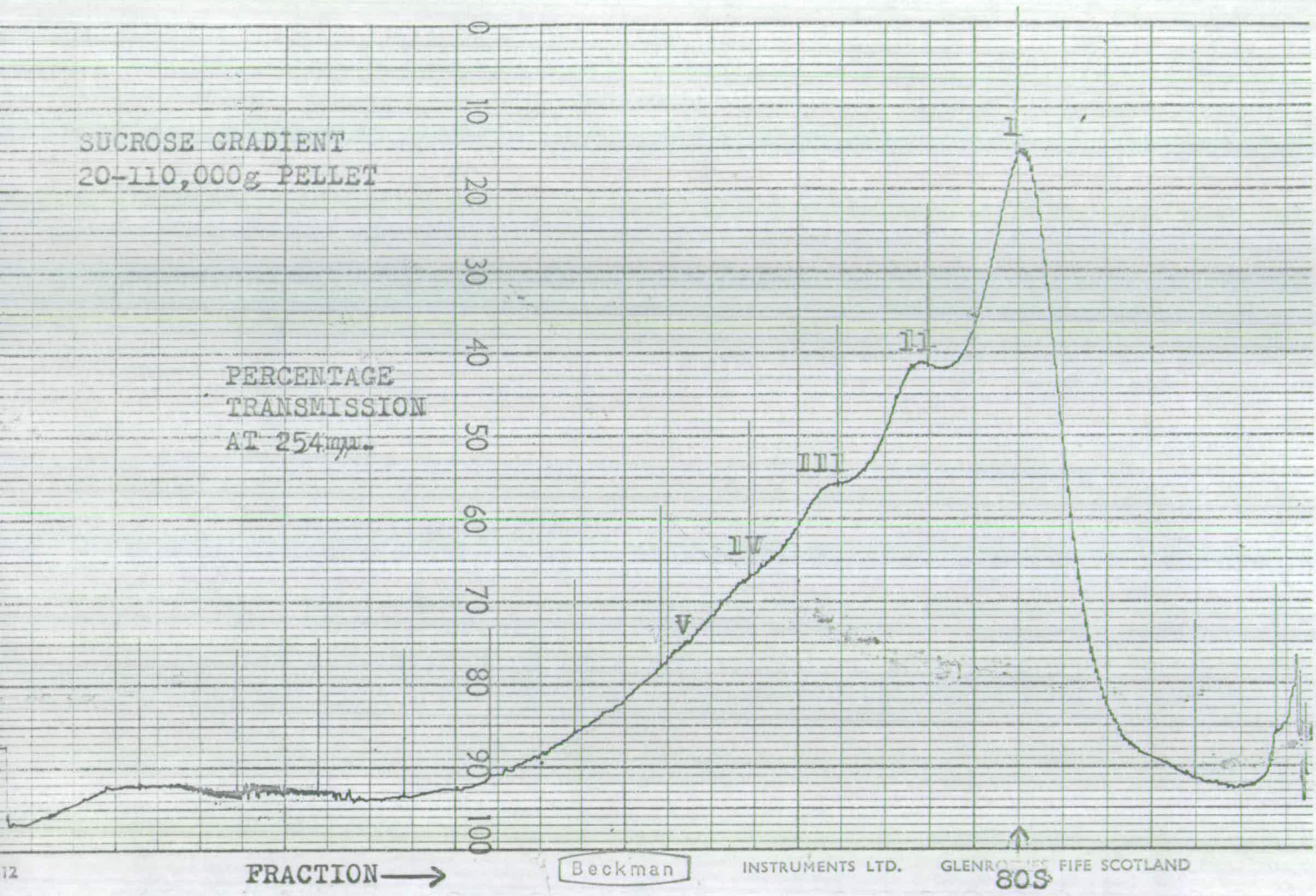


FIGURE 8 Absorbancy profile of a paramecium isolated ribosome preparation fractionated by centrifugation on a sucrose density gradient. The effluent was monitored by passage through a flow cell recording A_{254}mμ. Polysome peaks are numbered II to V.

layer gave a reading of absorbance at 260 mμ which increased towards the top of the tube. This region was visibly pink.

The sedimenting material was calibrated by centrifuging reticulocyte ribosome preparations, kindly supplied by Dr J.O. Bishop, through identical gradients. Reticulocyte ribosome fraction P2-4 (Bishop, 1965) consists largely of single ribosomes (monosomes), approximately 70% monosomes, 10% dimers and 20% subunits. This material has a peak sedimentation coefficient of 78S. When compared with the paramecium preparations, it was found that the reticulocyte monosome peak corresponded with the main peak in the paramecium gradients, and that the rates of sedimentation were identical. It was concluded that the peak at approximately 78S in the paramecium gradients consisted of single ribosomes whereas the shoulders occuring down the gradient represent ribosomal aggregates (polysomes) of increasing size. In continuous traces (Figure 8) it was possible to identify individual polysomal peaks up to the size of quadramers, or pentamers. In this preparation the interface peak has been removed by centrifuging at 20,000g for 20 min., and the ribosomes have been pelleted by centrifuging at 110,000g for 60 min. and resuspended by gentle homogenation before applying to the gradient.

The ratio of absorbance at 260 mμ to the absorbance at

Facing p.28

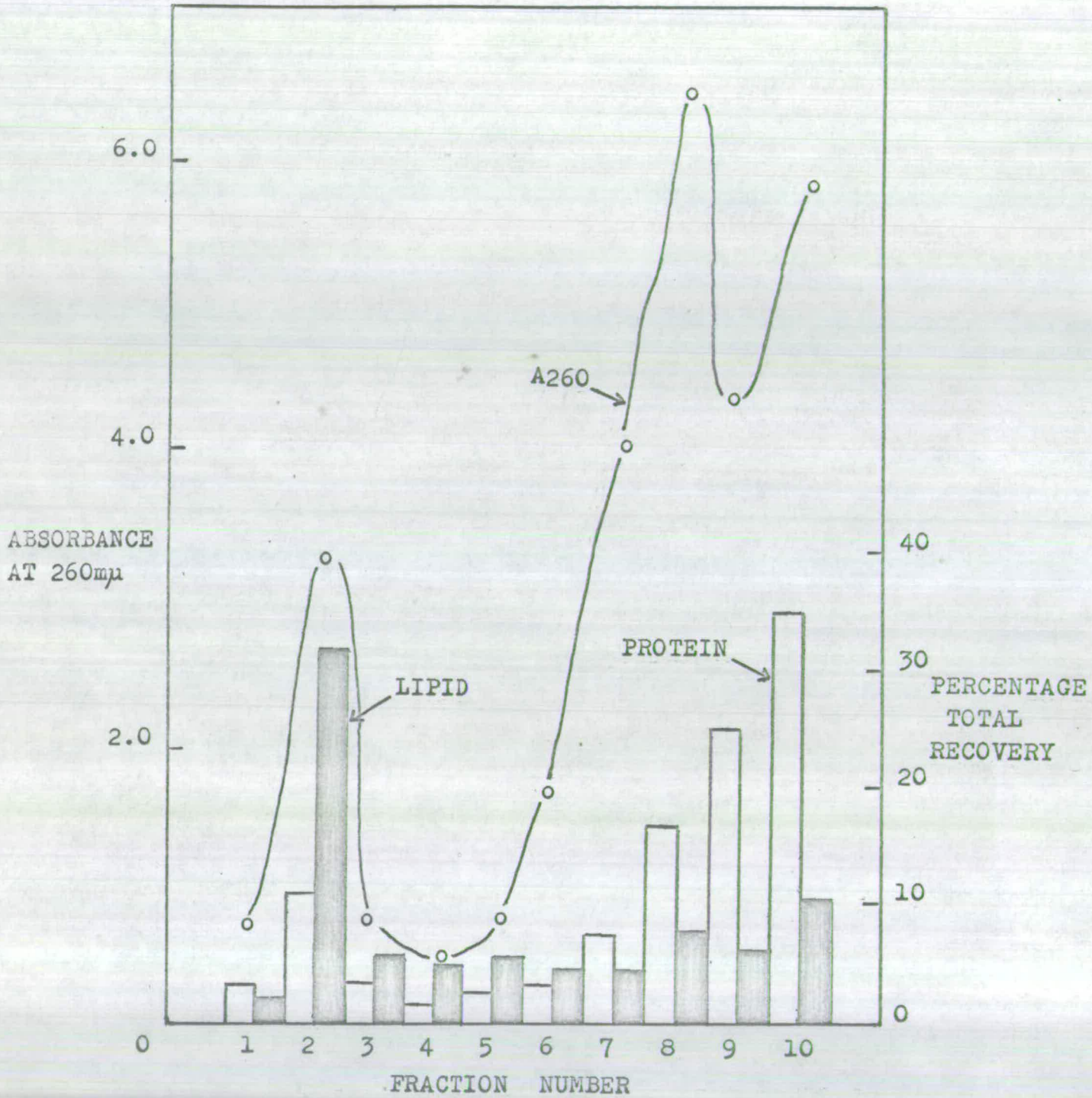


FIGURE 9 Composition of sucrose gradient fractions. Protein and lipid are expressed as percentages of total recovery. Total recovery from the gradient was 38.7mg. and 2.1mg. for protein and lipid, respectively.

235 mμ is a measure of the amount of RNA to protein in the sample. The value of 1.4 is characteristic of mammalian ribosomes prepared without detergent treatment (Kuff and Zeigel, 1960). As can be seen from Figure 7, the highest A260/A235 value is reached in the region of the monosome peak and smaller sized polysomes. The value of 1.5 obtained here indicates that these ribosomes are free from any contaminating protein. Other fractions, the soluble material at the top of the tube, the region of larger polysomes and the interface peak gave much lower values and it was concluded that these fractions contained relatively more protein to RNA. There was a small peak of A260/A235 corresponding to the interface peak.

The gradient fractions were analysed for protein and lipid, the latter being a rough indication of the presence of membranous material. The results are shown in Figure 9. The values for each fraction are expressed as percentages of total recovered component. A total of 38.7 mg., protein and 2.1 mg. lipid were recovered from the gradient. While protein concentration in general increased towards the top of the tube, the percentage of total lipid was greatest (32%) in one fraction, the interface fraction. This fraction also contained a smaller peak (10%) of the total protein. Small amounts of lipid were recorded in all other fractions.

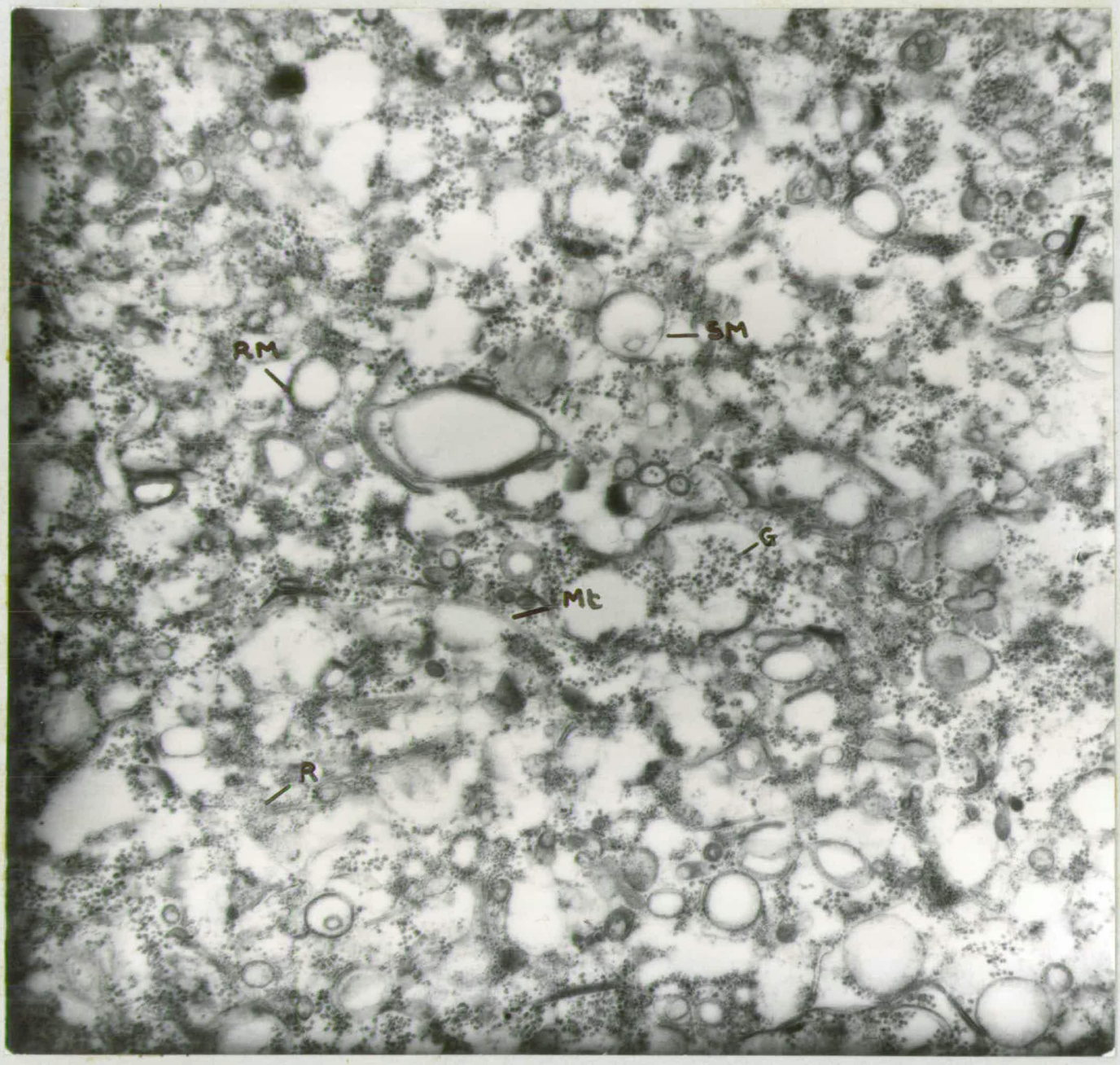


FIGURE 10. Electron micrograph of the membrane fraction pellet.
x 30,000.

KEY : G - glycogen granules
Mt- microtubule
R - ribosome
RM - 'rough' membrane
SM - 'smooth' membrane

These results are considered to be consistent with the view that the material sedimenting at the interface of the dense sucrose cushion and the sucrose gradient contains material qualitatively different from the other material in the gradient, the former containing membranous elements.

2. Electron microscopy of gradient fractions.

This study was undertaken for two main reasons - to confirm the nature of the fractions suggested by the chemical analysis and to check on the purity of these fractions. Pooled sucrose gradient fractions were pelleted and examined with the electron microscope. I am indebted to Mr R.E. Sinden for cutting the sections and taking the electronmicrographs. Figures 10 and 11 show representative views of the interface peak pellet (fractions 2 to 6 in Figure 7) and the polysomal region pellet (fractions 12 to 15 in Figure 7) respectively. Figure 10 confirmed that the interface peak did, in fact, contain numerous membrane-bounded vesicles of various sizes, while Figure 11 shows that the polysomal material is free of any contaminating membranous material. The large unattached particles in the membranous preparation are thought to be glycogen granules and the long fibres are probably micro-tubules. A few ciliary fragments may also be present although none appear in Figure 10. Free ribosomes are few

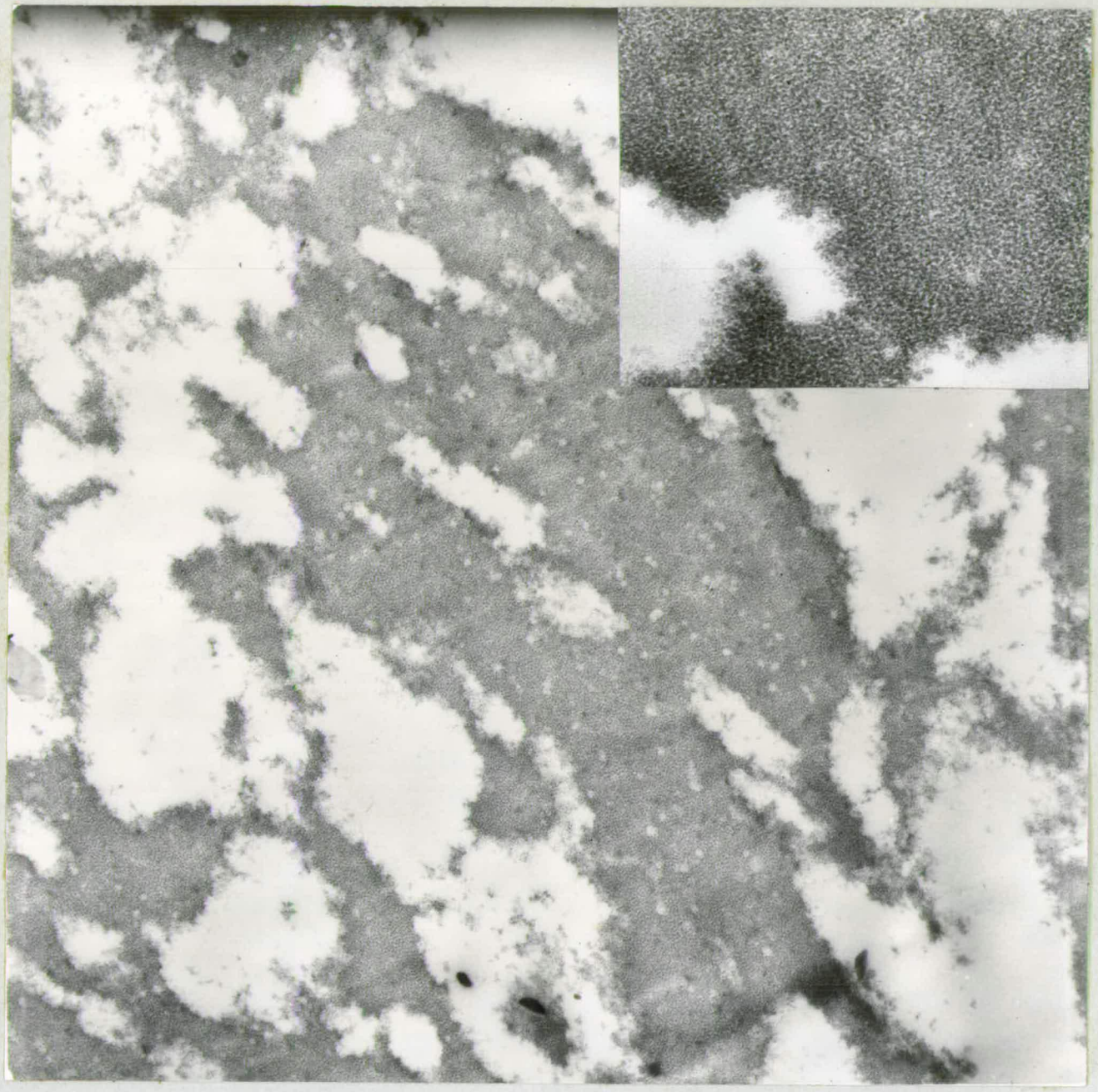


FIGURE 11. Electron micrograph of the polysome fraction pellet. x 30,000. Inset shows the same preparation x 60,000.

in this preparation, but some of the membranes can be seen to have attached particles, often in large clusters, of the size of ribosomes. These membrane-bound ribosomes are probably derived from something analogous to endoplasmic reticulum in paramecium cells, which, in fact, has been seen in whole cell preparations. The polysomal material appears to be homogeneous in nature.

3. Naming of gradient fractions.

From the work outlined above, and on the basis of related studies with other cell types, the components sedimenting to occupy different regions of sucrose gradients were named as follows, with reference to Figure 7 : (a) fractions 2 to 7 - membrane (b) fractions 8 to 15 - polysome (c) fractions 16 and 17 - monosome (d) fractions 18 to 21 - soluble. For convenience, sucrose gradient fractions will be referred to, henceforth, by these names.

Part II : Immobilization Antigen Synthesis by Living Paramecia.

1. Radioactive labelling of living cells.

The first approach in the study of immobilization antigen synthesis was to use living paramecia to incorporate the labelled amino acid and to examine homogenates or sub-cellular fractions for newly synthesised i-antigen. Since the conditions for axenic culture of Paramecium aurelia, variety 1, have not been obtained, the first problem was to find conditions suitable for the uptake of a radioactive protein precursor. In the first instance, total incorporation into acid insoluble material was taken as indicative of the success of the system in being potentially capable of i-antigen synthesis.

Exogeneous labelling of paramecia was found to be unreliable for two reasons :

(a) Paramecia did not always readily incorporate the C^{14}-labelled amino acid when supplied exogeneously in a buffered salts solution. For success with this procedure, the cells had to be in an active condition and had to be harvested by very gentle manipulation.

(b) Traces of bacteria, which are always present even after extensive washing of the paramecia, incorporate far more readily than do paramecia. However, if a bacteriostatic drug, chloramphenicol, was added to give a final concentration

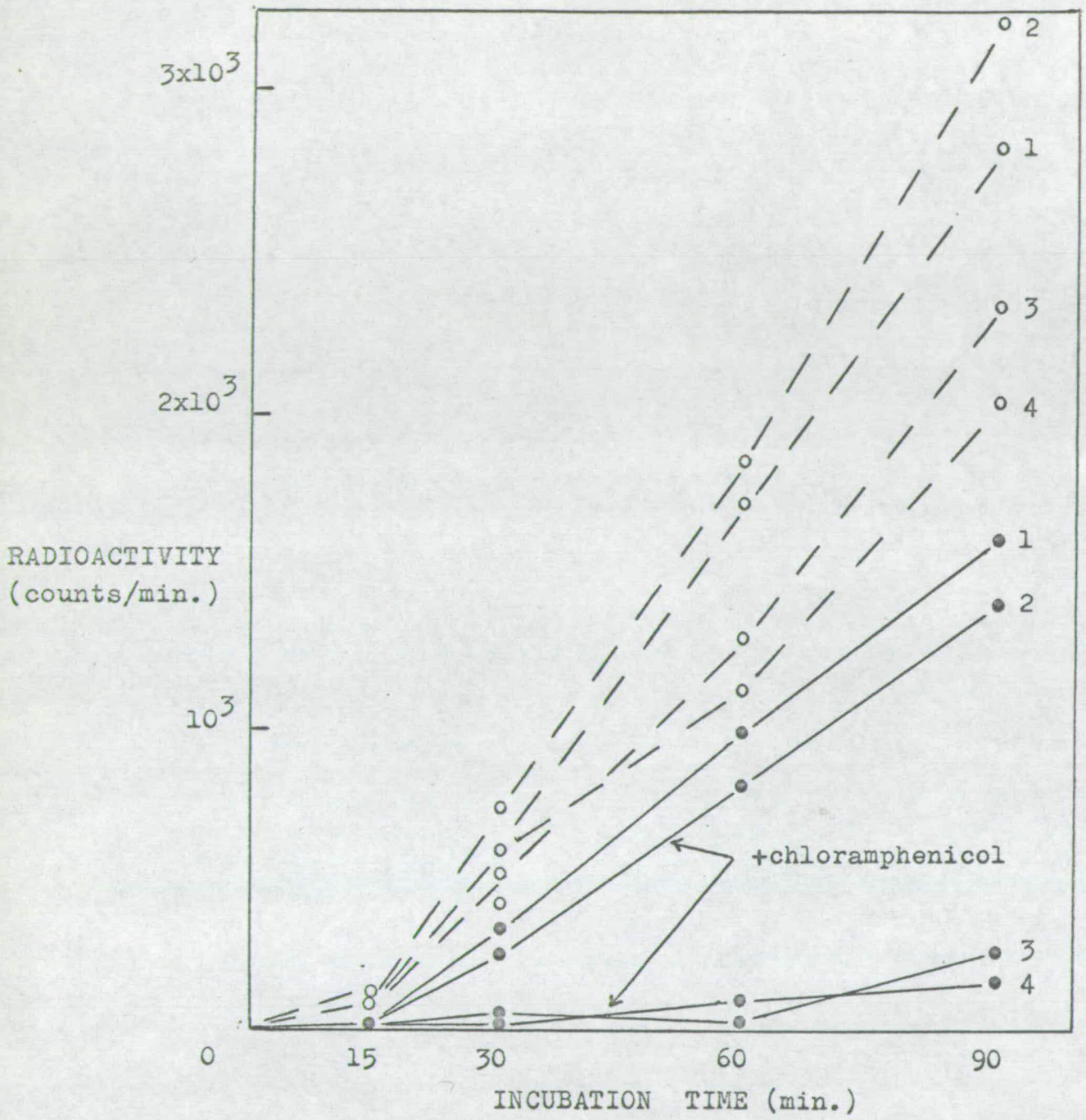


FIGURE 12. Incorporation of C^{14}-phenylalanine into acid insoluble material by a suspension of living Paramecium aurelia, with and without the addition of 100 µg./ml. chloramphenicol. Each assay represents 0.01 ml. cells and 0.05 µC C^{14}-phenylalanine (10 µC/µmole) in a total volume of 0.2 ml. buffered salts solution. The results of four separate experiments are shown.

of 100 µg./ml., the rate of incorporation was lowered. Since chloramphenicol, at concentrations above 10 µg./ml., inhibits protein synthesis in bacteria by 95% to 100% (reviewed Brock, 1961) and incorporation of a labelled amino acid into protein in Escherichia coli is inhibited by more than 85% in the presence of 50 µg./ml. chloramphenicol (Wisseman et al., 1954), it would seem likely that the Aerobacter aerogenes present in the paramecium suspensions, containing 100 µg./ml. chloramphenicol, would be largely inhibited from incorporating labelled amino acid.

Figure 12 shows the effect of chloramphenicol in four separate experiments. In two cases there was no significant chloramphenicol-resistant incorporation into acid insoluble material, whereas, in the other two cases, the chloramphenicol-resistant incorporation accounted for between 50% and 60% of the total incorporation. This level of chloramphenicol-resistant incorporation must be due to protein synthesis by the paramecium cells. However, the unreliability in obtaining labelled paramecia and the relatively low rate of incorporation achieved (2% to3% of the supplied labelled amino acid in 90 min.) prompted the investigation of other methods.

The labelling of paramecia by feeding them radioactive bacteria appears to be a more effective method of obtaining material suitable for the study of i-antigen synthesis. In

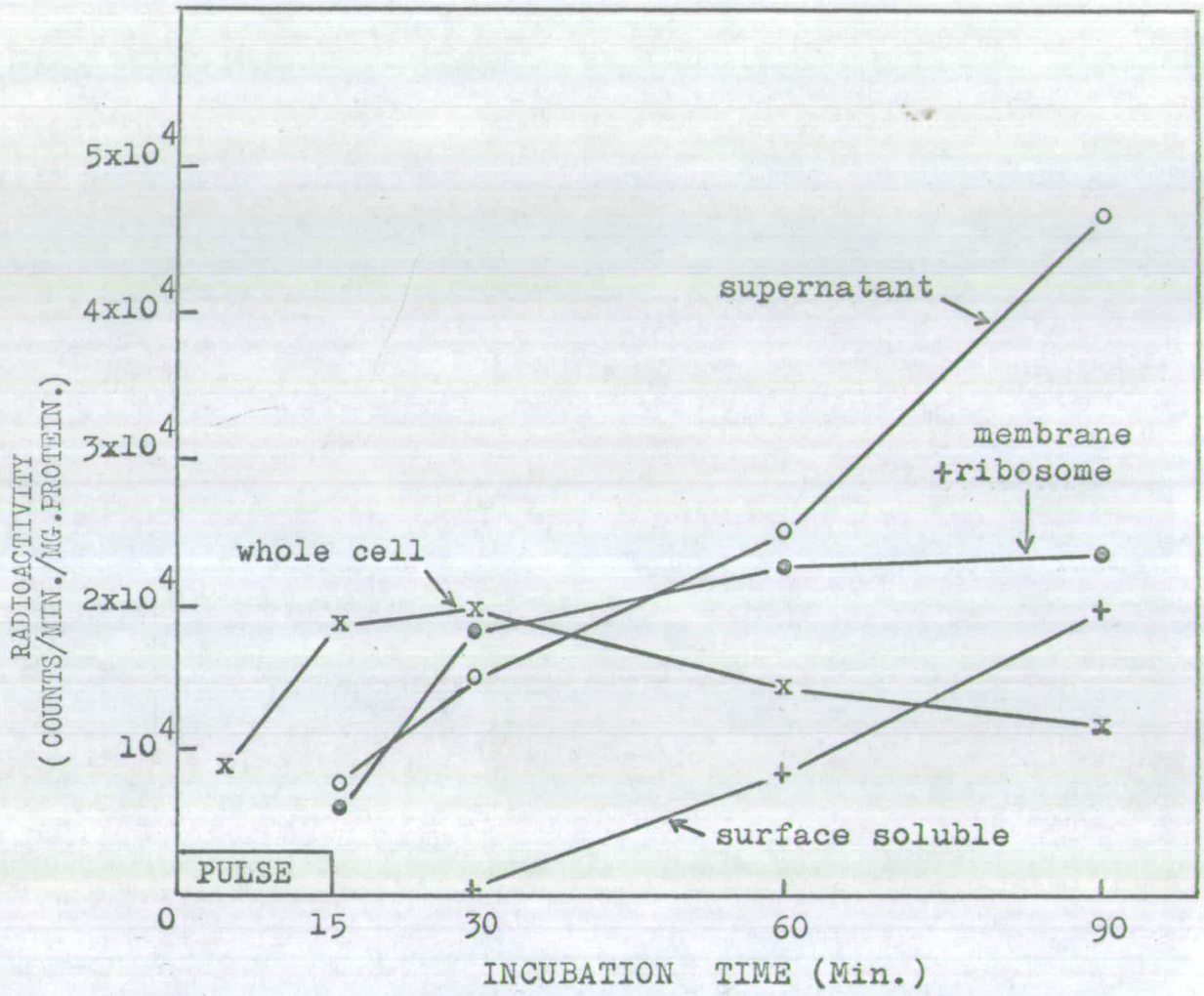


FIGURE 13 The distribution of radioactive protein at various times after the addition of C^{14}-leucine labelled bacteria to a suspension of Paramecium aurelia. The paramecia were removed from the labelled bacteria after 15min., washed and resuspended ingrowth medium containing unlabelled bacteria. Samples were homogenized after various times of incubation and acid-insoluble radioactivity was estimated for various preparations.

x——x whole washed paramecia.
●——● 10-110,000g pellet (membrane + ribosome).
o——o 110,000g supernatant.
+——+ salt/alcohol soluble surface material.

fact, Berger and Kimball (1965) found that tritium-labelled thymidine was specifically incorporated into paramecium DNA only when supplied by way of bacteria. It was thought that the advantages gained by using the normal feeding mechanism in supplying metabolites to paramecia would outweigh the disadvantages of having labelled bacteria present in the incubation mixture.

The kinetics of ingestion, by paramecia, of radioactively labelled bacterial protein, its digestion and redistribution in association with various paramecium cell fractions, are shown in Figure 13. The whole cell values include acid insoluble radioactivity incorporated into paramecium protein, together with radioactivity due to ingested but undigested bacterial protein. Under the conditions employed, ingestion of bacteria was rapid, and 15 min. feeding was long enough to obtain sufficient radioactivity inside the paramecia (approximately 50% of the radioactivity supplied. See Table 2). The paramecium cells were then washed free of labelled bacteria and resuspended in medium containing unlabelled bacteria. For the next 60 min., total acid insoluble radioactivity decreased to about half the original value, presumably due to digestion of bacterial protein. At about 90 min. after the start of the experiment, total labelled protein began to increase with time, due to the digestion process being over-

taken by the synthesis of bacterial breakdown products into paramecium protein. The increase in labelled protein levelled off after a further 30 to 60 min. (not shown in Figure 13). Immediately after the 'pulse' of feeding paramecia labelled bacteria, radioactivity was detected in paramecium sub-cellular fractions. The fractions examined were the 10 - 110,000g precipitate (membrane plus ribosome) and the 110,000g supernatant (soluble fraction). The highest specific activity in the early stages (till about 45 min.) was associated with the membrane/ribosome pellet, but after 30 min. the activity of this fraction levelled off. The activity of the soluble fraction increased exponentially, and after 60 min. was the fraction with the highest specific activity. Between 30 and 60 min., protein removed from the surface of the cells by salt/alcohol treatment became labelled and incorporation increased up to 90 min. but at a lower rate than the soluble fraction.

These results are consistent with the view of proteins being synthesised on ribosomes, which tend to reach equilibrium with the available labelled amino acid, the proteins later being released into the cytoplasm where they accumulate. Some of the protein may then be transported to sites on the surface of the cells.

The efficiency of the labelling procedure is outlined in Table 2. It can be seen that about 20% of the original

STAGE	TOTAL RADIOACTIVITY (counts/min.)	PERCENTAGE ORIGINAL ACTIVITY (%)
C-14 leucine	10.0×10^5	100
bacterial protein	8.4×10^5	84
(after incubation	8.1×10^5	81
for 60min.)	7.8×10^5	78
ingested bacterial	4.2×10^5	42
protein (after	4.0×10^5	40
feeding for 15min.)		
paramecium protein	2.1×10^5	21
(after "chase" of	1.9×10^5	19
60 min.)		
immobilization	1.8×10^3	0.18
antigen	1.0×10^3	0.10

TABLE 2 Efficiency in incorporation of 1 μC C^{14}-leucine through various stages in labelling antigen by way of labelled bacteria. Efficiency in counting 1 μC C^{14}-leucine filters was taken to be 45%.

radioactivity finds its way into paramecium protein. This level of incorporation is approximately 10 times that obtained in the same time when the label was supplied as an exogenous source.

2. Detection of labelled i-antigen.

Purified i-antigen was prepared from labelled paramecia to test whether or not the i-antigen was labelled under the experimental conditions described above. If the labelling of paramecia by way of bacteria could be shown to be a suitable procedure in labelling i-antigen, methods could then be investigated for the routine assay of labelled i-antigen. For the purpose of separating i-antigen from other labelled proteins, three techniques have been investigated : column chromatography, direct precipitation with specific antiserum and microimmunoelectrophoresis. Radioactivity was assayed in the separated antigen preparation by either liquid scintillation counting or autoradiography.

Paramecia were labelled by feeding them labelled bacteria as described above. After 2 hr., the labelled cells were chilled and added to 5 times as many unlabelled cells expressing the same serotype to act as 'carrier'. Immobilization antigen was extracted and purified by column chromatography. The elution peaks were assayed for radioactive protein and antigenic activity.

Facing p.36

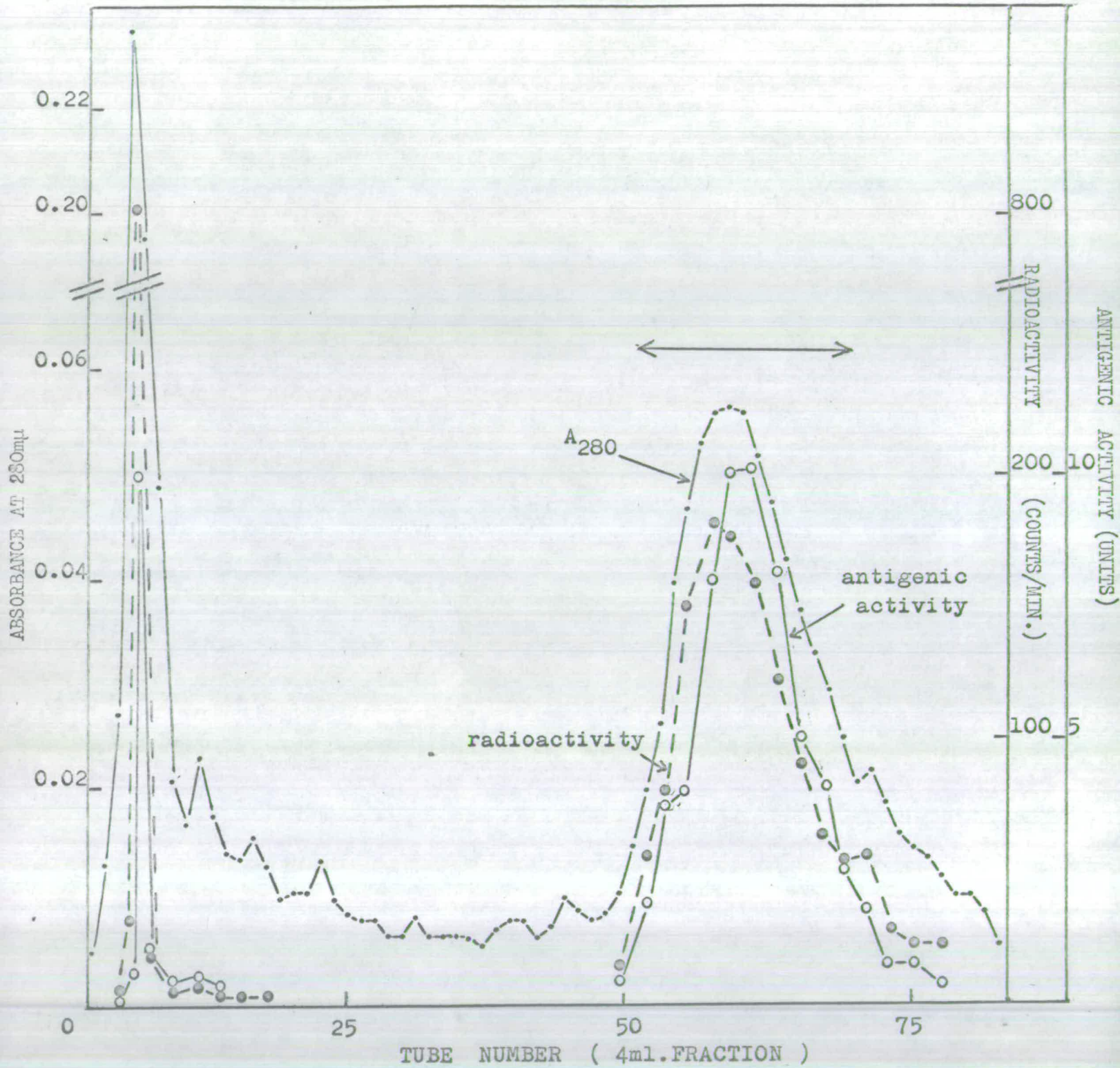


FIGURE 14 Elution diagram of C^{14}-labelled 90D antigen on a column of SE-Sephadex with a 400ml. gradient of 0.05M-sodium acetate pH4.2 to 5.2. The material within the arrows was pooled, dialysed and lyophilized.

The results are shown in Figure 14. There was a loss of 12% of the eluted antigenic activity in the wash peak where 28% of the eluted radioactive protein was found. However, at the usual elution point of i-antigen (at pH 4.8) the three assay peaks - of absorbance at 280 mμ, of radioactive protein and of antigenic activity are largely coincident. It was strongly suggested from these results that under the labelling conditions employed, radioactive i-antigen was formed. The efficiency of incorporation of the original C^{14}-leucine into paramecium i-antigen by way of bacteria is shown in Table 2. It can be seen that 0.5% to 1% of the radioactivity in paramecium protein is recovered as purified i-antigen. In this preparation the serotype of the purified i-antigen was 90D.

In order to investigate methods for the routine assay of radioactive i-antigen, and to further check the purity of the labelled i-antigen preparation by immunological criteria, the material from the peak fractions was dialysed against distilled water, pooled and lyophilized. Part of this material was redissolved in 0.9% NaCl and treated with various concentrations of homologous (anti-90D) and heterologous (anti-90G) antiserum prepared against purified i-antigens. All tubes were made up with normal rabbit serum to give a standard volume of added serum and after incubation the rabbit serum was precipitated with an excess of sheep-anti-rabbit

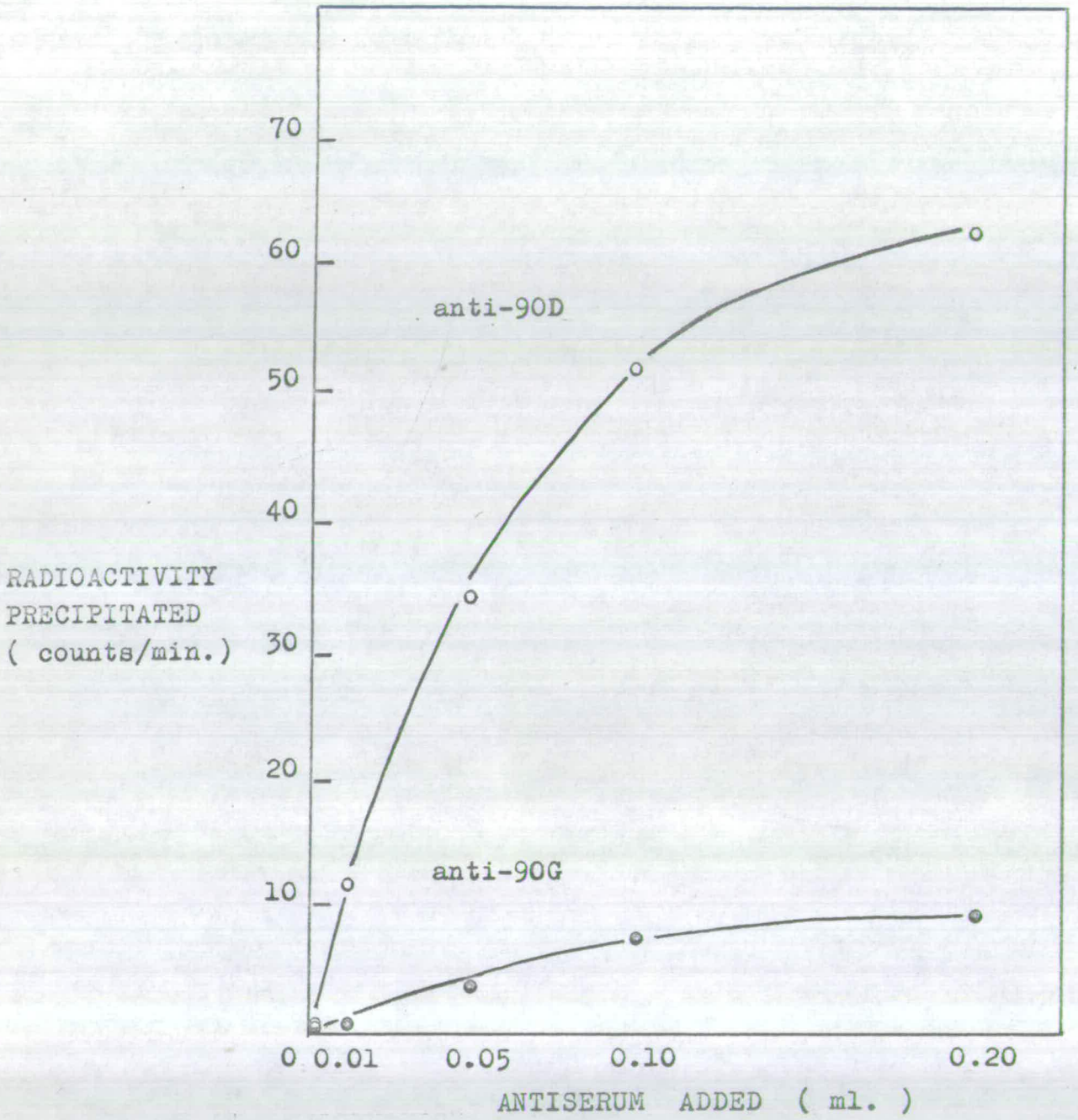


FIGURE 15 Precipitation of purified C^{14}-labelled 90D antigen by homologous (anti-90D) and heterologous (anti-90G) antisera. All tubes were made up to 0.2ml. with normal rabbit serum, incubated at 37°C for 1 hr., and treated with 0.2ml. sheep anti-rabbit serum. Precipitates were collected by centrifugation at 4000g for 30 min.. The pellet was resuspended and recentrifuged then the protein was precipitated washed and counted.

serum. This procedure ensured an equal amount of precipitation in all tubes without sacrificing specificity in precipitating i-antigens. (see Methods) The precipitates were collected by centrifugation, redispersed in saline and the protein present was precipitated and counted. The results are shown in Figure 15. As can be seen, there was a specific precipitation with homologous antiserum. The degree of cross-reaction with heterologous antiserum was about 15%. Since purified labelled i-antigen was used in this experiment, the degree of cross-reaction obtained is expected to be the lowest possible by this method.

Another part of the purified 90D antigen was redissolved, along with an equal amount of heterologous 90G purified antigen, in a small volume of 0.025M borate buffer at pH 8.2. This material was then applied to the wells of agar coated microscope slides and subjected to electrophoresis for 3 hr. at 20 volts/cm.. Anti-90D and anti-90G sera were then applied as soaked filter paper strips, longitudinally on either side of the wells, and the slides were left in a humidity chamber for 48 hr. to allow precipitin arcs to form between the diffusing antisera and the diffusing i-antigens. Precipitin arcs were formed with both antisera since 'carrier' heterologous i-antigen had been added to give a control. The arcs were slightly displaced towards the cathode end of the slide, the slow migration of i-antigen in this direction probably being

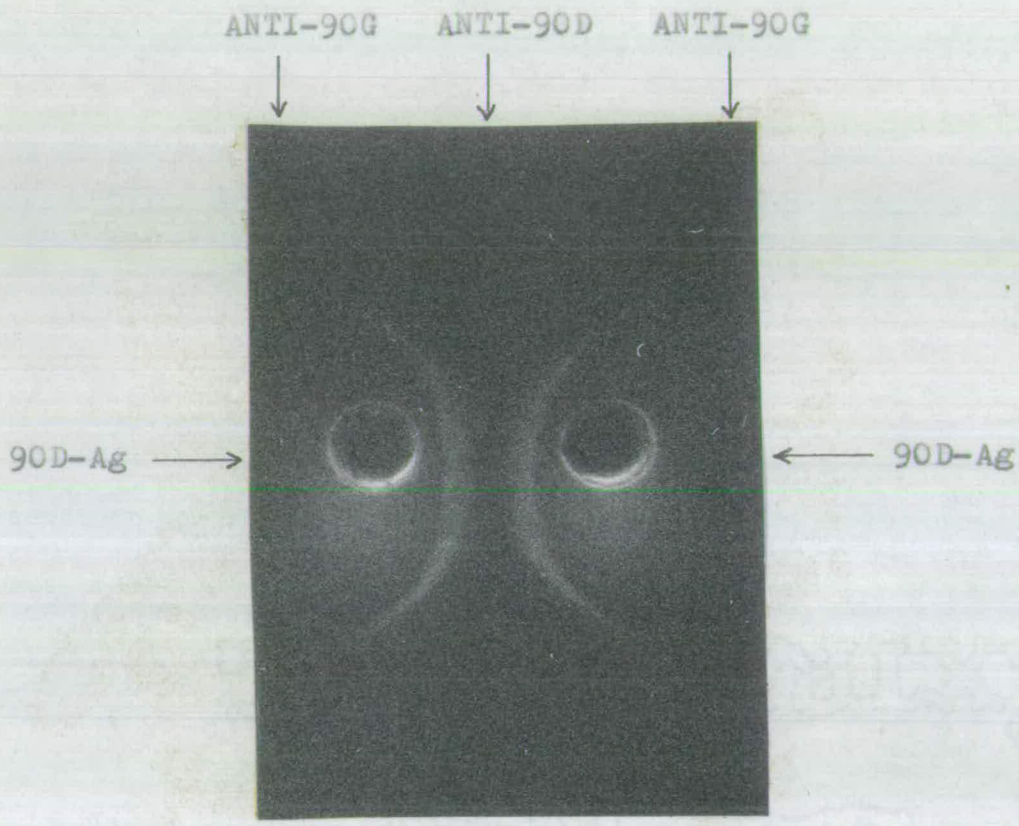
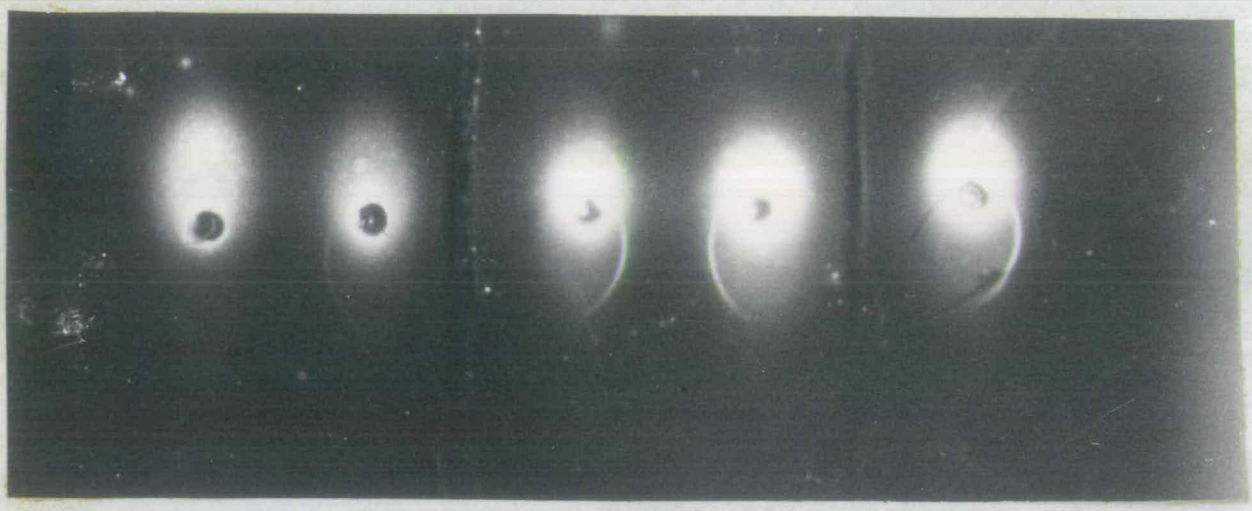


FIGURE 16. Autoradiograph of immunoelectrophoresis of purified C^{14}-labelled 90D antigen. Electrophoresis was for 3 hr. at 20 volts/cm. in 0.025M borate buffer pH 8.2. The preparation was then exposed in darkness in contact with X-ray film for six weeks.

due to endosmosis. The slides containing the precipitin arcs were then washed thoroughly in 0.9% saline for 2 days, dried, and exposed in contact with an X-ray film for six weeks in total darkness. An autoradiograph is shown in Figure 16. It can be seen that only the arcs formed against homologous (anti-90D) serum are radioactively labelled. There is very little precipitation of radioactivity in the region of the well indicating that little i-antigen was denatured and that other unstable labelled proteins were not present in the preparation. There was no observable cross-reaction with heterologous serum using this technique.

In conclusion, immobilization antigen is synthesised by living paramecia under the experimental conditions employed. Labelled i-antigen can be purified by column chromatography but since this technique requires relatively large amounts of labelled material, and takes considerable time to perform, direct specific precipitation of radioactivity by homologous antiserum and immunoelectrophoresis / autoradiography have been investigated for their suitability in routine assays of radioactive i-antigen. Of these techniques, direct precipitation has been found to be quick and simple to perform, but the degree of specificity is not very satisfactory with certain types of preparation (see Table I), whereas immunoelectrophoresis / autoradiography took considerable time to perform (6-7 weeks)



time (min.)	0	30	60	120	180	unlabelled i-Ag
intensity		(+)	++	+++	++++	marker

FIGURE 17. Autoradiograph of immunoelectrophoresis analysis of labelled i-antigen at various times after feeding paramecia on C^{14}-labelled bacteria. Conditions were similar to those for Figure 16 except that here whole cell homogenates were used. Increasing intensity of radioactive arc is indicated by increasing numbers of plus signs.

but enable a far more specific reaction. It must be remembered, however, that the latter method gave no direct quantitative result.

3. Kinetics of i-antigen synthesis.

In order to examine the time course of synthesis of i-antigen under bacterial labelling conditions and to test for the distribution of newly synthesised i-antigen in different cell fractions during early stages of labelling, paramecia were labelled by way of bacteria and radioactive i-antigen was assayed by the immuno-electrophoresis / autoradiography method.

Using this method, the increase of radioactivity in i-antigen with increasing time is shown in Figure 17. An attempt to be quantitative has been made by allocating an increasing number of plus-signs to precipitin arcs giving an increasing intensity of image on autoradiography. It was found that labelled i-antigen could be detected at 30 min. after the addition of the radioactive bacteria, and the intensity of labelling increased during the 3 hr. of the experiment, the greatest increase being noted between 30 min. and 60min..The large amount of precipitated radioactivity in the vicinity of the well generally occurs when the applied material is not purified to some extent for i-antigen: in this case the preparation is simply a cell lysate.

The analysis of separated sub-cellular fractions for

FRACTION	TOTAL VOLUME (ml.)	RESULTS: INTENSITY OF PRECIPITIN ARC (Ip)	RESULTS: INTENSITY OF AUTORADIOGRAPH (Ia) 30	RESULTS: INTENSITY OF AUTORADIOGRAPH (Ia) 90	DERIVED ARBITRARY UNITS: TOTAL ACTIVITY (radioactivity/ml.original homogenate:Ia/ml.) 30	DERIVED ARBITRARY UNITS: TOTAL ACTIVITY (radioactivity/ml.original homogenate:Ia/ml.) 90	DERIVED ARBITRARY UNITS: SPECIFIC ACTIVITY (radioactivity/amount precipitate:Ia/Ip.) 30	DERIVED ARBITRARY UNITS: SPECIFIC ACTIVITY (radioactivity/amount precipitate:Ia/Ip.) 90
HOMOGENATE	2.5	+++	+	+++				
		+++	0	+++	0.8	6.6	0.1	0.9
		+++	0	++				
PELLICLE +CILIA	1.0	+++	0	+++				
		+++	0	+++	0	2.7	0	0.9
		+++	0	++				
MEMBRANE	0.5	+++	+	++				
		++	+	++	0.3	0.8	0.3	0.7
		++	0	+				
RIBOSOME	0.5	++	0	+				
		+	0	+	0	0.3	0	0.5
		+	0	0				
SOLUBLE	2.5	+	0	+				
		+	0	+	0	2.5	0	1.0
		+	0	+				

TABLE 3 Summary of results obtained after microimmunoelectrophoresis and autoradiography of labelled paramecium homogenate and cell fractions. Paramecia were incubated with C-14-leucine labelled bacteria for 15min., then washed and transferred to growth medium containing unlabelled bacteria for a further 30 or 90min.. After homogenization and fractionation aliquots were subjected to immunoelectrophoresis. The results were scored as 0, +, ++ or+++ indicating the relative intensity of the precipitin arcs and of their autoradiographic image (after four weeks exposure). The results of three experiments are shown. Each value represents a duplicated assay.

Facing p.40

radioactive i-antigen at two times after the start of the experiment is shown in Table 3. (The results shown are taken from three separate experiments, each assayed in duplicate.) Here, not only have pluses been assigned, but numerical units have been derived to give some idea of the specific activity as well as the total activity of each fraction. These results indicate that the only fraction with detectable associated i-antigen activity after 15 min. labelling and a 30 min. chase is the membrane fraction. After 90 min. chase, labelled i-antigen is detectable in all fractions, perhaps due to fragmentation of i-antigen containing structures or contamination of preparations with solubilized labelled i-antigen. However, the greatest increase of specific activity between 30 min. and 90 min. was noted in the pellicle and cilia fraction and the soluble fraction (more than ninefold) whereas the increase in specific activity in the membrane fraction was least (only twofold).

The early appearance of newly synthesised i-antigen in the membrane fraction could not be due to contamination since it was the most labelled fraction : it must be due to either specific adsorption of antigen to this fraction during homogenization or else must represent the particular site of recently synthesised i-antigen. However, the smaller increase of i-antigen activity with time in this fraction indicates that it does not include the cellular material mainly associated with

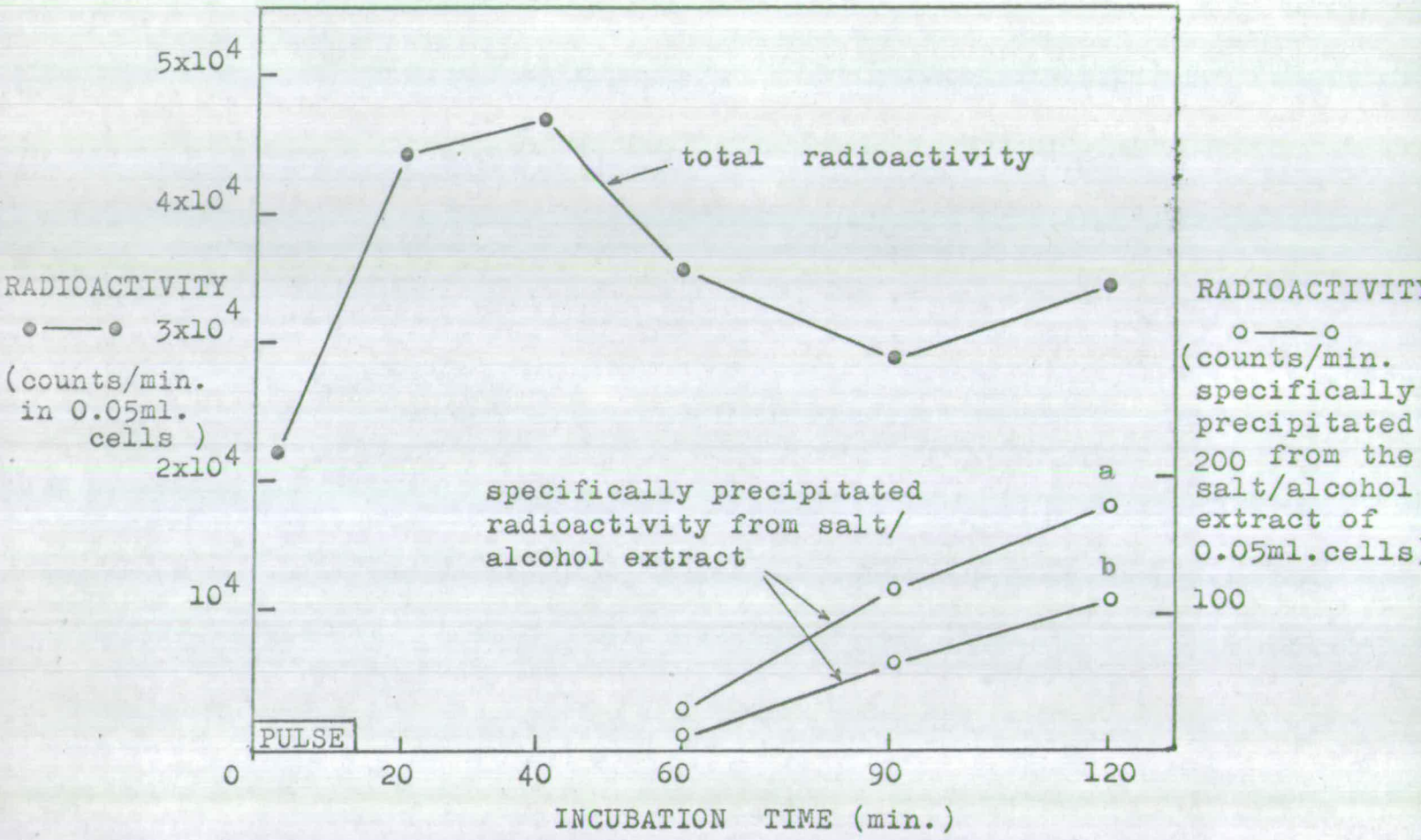


FIGURE 18 The appearance of labelled i-antigen extractable from the surface of the cell in relation to the incorporation of labelled bacteria by living Paramecium. The i-antigen assays from two experiments are shown (a and b).

the accumulation of newly synthesised i-antigen. On the other hand, the fraction including the bulk of the pellicle and cilia had no detectable labelled i-antigen at the earlier time, yet later became the fraction with the highest amount of labelled i-antigen.

These results, showing the kinetics of i-antigen synthesis and the distribution of this protein between the various sub-cellular fractions, are similar to those obtained already for total protein (Figure 13).

In an attempt to measure the earliest appearance of labelled i-antigen on the surface of the pellicle and cilia, without running the risk of contamination by solubilized labelled i-antigen on homogenization, the salt/alcohol extraction procedure was used. Since more than 90% of the surface antigen can be solubilized by salt/alcohol treatment (Preer, 1959b) and since this material gives a fairly specific precipitin reaction (see Table I), paramecia treated with salt/alcohol at various times during labelling would give a good estimate of radioactive surface i-antigen when assayed by the precipitin method. The results are shown in Figure 18. In two experiments the earliest appearance of labelled i-antigen on the surface of the pellicle and cilia was at 60 min..

Although the results shown in Figures 17 and 18 are not directly comparable, a different extraction procedure and a

different assay method were used in each type of experiment, they indicate that i-antigen is synthesised internally in the cell, in association with membranous material, and only later appears on the surface of the pellicle and cilia.

4. Site of i-antigen synthesis.

It has been indicated from the studies on the kinetics of i-antigen synthesis, that the cell fraction showing the earliest signs of labelled i-antigen was the membrane fraction. Since this material was contained in the 10,000g supernatant, it was decided to further examine this supernatant by separation on sucrose gradients, then to test gradient fractions for specifically precipitated counts. This technique would provide a greater degree of separation of cellular components, particularly in the separation of membrane, ribosomes of different size and soluble material.

Supernatants, derived from homogenized labelled paramecia, were layered on linear sucrose gradients. After centrifugation, fractions were analysed for absorbance at 260 mμ., total radioactive protein and specifically precipitated radioactivity. The specific reaction was taken to be the amount of radioactivity precipitated by homologous antiserum, minus the amount of radioactivity precipitated by a heterologous antiserum (control), under the conditions described in Methods.

facing p.42

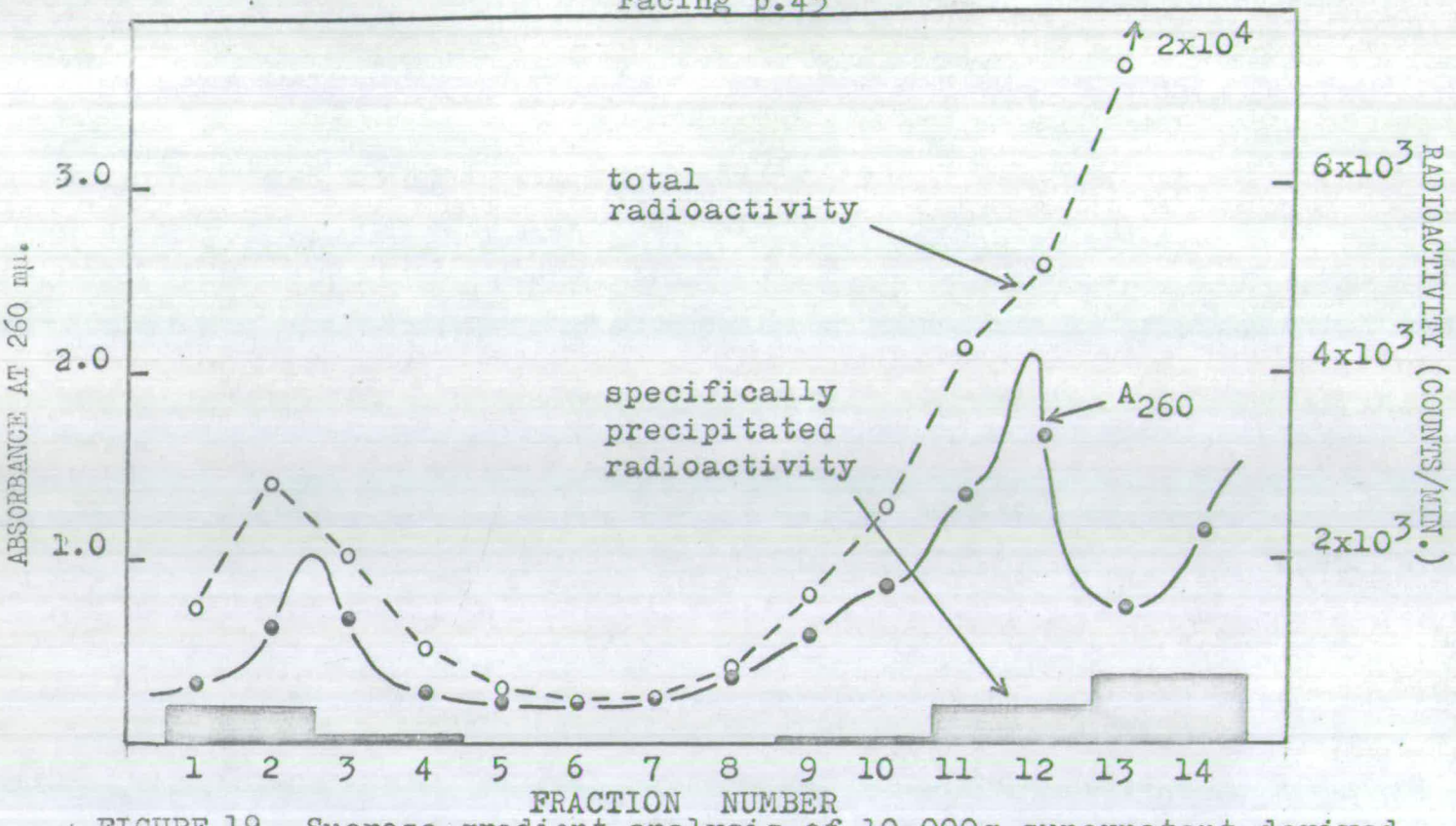


FIGURE 19 Sucrose gradient analysis of 10,000g supernatant derived from paramecia incubated with C-14-labelled bacteria for 15min. followed by a 30min. "chase".

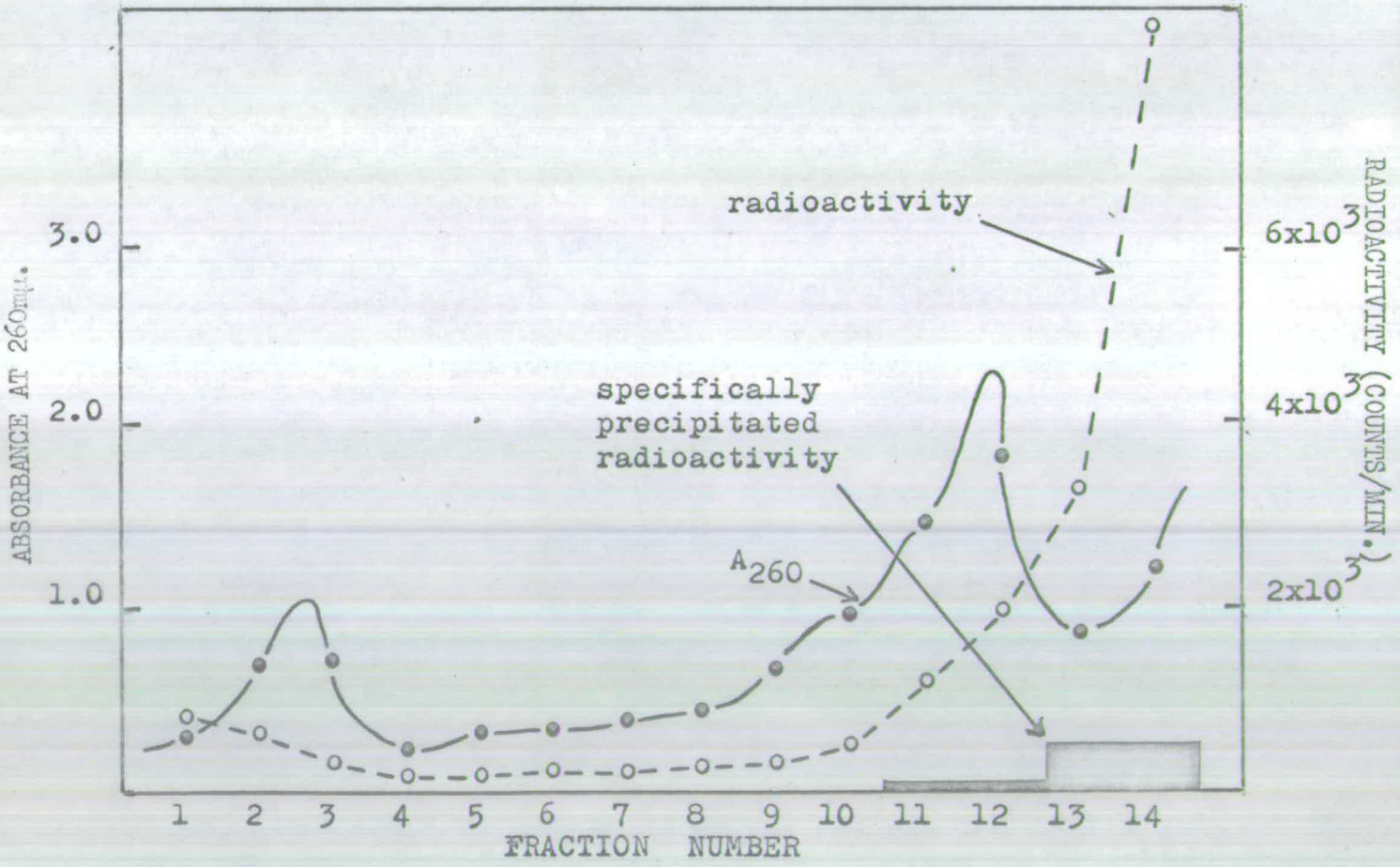


FIGURE 20 Sucrose gradient analysis of an unlabelled 110,000g pellet homogenized with labelled 110,000g supernatant. Labelling conditions as above.

Figure 19 shows the sucrose gradient analysis of a labelled paramecium 10,000g x 10 min. supernatant. Total radioactive protein had a profile distribution similar to the A260 profile, except at the top of the tube, where most of the labelled protein was found in soluble form. Specifically precipitated i-antigen activity has a bimodal distribution; in association with the membrane fraction which sediments to the gradient/dense sucrose interface near the bottom of the tube and at the top of the tube in association with the soluble material, and possibly with the ribosome fractions.

That there is indeed some i-antigen activity associated with free ribosomes and that the activity in the membrane fraction was not due to adsorption of soluble labelled i-antigen was demonstrated by the type of experiment shown in Figure 20. Here, labelled soluble material taken from the top of a gradient (corresponding to fractions 13 and 14 in Figure 19) was homogenized with an unlabelled 110,000g pellet and subjected to sucrose gradient analysis. In this case, the A260 profile was similar to that of the 10,000g supernatant preparation but the distribution of total radioactive protein and specifically precipitated radioactivity was more or less restricted to the top of the tube. There was no radioactive i-antigen in the membrane fraction and the relative amount of radioactive i-antigen associated with free ribosomes was less than in the

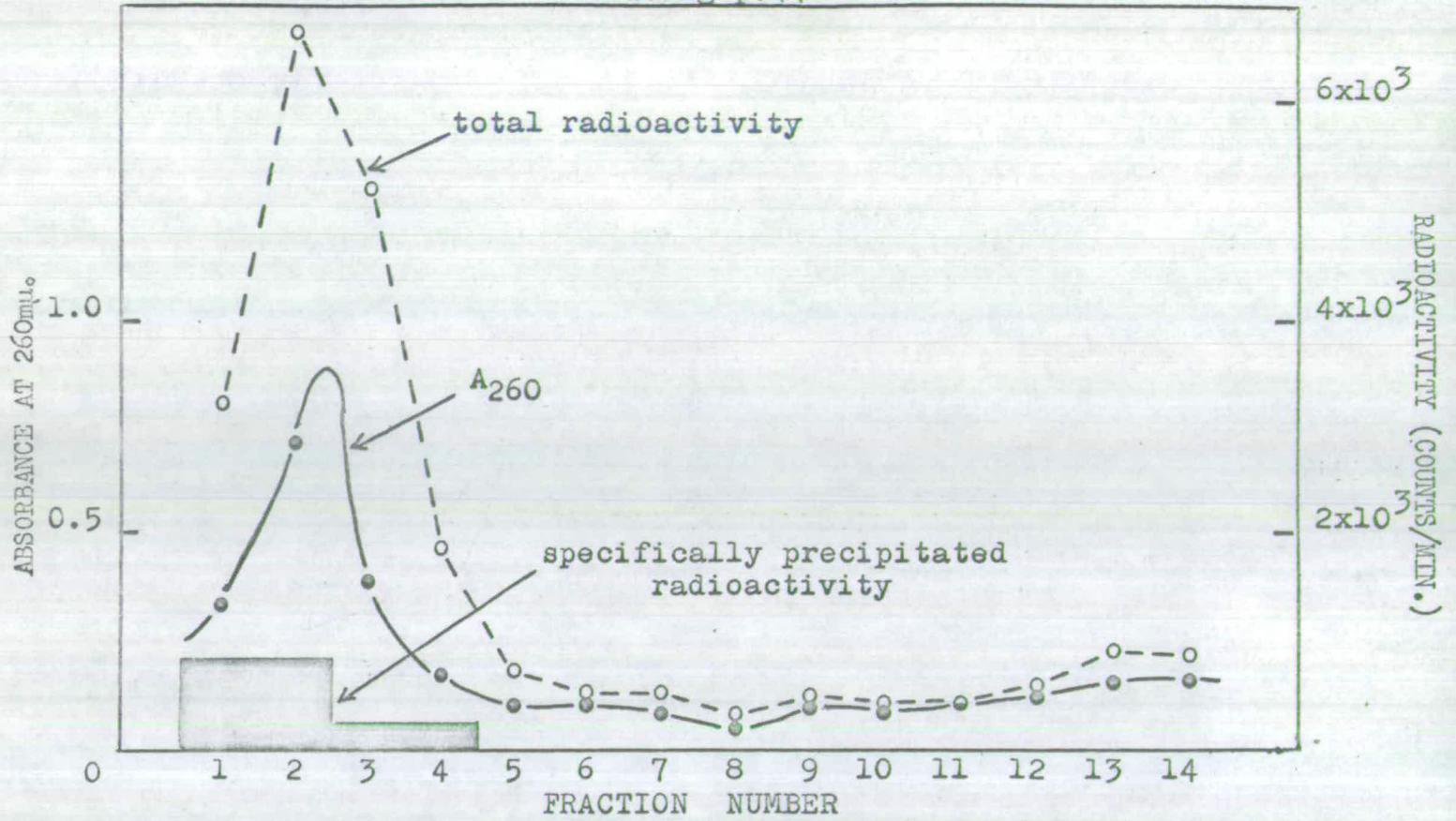


FIGURE 21 Sucrose gradient analysis of isolated labelled membrane fraction prepared by homogenizing a paramecium 10-20,000g pellet. Labelling conditions as for Figure 19.

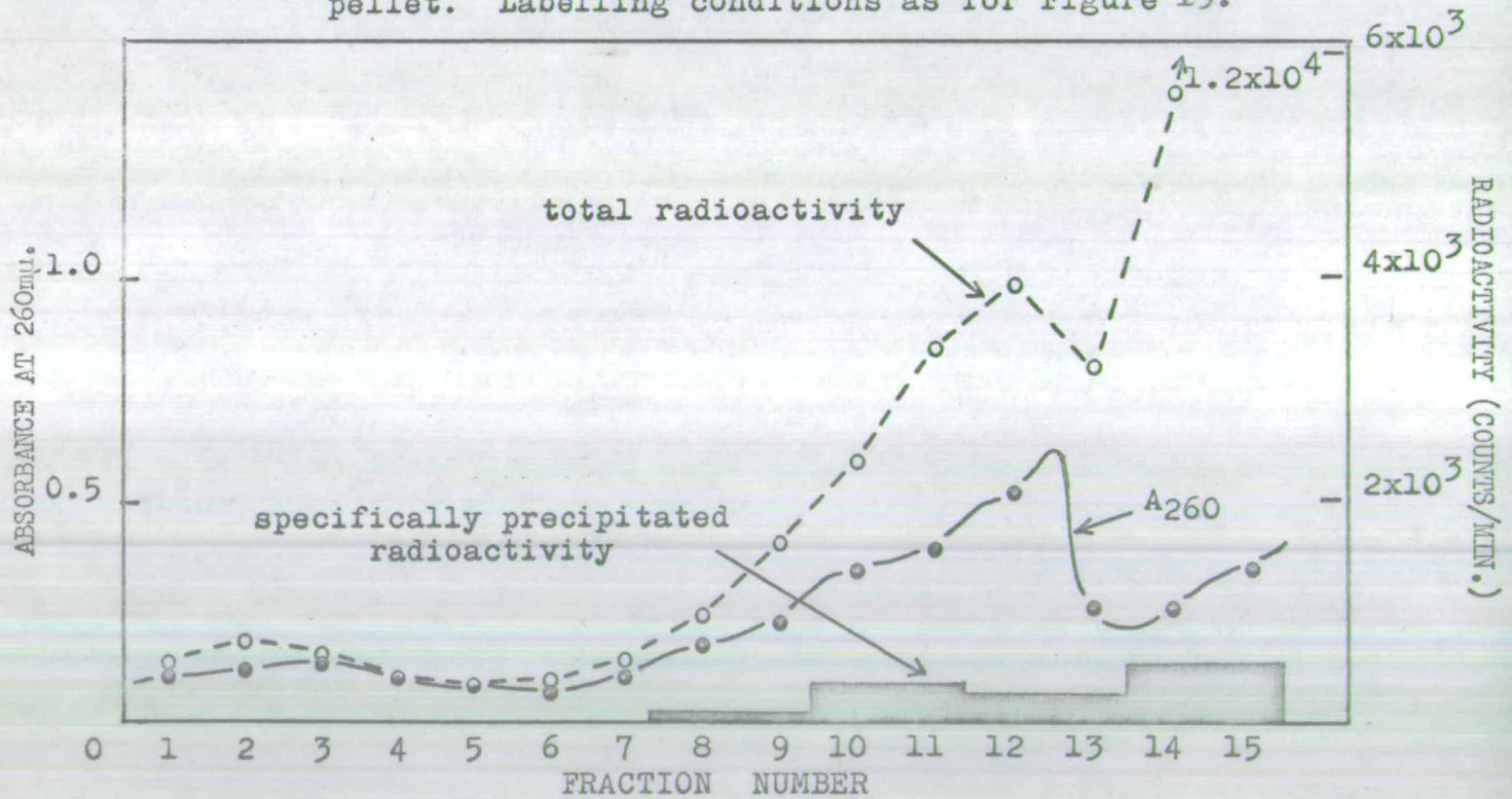


FIGURE 22 Sucrose gradient analysis showing the effect of 0.5%DOC on the labelled membrane fraction. The material used in the above gradient was treated with 0.1 volume of 5%DOC at 0°C prior to centrifugation.

previous experiment. These results indicate that both the membrane fraction and the free ribosomes have some radioactive i-antigen genuinely associated with them.

In order to examine the membrane material in more detail, this fraction could be isolated by collecting the pellet formed on centrifuging a 10,000g x 10 min. supernatant at 20,000g x 20 min.. On resuspending the pellet and centrifuging through a sucrose gradient, the material formed a single peak of absorbance at 260 mμ. and total radioactive protein both on the dense sucrose/gradient interface (Figure 21). As in earlier experiments, this peak fraction contained specifically precipitated radioactivity which amounted to approximately 6% of total radioactive protein in the membrane fraction.

On treating this same isolated membrane material with 0.5% sodium deoxycholate (in homogenization buffer), at 0°C before sucrose gradient analysis, the result shown in Figure 22 was obtained. Here, the peak observed in the previous gradient had been removed and the absorbing material and radioactivity was found nearer the top of the tube. The distribution of both A260 and total radioactive protein closely resembled a distribution of polysomes and monosomes with material solubilized at the top of the tube. Indeed, the A260 peak had a sedimentation coefficient of approximately 80S. Most of the radioactivity was detected at the top of the tube but

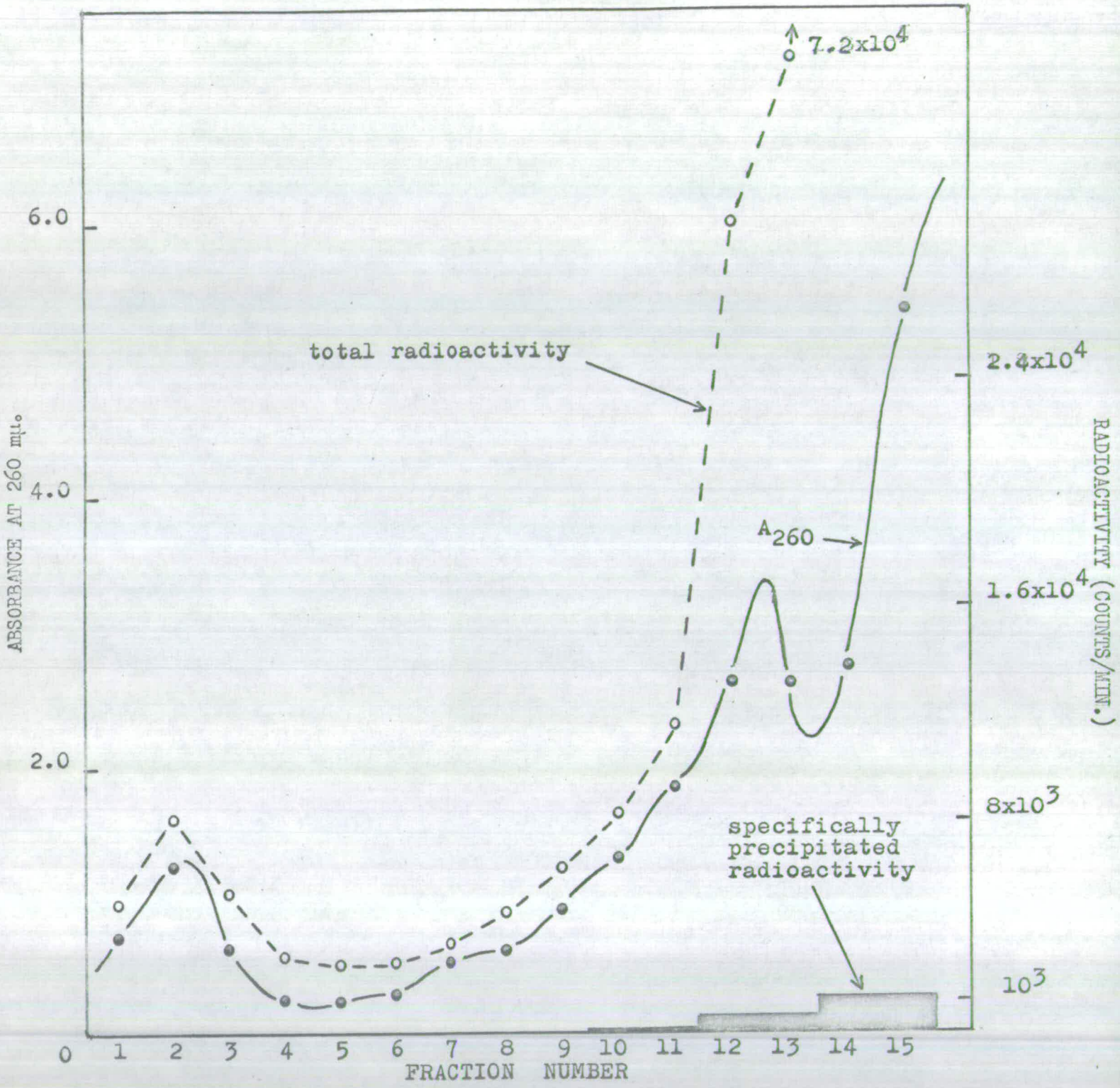


FIGURE 23 Sucrose gradient analysis of a DOC treated 10,000g pellet derived from labelled paramecia. The treated material was centrifuged at 10,000g before the supernatant was layered on the gradient. Conditions of labelling as for Figure 19.

radioactive i-antigen was found over a large part of the gradient. Less than half of the radioactive i-antigen appeared to be in the form of soluble protein while the peak of bound i-antigen activity occurred in the polysome region. The fact that all of the i-antigen activity was not solubilized on DOC treatment suggested that some 'nascent' i-antigen was present on ribosomes. This 'nascent' i-antigen was, however, sufficiently completed to be detected by specific antiserum precipitation. The soluble activity presumably represented the completed i-antigen molecules.

In order to estimate how much labelled i-antigen was sedimenting due to centrifugation at 10,000g x 10 min. and was therefore escaping detection, the pelleted material was agitated in the presence of 0.5% DOC, recentrifuged at 10,000g x 10 min. and the supernatant was subjected to sucrose gradient analysis. Figure 23 shows the gradient analysis of material prepared in this way. In this case, all of the i-antigen activity was detected in the monosome and soluble region of the gradient. The total amount of solubilized i-antigen activity amounted to about as much as was found in the membrane fraction derived from the same cell volume. However, the specific i-antigen activity in terms of i-antigen counts / protein counts was far greater for the membrane fraction than for the debris pellet (6% and 1.5% respectively). It would appear then, that the membrane fraction is representative of the main site

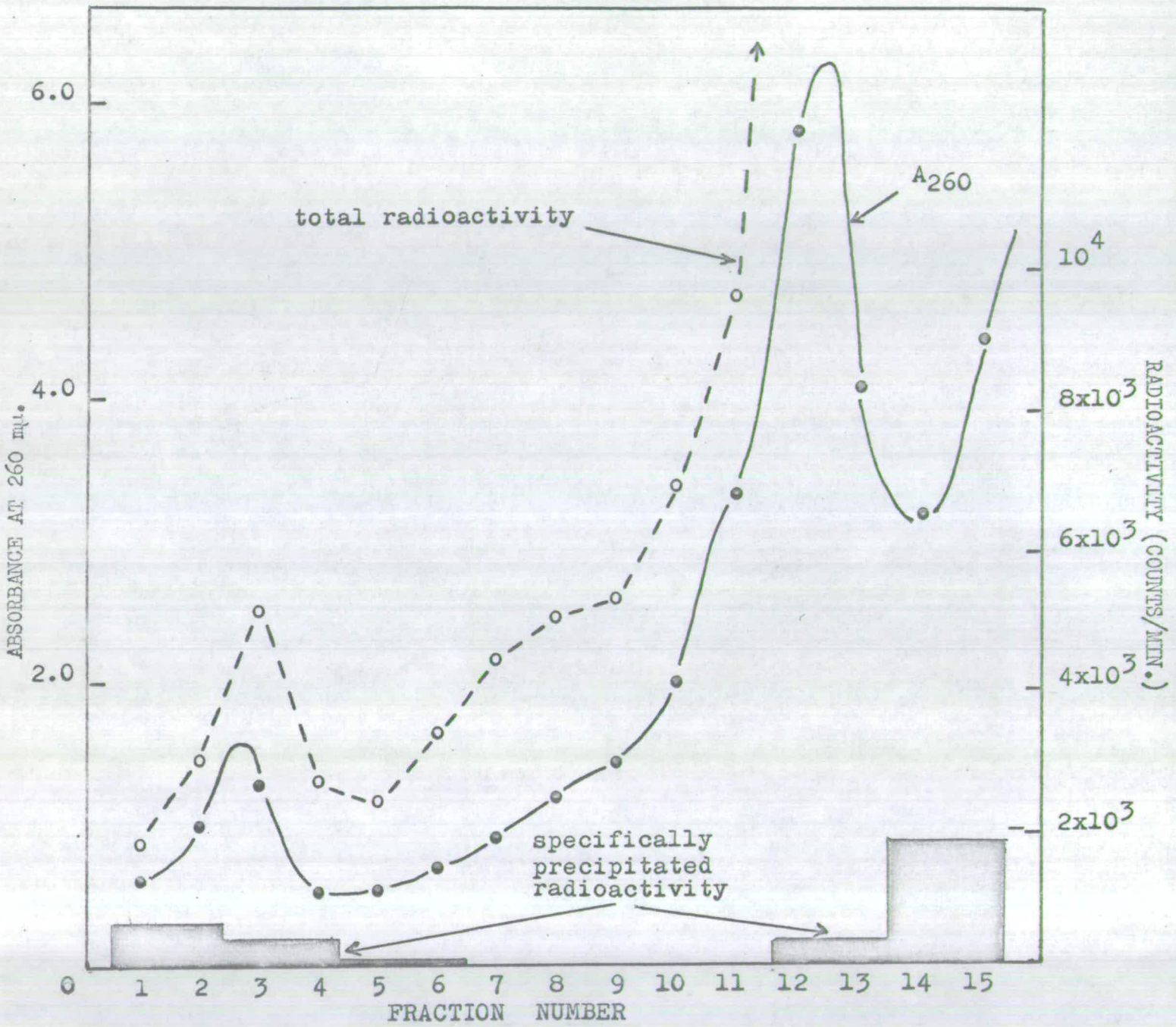


FIGURE 24 Sucrose gradient analysis showing the effect of homogenizing a labelled 20,000g supernatant fraction with an unlabelled 20,000g pellet (membrane fraction). The labelled preparation was derived from paramecia labelled via bacteria as for Figure 19.

of newly synthesised i-antigen.

Although there appeared to be little contamination of the membrane fraction with labelled soluble i-antigen (see Figure 20), the possibility existed of an association of free ribosomes containing radioactive 'nascent' i-antigen with the membrane fraction. Two possibilities existed : adsorption of these ribosomes to membranous elements or an aggregation of ribosomes resulting in sedimentation along with the membrane fraction.

On homogenizing a labelled paramecium 20,000g supernatant with an unlabelled 10 - 20,000g pellet, it was found, after density gradient centrifugation, that some of the labelled i-antigen activity had sedimented in the region of the membrane fraction (Figure 24). Since this material was originally unlabelled, some aggregation of the material in the labelled ribosome region had occurred, or else ribosomes containing 'nascent' i-antigen polypeptides had adsorbed on to components in the membrane fraction. The latter view was favoured because of the previous evidence of deoxycholate released ribosomes from the membrane fraction. Since the antiserum precipitation was specific, this increase in sedimentation of labelled i-antigen units was not considered to be due to denaturation.

In summary, the labelling of Paramecium aurelia by way of labelled bacteria provided a convenient method for the study of

i-antigen synthesis. Labelled i-antigen was detected 30 min. after the addition of the labelled source and was found to be mainly associated with the membrane fraction. Labelled i-antigen was not detected on the cilia and pellicle until 60 min. after the addition of the labelled source. The early association of labelled i-antigen with the membrane fraction, together with the evidence of membrane-ribosome bound labelled i-antigen suggests that this fraction represents the site of i-antigen synthesis.

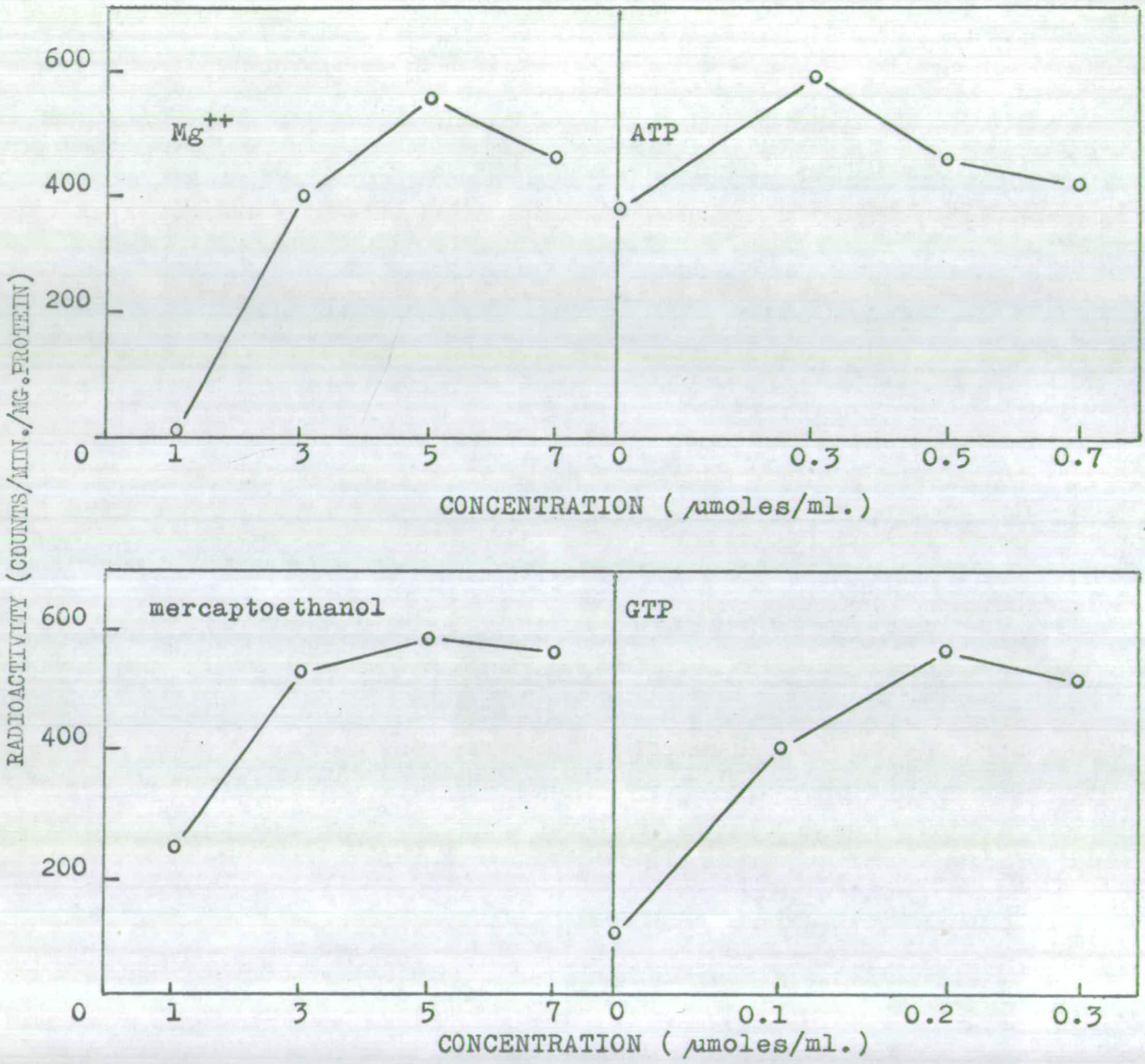


FIGURE 25 The effect of different Mg^{++}, 2-mercaptoethanol, ATP and GTP concentrations on the incorporation of C-14-leucine into TCA insoluble material by dialysed paramecium 10,000g supernatant. The reaction mixture contained in 1ml.(unless varied as above): 50µ moles Tris-HCl buffer (pH7.6), 25µ moles KCl, 5µ moles $MgCl_2$, 5µ moles 2-mercaptoethanol, 0.3µ moles ATP, 10µg creatine phosphokinase, 5µ moles creatine phosphate, 0.2µ mole GTP, 6mµ moles(1µc) C-14-leucine, 10mµ moles each of 17 amino acids, 0.2ml.(2.88mg.) 10,000g supernatant. Incubation was at 30°C for 20min.

Part III : Immobilization Antigen Synthesis by Paramecium Cell-free Systems.

The limitations in studying immobilization antigen synthesis by living paramecium were obvious: the relatively long labelling periods, the presence of labelled bacteria and the likely contamination of various sub-cellular components with labelled i-antigen by homogenizing the cells after labelling. Although many drawbacks could be envisaged, particularly the seeming complexity of organization in i-antigen synthesis, it was considered to be worth while to investigate the possibilities of a cell-free system.

Dialysed cytoplasmic extracts of Paramecium aurelia were supplemented with an energy source and amino acids and they were tested for their ability to incorporate a C^{14}-labelled amino acid into acid insoluble material. If some of the labelled material could be identified as i-antigen then a model system for the study of the components involved in antigen synthesis would be established. Such a study would provide a useful comparison with the view of i-antigen synthesis gained from the studies with living cells.

1. Requirements for amino acid incorporation.

Incorporation by paramecium 'post-mitochondrial' (10,000g x 10 min.) supernatants was found to be stimulated by an

	INCORPORATION	
	counts/min. per mg.protein	%complete system
Complete system	705	100
" " - energy-generating system	568	81
" " - complete energy system	178	25
" " - 17 amino acids	267	38
" " - membrane/ribosomes		5
" " - soluble fraction		12
" " + yeast s-RNA (60 µg/ml.)	763	108

TABLE 4. Requirements for incorporation of C^{14}-leucine into protein by paramecium 10,000g supernatant. The components of the incorporation mix unless omitted (or added) as above, were as described for Figure 25. Incubation was at 30°C for 20 min.. Membrane/ribosomes were separated from the soluble fraction by centrifuging at 110,000g for 60 min., and activity was expressed as %reconstituted system.

		INCORPORATION	
		counts/min. per mg.protein	%complete system
Complete system		738	100
" " + Ribonuclease	20 µg/ml.	-6	0
" " + ActinomycinD	50 µg/ml.	703	95
" " + Puromycin	75 µg/ml.	350	47
"	200 µg/ml.	274	37
" " + Chloramphenicol	100 µg/ml.	721	98

TABLE 5. The effect of antibiotics on the incorporation of C^{14}-leucine into protein by paramecium 10,000g supernatant. The components of the incubation mix were as described for Figure 25. Incubation was at 30°C for 20 min..

ATP-generating system, GTP and mercaptoethanol, and entirely dependent upon the presence of Mg^{++}. Figure 25 shows the effect of varying concentrations of added ATP, GTP, mercaptoethanol and Mg^{++} on the incorporation of C^{14}-labelled leucine into acid insoluble material by paramecium 10,000g supernatant. Maximum incorporation was observed in the presence of 0.3 µ.mole ATP, 0.2 µ.mole GTP, 5 µ.moles mercaptoethanol and 5 µ.moles $MgCl_2$, all in a total volume of 1 ml..

The paramecium supernatants were dialysed against dialysis buffer for several hours at 2-3°C in order to standardize the concentration of dialysable components before incubation. Even after dialysis for 3-4 hr., it appears that sufficient amino acids remain in the paramecium extract to allow some synthesis. As for soluble-RNA, it seems that there is an adequate supply in the extract: at least added yeast S-RNA did not substantially increase the incorporation capacity of the system. The effect of ommitting various components of the cell-free incorporating system is shown in Table 4. Both the membrane/ribosome material and the soluble fraction, as well as an energy system, were required for incorporation.

2. Effect of inhibitors on amino acid incorporation.

The influence of various drugs on the incorporation of C^{14}-labelled leucine into acid insoluble material was studied.

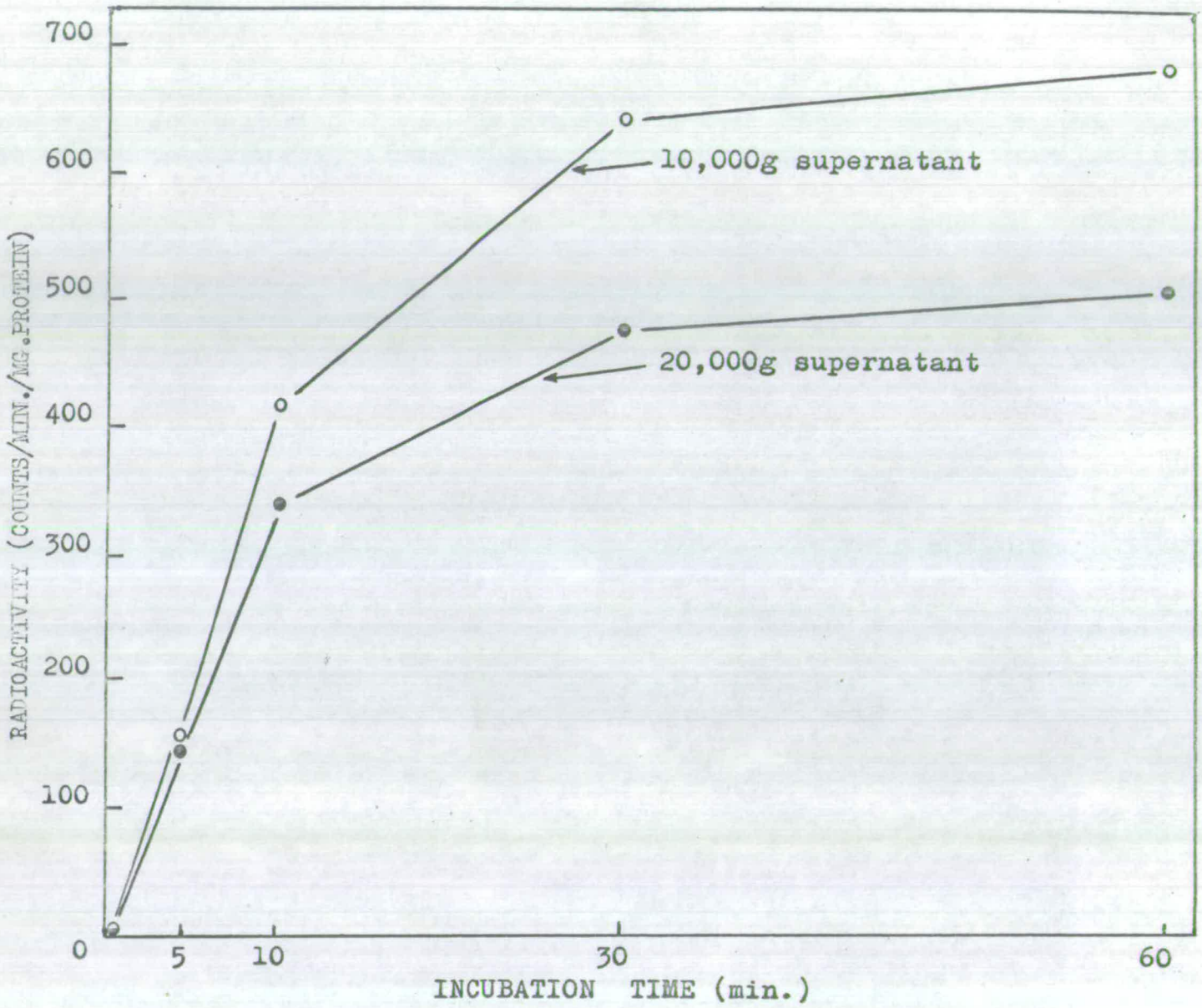


FIGURE 26 Incorporation of C-14-leucine by dialysed paramecium supernatants. Paramecium 10,000g supernatant contained 12.0mg. protein/ml. whereas the 20,000g supernatant derived from it had a protein concentration of 9.6mg./ml.. Supernatants were dialysed against dialysis buffer at 0°C for 3hr.. Each assay contained in 1ml.,2.40mg. 10,000g supernatant or 1.92mg. 20,000g supernatant and 6mμ moles (1μC) C-14-leucine. Other components of the incorporation mix are as described for Figure 25. Incubation was at 30°C.

The results are summarized in Table 5. Under the condition employed here, chloramphenicol and actinomycin D were found to be practically ineffective as inhibitors. Ribonuclease, on the other hand, always inhibited incorporation totally. Puromycin inhibited the system by between 47% and 37% depending upon the concentration used. These results are consistent with the known action of these drugs on protein synthesising systems.

3. Time course of amino acid incorporation.

Figure 26 illustrates the time course of the incorporation of C^{14}-labelled leucine into acid insoluble material by cell supernatants with and without the membrane fraction. The amount of incorporation per mg. protein was found to be higher in the fraction containing the membrane fraction (10,000g x 10 min. supernatant), indicating that this material accounts for some of its own incorporation or else stimulates incorporation by the rest of the system. After 30 min. incubation at 30°C, incorporation had virtually ceased in both systems.

The results from the last three sections, taken together, are a good indication that the amino acid incorporation observed, was genuine protein synthesis which occurs by way of a pre-formed messenger-RNA. These results are similar to those obtained

with other systems and their significance is discussed fully in a later section.

4. Detection of labelled i-antigen.

The conditions favouring protein synthesis in general were taken to be those best suited for i-antigen synthesis. Since i-antigen activity has been found in association with both membrane and free ribosome fractions derived from labelled paramecia, both of these fractions were tested in the cell-free system for their ability to synthesise i-antigen.

Two modifications were made to the cell-free system. To increase the efficiency in labelling newly synthesised paramecium protein, C^{14}-labelled protein hydrolysate (640 µC/mg.) was substituted for C^{14}-leucine. The comparative efficiencies are shown in Table 6. Also, since there was little release of labelled protein in the cell-free system, i.e. the 110,000g supernatant contained less than 20% of the labelled protein after 30 min. incubation, an attempt was made at artificial release of 'nascent' protein with the use of puromycin. This drug is known to act by causing a break in the growing polypeptide chain.

Puromycin (200 µg/ml.) was added to the mix after 10 min. and incubation at 30°C was continued for a further 10 min.. The incubation mix was then chilled and treated with sodium

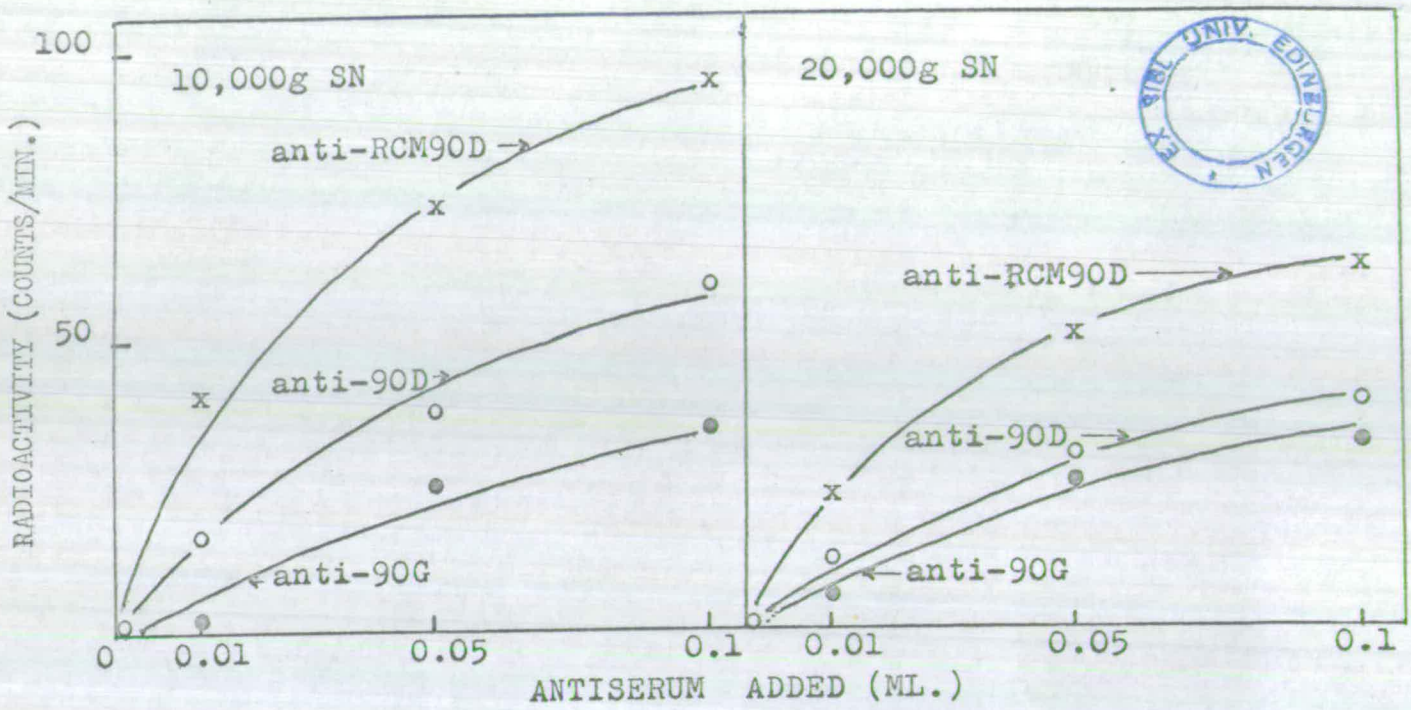


FIGURE 27 Precipitation of puromycin released protein derived from Paramecium 10,000g and 20,000g labelled supernatants. Conditions as for Figure 25 .

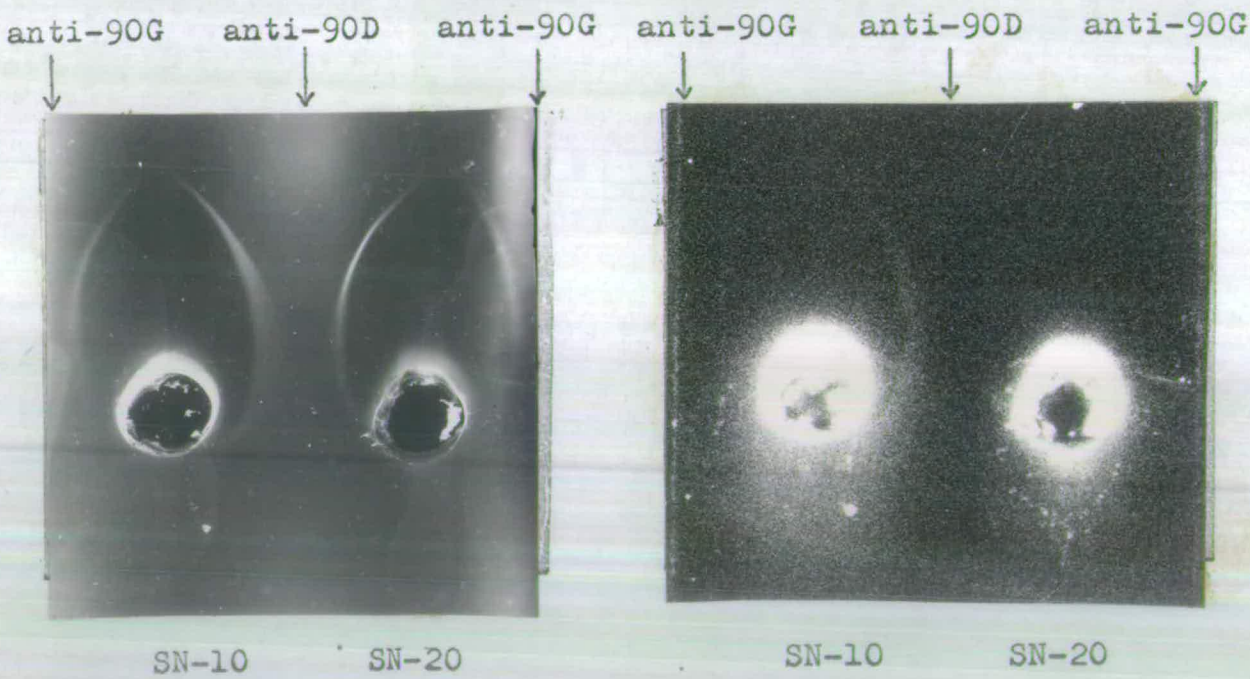


FIGURE 28 Immunoelectrophoresis and autoradiography of puromycin released protein derived from Paramecium 10,000g and 20,000g labelled supernatants. Conditions as for Figure 25 .

deoxycholate (0.5%) to solubilize the membrane material. Under these conditions, about 60% of the labelled protein was found in the supernatant after centrifugation at 110,000g for 90 min.. This 'released' labelled protein was then tested for i-antigen activity.

Figure 27 shows the effect of antisera in precipitating 'released' radioactive protein from a preparation derived from cells expressing 90D serotype. Anti-90G serum was used as the control. Anti-RCM-90D was serum prepared against reduced carboxymethylated 90D antigen (Jones, 1965). This antiserum may detect i-antigen more closely resembling subunits, while the anti-90D serum probably detects the more completed i-antigen molecule. It can be seen from the figure that there is a high level of unspecific precipitation of radioactivity in preparations derived from supernatants both including (10,000g) and excluding (20,000g) the membrane fractions. Material derived from the 10,000g supernatant gave a precipitation, by homologous (anti-90D) serum, of approximately double the control, while the effect of antiserum against RCM-i-antigen was about three times that of the control. On the other hand, material derived from the 20,000g supernatant gave little or no specific precipitation with anti-90D serum, but anti-RCM-90D serum gave some specific precipitation amounting to less than twice the control reaction.

That the anti-RCM-90D serum was specific for labelled

material derived only from 90D-type cells was tested by labelling a heterologous (90G) stock of paramecium and measuring the precipitation of radioactivity, as above, by this antiserum, anti-90D and anti-90G. In this case, the anti-90G serum was shown to have the specific effect while the anti-90D and anti-RCM-90D sera caused an equal amount of, presumably, non-specific precipitation.

Puromycin 'released' protein was also tested for labelled i-antigen by the immunoelectrophoresis/autoradiography technique (Figure 28). Carrier 90D and 90G purified i-antigens were added to preparations derived from both 10,000g and 20,000g incubated supernatants. Precipitin arcs were formed with both anti-90D and anti-90G sera against the two samples, but only one of the four arcs contained radioactive material; that of the 10,000g supernatant against anti-90D serum. Labelled i-antigen was only detected by this method in labelled material derived from supernatants including the membrane fraction.

It was concluded that cell-free Paramecium supernatants were capable of incorporating labelled amino acid into i-antigen. The labelling, and presumably the synthesis, of i-antigen was greatly, if not entirely, dependent upon the inclusion of the membrane fraction. However, preparations with and without the membrane fraction seemed capable of synthesising protein specifically precipitated by antiserum against reduced

carboxymethylated i-antigen. The significance of these results, together with some other results, will be discussed later.

5. Site of i-antigen synthesis.

The conclusion, that labelled 'native' i-antigen was mainly detected in paramecium supernatants containing the membrane fraction, was supported by the apparent importance of this fraction on the distribution of labelled i-antigen when the cell-free incorporating material was analysed by sucrose gradient centrifugation.

For sucrose gradients, C^{14}-leucine was used as radioactive label since it gave a lower background than did C^{14}-protein hydrolysate, which tended to adsorb on to the filters..

Figure 29 shows the distribution of total radioactivity and specifically precipitated radioactivity after 10 min. incubation at 30°C. Identical treatment, except omission of incubation, served as a control gradient which gave little incorporation of radioactivity. As expected, incubation prior to gradient analysis resulted in a shift (of the material absorbing at 260 mμ.) towards the monosome region, compared with unincubated samples (see Figure 30). This feature presumably represents polysome breakdown by endogenous degradative enzymes. In order to characterize polysomal size, gradients were calibrated with respect to approximate

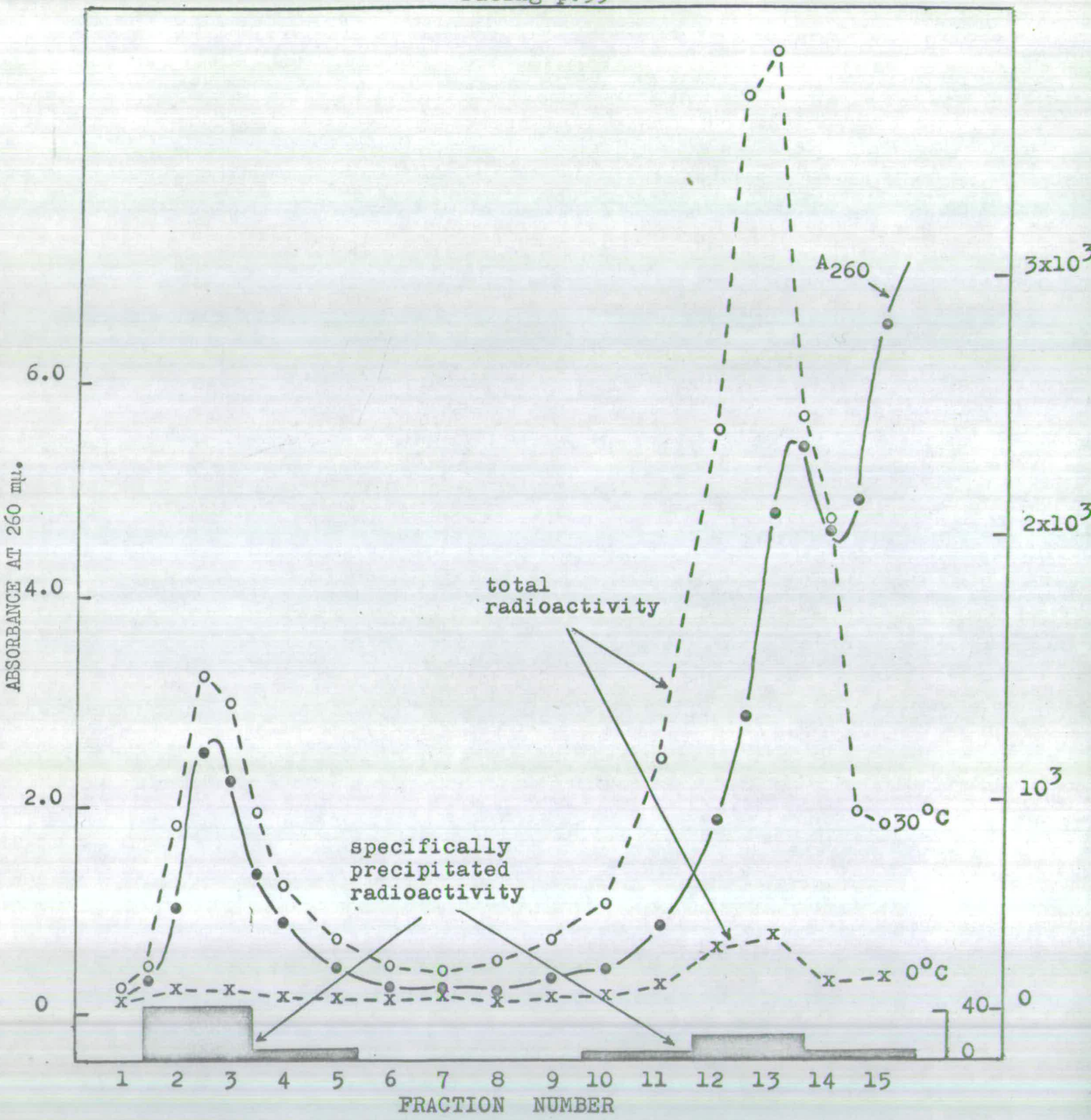


FIGURE 29 Sucrose gradient analysis of an "in vitro" amino acid incorporating paramecium 10,000g supernatant. Incubation time in the presence of 6mμ moles (1μC)/ml. C-14-leucine was for 10min. at 30°C. Incorporation at 0°C is also shown.

sedimentation coefficients on the basis of direct proportionality between sedimentation coefficient and distance travelled from the top of the gradient (Martin and Ames, 1961) using the position of the monosome peak as a standard of 80S.

In Figure 29, peaks of radioactivity were found to correspond to both the free ribosome and membrane A260 peaks, but there appeared to be little released soluble protein. Specifically precipitated radioactivity amounted to approximately 0.5% of the total radioactive protein and was mainly found in the membrane fraction. Some of the i-antigen activity was, however, found in association with free ribosomes. Since, in some experiments, this amounted to 35% of the total radioactive i-antigen, it would appear to be a significant factor. However, the question of the membrane fraction as the sole site of i-antigen synthesis could not be answered by this type of experiment since free ribosomes with associated i-antigen activity could be breakdown components. On the other hand, free ribosomes could represent the synthetic sites, the membrane fraction merely serving to bring together and order the synthesised polypeptides into identifiable i-antigen.

In order to see if the distribution of radioactive i-antigen in gradient analysis of incubated paramecium supernatant truely reflected the synthetic potential of each of the gradient fractions, separated gradient fractions were incubated with the

Facing p.56

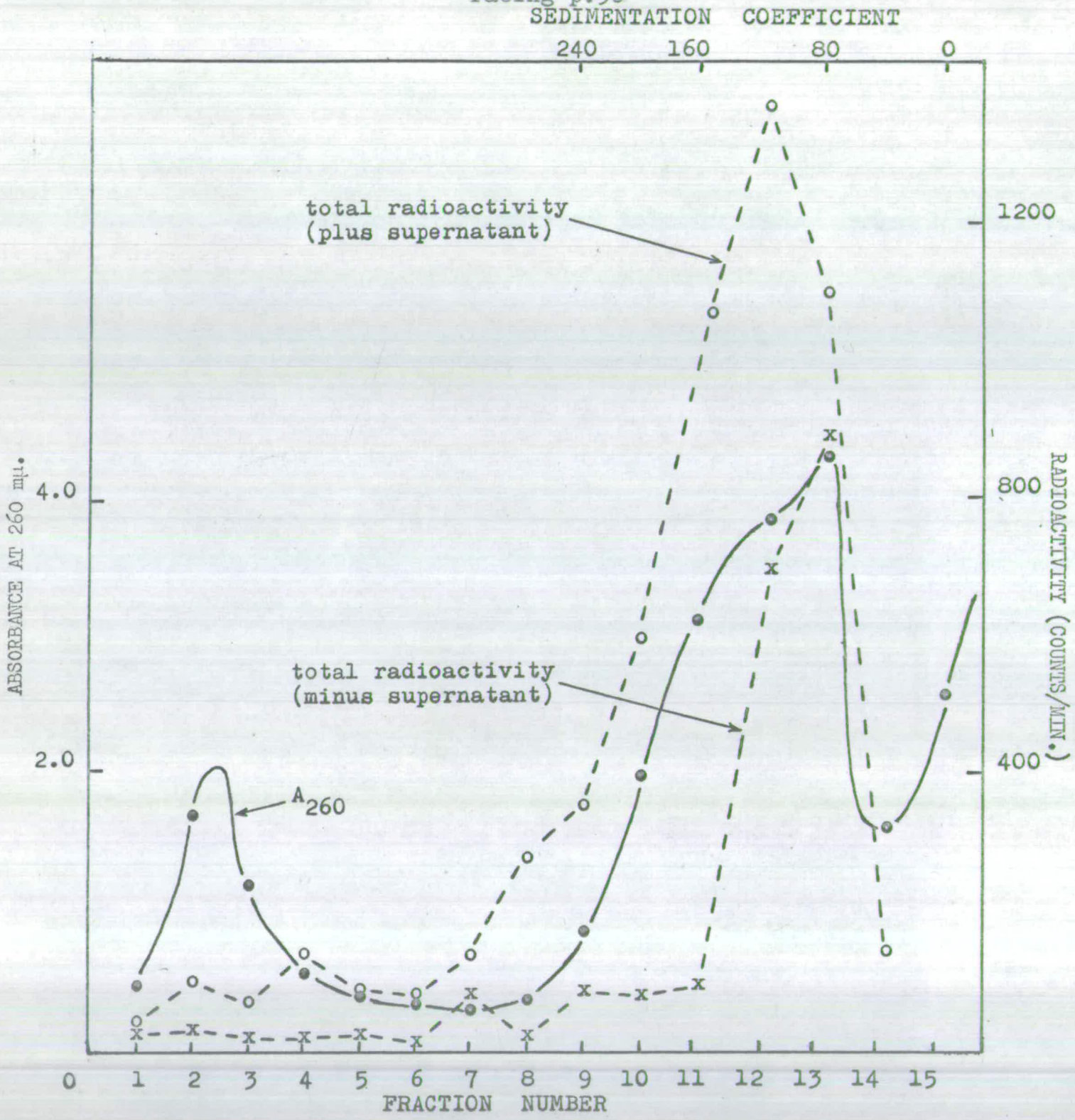


FIGURE 30 Sucrose gradient analysis of paramecium 10,000g supernatant showing protein synthetic ability of separated fractions. Each fraction was tested with and without the addition of soluble material from fraction 15, for its ability to incorporate C-14-leucine into TCA insoluble material.

components of the amino acid incorporating system. The results are shown in Figure 30. The gradient fractions were divided in two and incubated with and without the addition of soluble components from the top of the gradient. As can be seen from the radioactivity profiles, the addition of soluble material was essential for incorporation by the polysome fractions. Fractions near the top of the tube did not require added soluble material presumably because these fractions already contained sufficient soluble fraction as contaminent. The surprising feature was that there seemed to be no synthetic activity in the membrane fractions. At first, this was believed to have been due to the inhibitory action of the dense sucrose present in these fractions. However, dialysis of the fractions did not restore synthetic ability and it appeared that radioactivity found in the membrane fraction in earlier experiments was due to adsorption of protein already synthesised by free ribosomes. Experiments involving variations of membrane concentration to soluble material showed that this interpretation was not correct and that the membrane fraction, as opposed to the free ribosome fractions, exhibited a narrow concentration optimum for successfully synthesising protein. The effect of varying the fraction concentration is shown in Figure 31. The inhibition of synthesis by high membrane concentration is probably due to the effect of degradative enzymes present in this material.

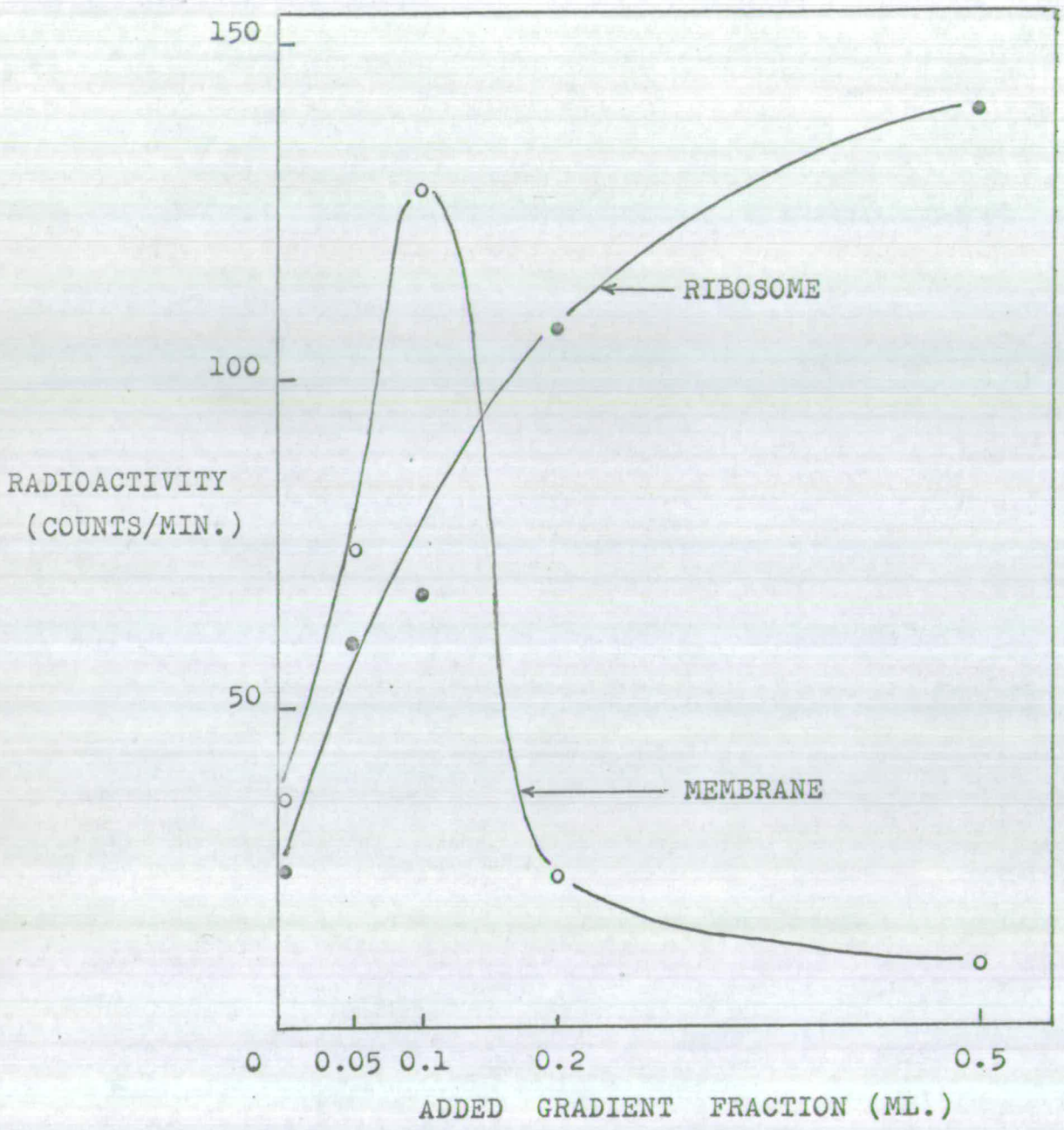


FIGURE 31 Incorporation into protein by increasing concentrations of membrane and ribosome. Dialysed sucrose gradient fractions were added to the amino acid incorporating mix including 0.1ml. supernatant derived from the gradient top fraction. The membrane and ribosome gradient fractions were chosen to give equal absorbance (2.0) at 260mμ. The total volume was 1ml. and incubation was at 30°C for 20min..

Since each preparation varied in a number of respects, the optimum concentration of membrane fraction was calculated each time it was used in incorporation studies. Figure 31 shows one such calibration and is expressed in arbitrary units.

Figure 32a shows the ability of sucrose gradient fractions, adjusted to optimal concentration conditions, to synthesise protein immunologically recognisable as i-antigen. Since antiserum against reduced carboxymethylated i-antigen has been shown to have a specific effect in the precipitation of some labelled protein, the incubated sucrose gradient fractions were tested with both anti-90D and anti-RCM-90D sera. As can be seen from the figure, both types of antiserum reacted with labelled material in the membrane fraction where 81% of the i-antigen activity was found. However, the spectrum of precipitation by the two antisera in the polysome region was somewhat different: anti-90D reacted with gradient material sedimenting about 240S while anti-RCM-90D reacted with gradient material mainly between 240S and 120S.

The effect of deoxycholate treatment prior to gradient separation resulted in the distribution of synthetic ability shown in Figure 32b. Material sedimenting as membrane, and the synthetic ability attributed to it, was largely removed by this treatment. However, synthesis by the free ribosomes

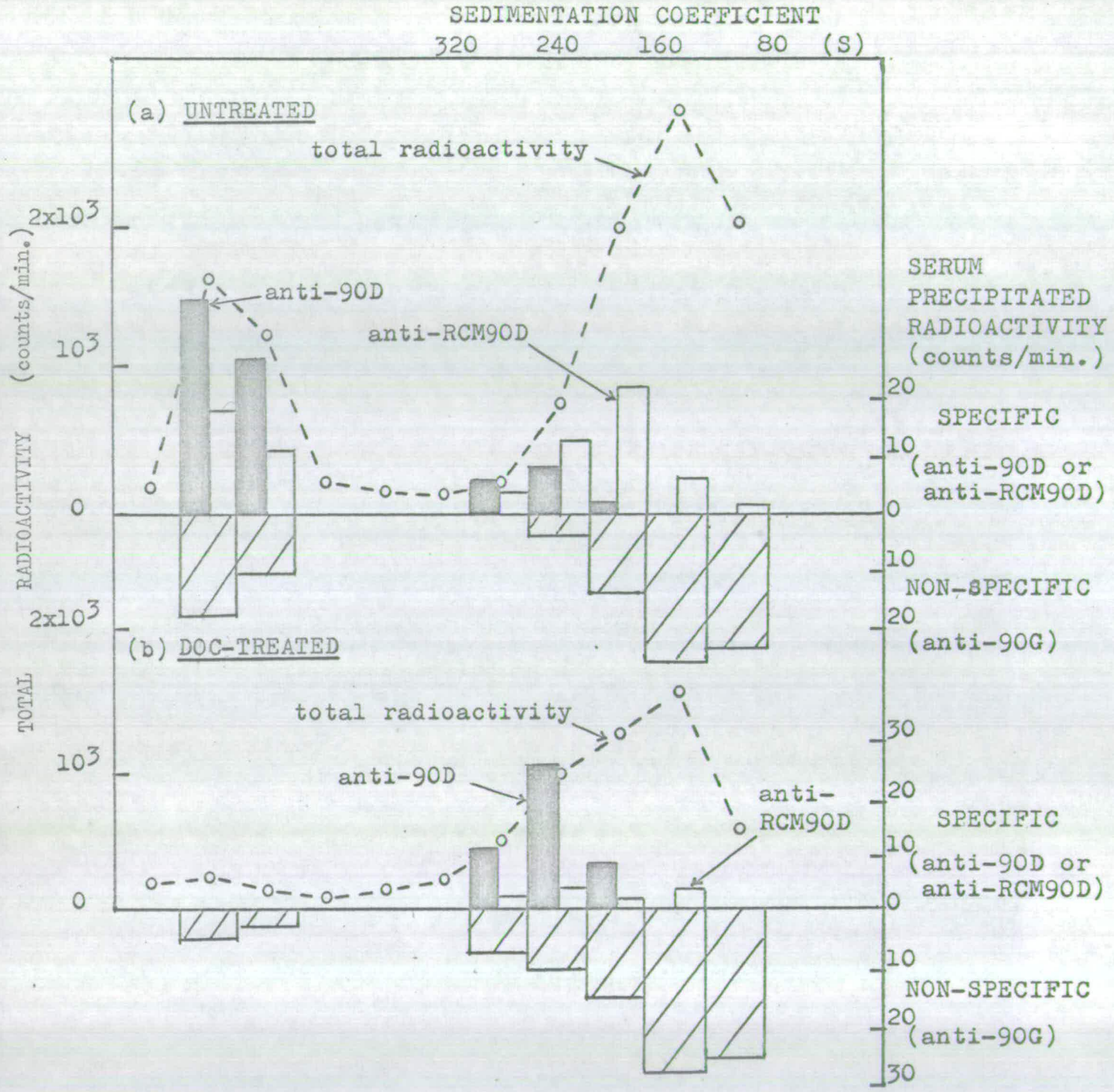


FIGURE 32. Sucrose gradient analysis of paramecium 10,000g supernatants, (a) untreated and (b) treated with 0.5% deoxycholate, showing the ability of separated fractions to synthesise i-antigen. Each fraction was incubated in the presence of C^{14}-leucine at 30°C for 30 min., assayed for total radioactive protein and serum precipitated radioactivity - two specific antisera and one non-specific antiserum (control). were used. Absorbance is not shown but sedimentation coefficients are indicated as references.

(plus the DOC-released ribosomes) was significantly reduced, particularly by the ribosomes near the top of the tube. This effect is presumably due to the adverse action of deoxycholate when contaminated fractions near the top of the tube are incubated in its presence. In spite of the inhibition of total synthesis, there was an increase in the precipitation of radioactivity by anti-90D serum in the region between 320S and 160S, and a general reduction in the radioactivity specifically precipitated by the anti-RCM-90D serum.

Although similar results were found in several experiments, the level of i-antigen labelling was low and made it particularly difficult to obtain significant results from sucrose gradient analysis. Since the i-antigen is unusually high in cystine content, it was decided to substitute S^{35}-labelled cystine as the labelled amino acid in place of C^{14}-labelled leucine in the hope that incorporation would be largely into i-antigen. Table 6 compares the efficiency of incorporating C^{14}-leucine, C^{14}-protein hydrolysate and S^{35}-cystine into i-antigen by the cell-free system. Although the use of C^{14}-protein hydrolysate gave i-antigen approximately three times more labelled than C^{14}-leucine labelled i-antigen, this labelled source, as already mentioned, gave a high degree of non-specific precipitation, particularly due to unincorporated labelled material not passing through the filters during washing of the protein precipitate. The use of S^{35}-cystine was found to be

STAGE	TOTAL RADIOACTIVITY (counts/min.)	PERCENTAGE ORIGINAL ACTIVITY (%)
C^{14}-leucine	10.0×10^5	100
paramecium protein	2.3×10^4	2.3
	2.7×10^4	2.7
i-antigen	0.9×10^2	~ 0.01
	1.1×10^2	"
C^{14}-protein hydrolysate	10.0×10^5	100
paramecium protein	7.7×10^4	7.7
	9.2×10^4	9.2
i-antigen	2.5×10^2	~ 0.03
	3.2×10^2	"
S^{35}-cystine	10.7×10^5	100
paramecium protein	1.8×10^4	1.7
i-antigen	4.2×10^2	~ 0.04

TABLE 6. Efficiency in incorporating 1 μC. C^{14}-leucine or C^{14}-protein hydrolysate or S^{35}-cystine into paramecium protein and i-antigen in the cell-free system. Conditions of labelling as for Figure 25. Efficiency in counting C^{14} on filters was 45%, S^{35} on filters was 48%.

more suitable for labelling i-antigen. In spite of the fact that total paramecium protein was less labelled when this radioactive source was used, i-antigen was approximately four times more labelled than C^{14}-leucine labelled i-antigen. (These values served as a guide only in the practical aspect of labelling i-antigen. No conclusions about the relative uptake of different amino acids can be obtained from these results since the isotopic concentration was different in each case and the level of endogenous amino acids in the cell supernatants was unknown.)

Figure 33 shows the effect of separated sucrose gradient fractions in incorporating S^{35}-labelled cystine into protein and i-antigen. Here, the i-antigen was more actively labelled and the results were more significant. However, the pattern of radioactivity was basically that already obtained in previous experiments using C^{14}-labelled leucine.

It was now evident that the membrane fraction was important not only as an early site of i-antigen, but as a site of synthesis. The significance of i-antigen activity in association with free polysomes could be interpreted in two ways: as breakdown products released from their natural location on homogenization, or as precursor steps in i-antigen synthesis. The particular reaction of smaller polysome aggregates with antiserum against i-antigen subunits and of larger polysome aggregates with antiserum against

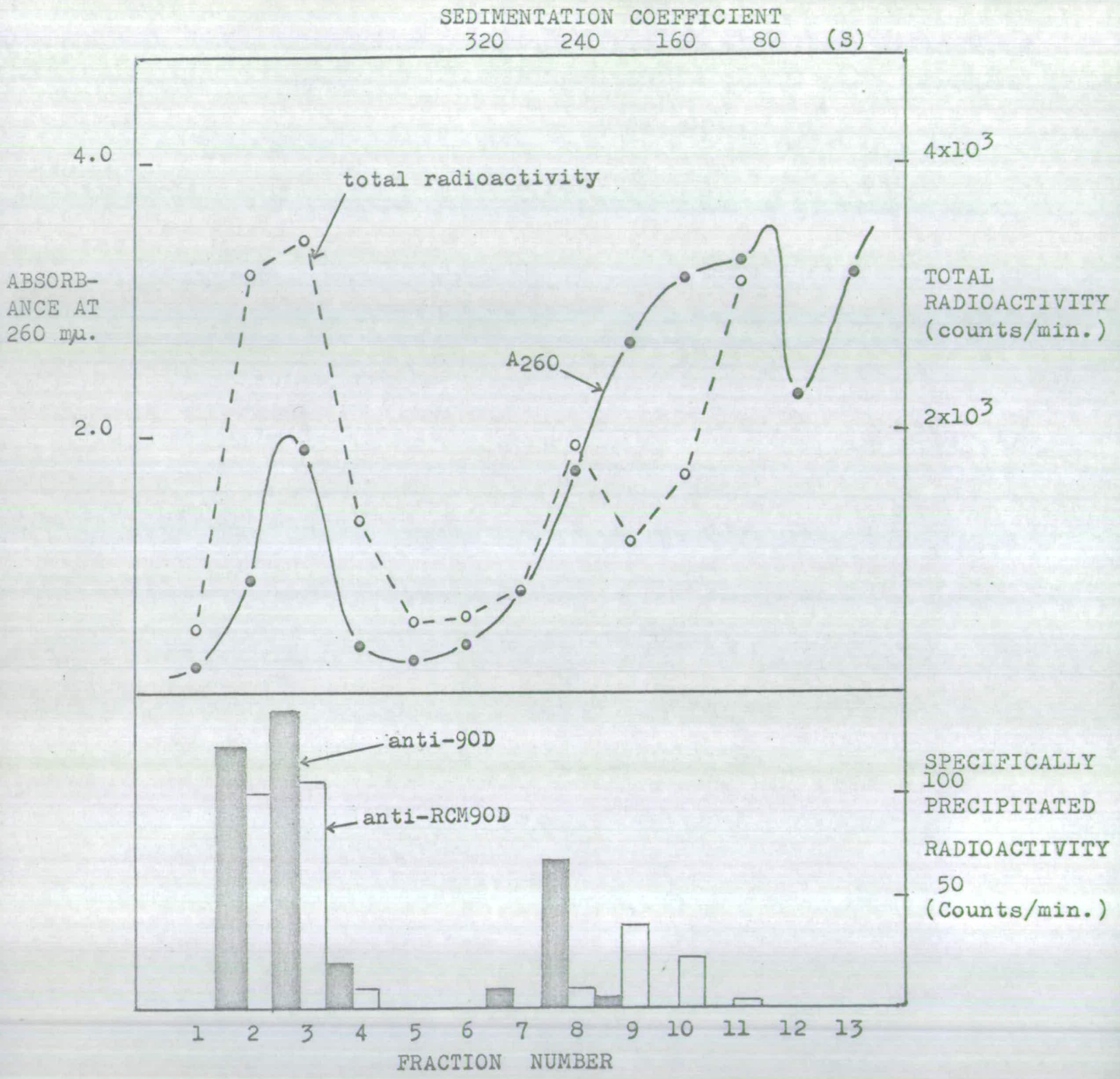


FIGURE 33. Sucrose gradient analysis of paramecium 10,000g supernatant showing the ability of separated fractions to synthesise i-antigen. Conditions were as for Figure 32 except that the labelled amino acid was S^{35}-cystine instead of C^{14}-leucine.

native i-antigen, together with the known specific adsorption of polysomes with associated i-antigen activity on to the membrane fraction, support this latter hypothesis. The functional integrity of the free i-antigen synthesising polysomes and their relatively narrow distribution in the gradient also suggests that they are not breakdown products.

6. Analysis of puromycin released i-antigen.

Immunological tests have revealed radioactive protein reacting specifically as i-antigen. However, no information is gained about the size or completeness of the antigenic molecules. Presumably, the presence of one antigenic site would be sufficient to effect complex formation with antibody. From earlier studies, it appeared that little released labelled protein was being formed, at least after 30 min. after the addition of the labelled amino acid. Since puromycin treated incubates gave a positive reaction with the immuno-electrophoresis/autoradiography test, even after 5 min. labelling, it was concluded that, at best, some of the reacting labelled material was near enough the size and charge of complete i-antigen to behave identically on electrophoresis and diffusion.

In order to find out more about the size of labelled antigenic molecules, puromycin released protein was analysed

(Facing p.61)

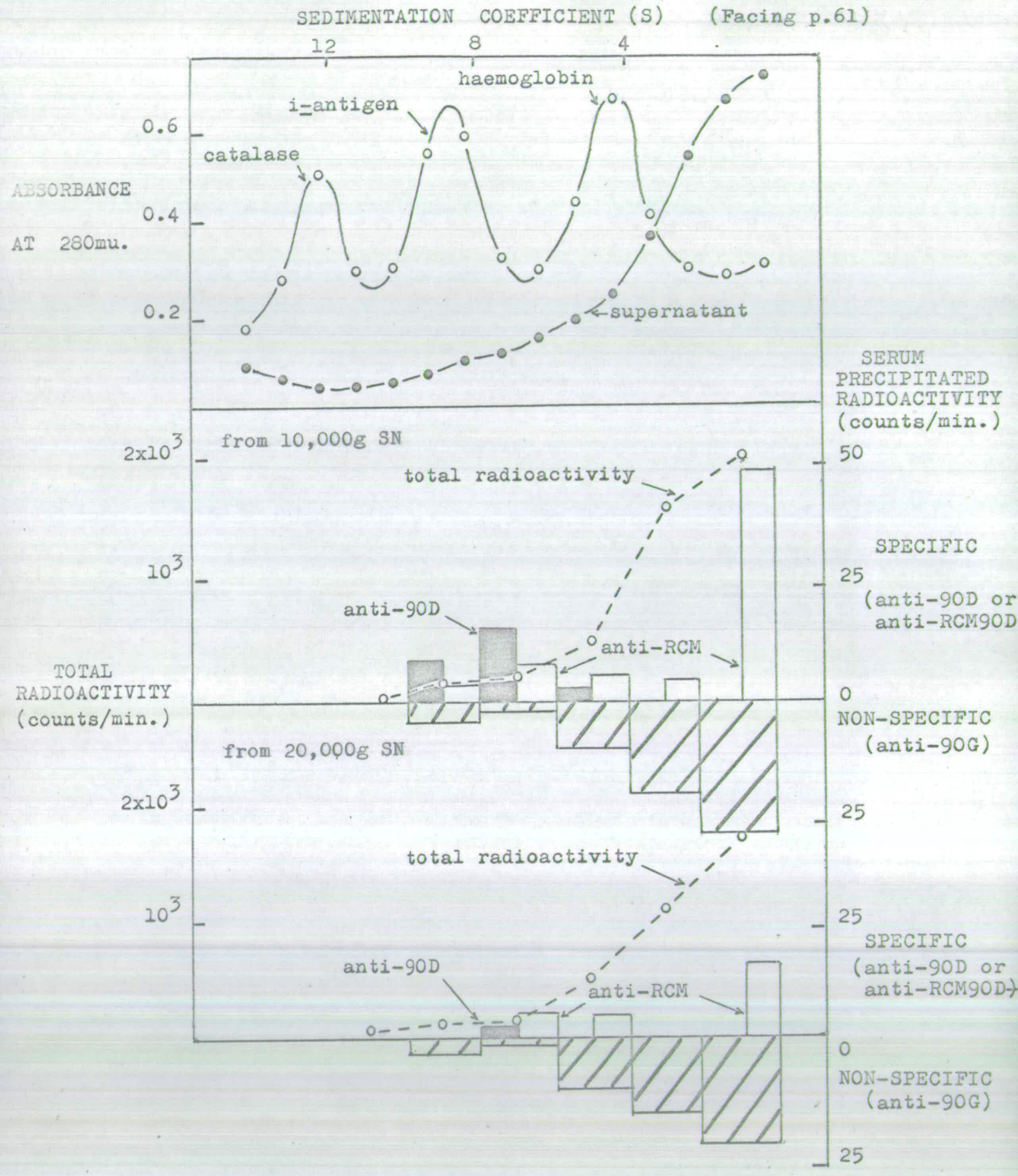

SEDIMENTATION COEFFICIENT (S)
12
8
4
haemoglobin
i-antigen
catalase
0.6
0.4
0.2
ABSORBANCE AT 280mu.
supernatant
SERUM PRECIPITATED RADIOACTIVITY (counts/min.)
from 10,000g SN
2x10^3
10^3
total radioactivity
anti-90D
anti-RCM
50
SPECIFIC
25
(anti-90D or anti-RCM90D)
TOTAL RADIOACTIVITY (counts/min.)
0
NON-SPECIFIC (anti-90G)
from 20,000g SN
2x10^3
10^3
total radioactivity
anti-90D
anti-RCM
25
SPECIFIC (anti-90D or anti-RCM90D)
0
NON-SPECIFIC (anti-90G)
25

FIGURE 34 Sucrose gradient analysis of puromycin-released protein derived from labelled paramecium supernatants. Paramecium 10,000g (including membrane) and 20,000g (excluding membrane) supernatants were labelled in the cell-free system as for Figure 25. After 5 min. incubation at 30°C, puromycin was added to a concentration of 200 µg./ml. and incubation was continued for a further 10 min.. The incubates were then chilled, treated with 100x 'cold' leucine and made up to 0.5% deoxycholate. After dialysis for 4 hr. against dialysis buffer at 2-3°C, the preparations were centrifuged at 110,000g for 90 min.. The pellets were discarded and the supernatants were layered on 5-20% sucrose gradients and centrifuged at 30,000 r.p.m. for 30 hr.. Fractions were collected and analysed for absorbance, total protein radioactivity and specifically precipitated radioactivity - the antisera used were anti-90D, anti-RCM90D and anti-90G (control). Catalase, purified i-antigen and haemoglobin served as gradient markers.

on sucrose gradients. Since puromycin release of polypeptides from ribosomes only occured effectively in the presence of cell sap, the sample material contained released plus soluble material. Figure 34 shows the distribution of absorbance at 280 mμ., total radioactive protein and specifically precipitated radioactive protein from incubated supernatant with and without the membrane fraction. Catalase, i-antigen and haemoglobin were used as gradient markers. As can be seen from the figure some labelled antigenic material sedimented through the gradient as far as the 8S i-antigen marker. This material, which was only found in the supernatant which originally contained membrane, reacted mainly with antiserum against native i-antigen. Material sedimenting through the gradient at a slower rate reacted mainly with antiserum against RCM-i-antigen. The activity released from the free ribosome preparation behaved almost entirely as the form reacting only with anti-RCM serum and sedimenting at rates slower than the marker i-antigen.

It appears from these experiments that the labelled material reacting specifically with antiserum against i-antigen has narrow limits of size distribution, about the 8S marker. This evidence, together with that of the identical behaviour of labelled i-antigen with native i-antigen on immunoelectrophoresis, indicates that synthesis must be complete or almost complete, and that aggregation of subunits has occurred,

before the molecule reacts with the antiserum. The reaction with anti-RCM-i-antigen occurs with material lighter than the i-antigen marker, this having a wide distribution through the gradient but mainly remaining at the top of the tube. This material may represent unaggregated subunits or polypeptides (at the top of the gradient) and small aggregations of subunits (through the gradient). It is not known whether or not aggregation occurs before synthesis is completed, but the failure of the cell-free system to release completed i-antigen might indicate that aggregation does, in fact, occur before synthesis is completed. This point will be discussed later.

7. Nature of i-antigen - ribosome - membrane association.

The sedimentation of radioactive i-antigen with the membrane fraction could be due to one of two possibilities; the polysomes responsible for i-antigen synthesis are attached to membranous elements, or the polysomes responsible for i-antigen synthesis are very large aggregates of over 100 ribosome units (based on sedimentation coefficient).

One method of distinguishing between the two possibilities is the behaviour of the radioactivity associated with the membrane fraction on prolonged centrifugation through 2M sucrose. This technique is known to permit the separation of free ribosomes (Petermann, 1964; Kuff, et al., 1966), the membrane material being of insufficient density to penetrate

GRADIENT FRACTION	2M SUCROSE FRACTION	PROTEIN RADIOACTIVITY		i-ANTIGEN RADIOACTIVITY		ABSORBANCE		
		counts/min.	%recovered	counts/min.	%recovered	A_{260}	%recovered	A_{260}/A_{235}
MEMBRANE	SUPERNATANT	693	87	42	>90	0.30	70	0.77
	PELLET	96	13	(1)	<10	0.13	30	0.72
FREE RIBOSOME	SUPERNATANT	370	15	(-2)		0.28	21	0.82
	PELLET	2088	85	10		1.08	79	1.39

TABLE 7 Distribution of radioactivity and absorbance after centrifugation of sucrose gradient fractions through 2M sucrose. The gradient fractions were derived from a gradient similar to the one shown in Figure 29, and correspond to fractions 1-3 and 12-13. The values for radioactive i-antigen in brackets do not vary significantly from background and are representative of values obtained in different experiments.

Facing p.63

the dense sucrose. Labelled gradient fractions (corresponding to tubes 2-3 and 12-13 in Figure 29) were layered as 3 ml. samples over 1.5 ml. 2M sucrose in gradient buffer. After centrifugation at 130,000g for 5 hr., a densely turbid band was present at the 2M sucrose/sample interface in tubes containing membrane fraction; little or no turbidity was seen at the interface in tubes containing free ribosome preparations. The contents of each tube were divided into two fractions; a supernatant consisting of the original sample zone, the interface and the upper 0.5 ml. of the 2M sucrose, and a pellet consisting of the residual 2M sucrose which was then agitated to suspend pelleted material. Supernatant and pellet fractions were then dialysed against dialysis buffer, made up to the same volume, and analysed for absorbance and radioactivity. The results are shown in Table 7.

In the case of the membrane fraction most of the absorbance at 260 mμ. (70%) and radioactivity (87%) failed to sediment through the dense sucrose. Most of the labelled protein thus appeared to be bound to the membrane material. Labelled i-antigen activity also failed to sediment, indicating that it was genuinely associated with the membrane material and not in the form of large unattached polysomes. A useful comparison was supplied by the free polysome fraction of which most of the A260 (79%), protein radioactivity (85%)

and labelled i-antigen sedimented. The absorbance ratio A260/A235 indicated that the sedimenting material from the polysome preparation was primarily ribosomal in nature.

In conclusion, the study of i-antigen synthesis using a Paramecium cell-free system largely confirmed the results obtained from similar experiments performed on living cells; that i-antigen is mainly synthesised on membrane-bound ribosomes but is also found, particularly in a suspected subunit form, on free polysomes. The cell-free system permitted the study of this system in far greater detail.

DISCUSSION

I The Organization and Function of Ribosomes.

It has been established in a number of instances that proteins are synthesised on clusters of ribosomes linked together by strands of messenger-RNA (Warner et al., 1962, 1963; Gierer, 1963). The information necessary for the manufacture of a protein is a function of the messenger-RNA molecule, the base sequence of which is related to the sequence of amino acids in the protein molecule (Brenner et al., 1961; Nirenberg and Matthaei, 1961). Three nucleotide bases are believed to be responsible for the coding of a single amino acid (Crick et al., 1961). Thus it is possible to consider protein synthesis in a quantitative manner.

This mechanism of protein synthesis, by ribosomal aggregates or polysomes (reviewed, Campbell, 1965), has been demonstrated in bacterial, protozoan, mammalian and plant systems, and results in the formation of proteins as diverse as haemoglobin (Warner et al., 1962, 1963; Marks et al., 1962; Gierer, 1963), γ-globulin (Scharff and Uhr, 1965; Norton et al., 1965; Schapiro et al., 1966; Becker and Rich, 1967; Williamson and Askonas, 1967), β-galactosidase (Zipser, 1963; Kiho and Rich, 1964, 1965), collagen (Kretsinger et al., 1964; Malt and Speakman, 1964; Manner et al., 1967) and viral coat protein

(Scharff et al., 1963). Most of these proteins appear to be synthesised on polysomes of fairly uniform size i.e. there appears to be a relation between the size of the protein molecule and the size of the polysomal cluster involved in its synthesis. For example, the haemoglobin polypeptide has a molecular weight of about 17,000 and is synthesised on clusters of 5 to 6 ribosomes (Gierer, 1963) while β-galactosidase has a sub-unit with a molecular weight of about 135,000 and is synthesised on a cluster of about 50 ribosomes (Zipser, 1963; Kiho and Rich, 1964, 1965). This relationship which is a function of the length of messenger-RNA coding for the polypeptide, is later shown diagramatically (Figure 35).

In those cells synthesising protein for secretion, e.g. liver cells and tumour cells, the majority of ribosomes is attached to the membranes of the endoplasmic reticulum (Palade, 1958; Porter, 1961). These bound ribosomes also seem to be arranged in polysomal clusters on the membranes (de Petris and Karlsbad, 1965; de Petris, 1967) and appear to be active in the synthesis of polypeptides (Henshaw et al., 1963; Kuff et al., 1966). Upon cell fractionation, the endoplasmic reticulum is fragmented into vesicles which are isolated as the microsomal fraction (Palade and Siekevitz, 1956). As for transport, it appears that secretory proteins pass directly from the attached ribosomes into the cisternal cavities across

the endoplasmic reticulum membrane (Redman et al., 1966; Redman and Sabatini, 1966; Sabatini et al., 1966).

Membrane-bound ribosomes have also been found to occur in yeast (Hauge and Halvorson, 1962) and bacteria. Bacterial membrane-bound ribosome protein synthesis has been described in fractionation experiments involving both labelled cells (Hendler and Tani, 1964; Hallberg and Hauge, 1965) and cell-free systems (Schlessinger, 1963; Moore and Umbreit, 1965) as well as on electron microscope examination of bacterial sections (Abrams et al., 1964; Hendler et al., 1964; Schlessinger et al., 1965).

It has been found that ribosomes attached to membranes are strongly bound in both animal (Kuff et al., 1965; Sabatini et al., 1966) and bacterial (Aronson, 1966) systems. Sabatini et al. conclude that :

"a) the ribosomes which are strongly attached are, because of this situation, more active in protein synthesis.
or b) the presence of the product of synthesis on the ribosomes is what makes them stick to the membrane."
However, Aronson concludes that the presence of nascent polypeptides is the critical factor in establishing association.

Thus, all proteins, apart from small polypeptides such as antibiotics (Mach, 1963) and cell-wall polymers of bacteria (Salton, 1964) are synthesised on ribosomes. These ribosomes

are organized in polysomal clusters either free in the cytoplasm or attached to membranes. The association of polysomes with membranes may contribute to the stability of the messenger-RNA (Schlessinger, 1963; de Petris et al., 1963; Aronson, 1965) or may be a factor in establishing the location of proteins in the cell (Marr, 1960; Neu and Heppel, 1964) or for secretion (discussed above).

This preliminary review of the type of protein synthesising systems serves as a useful comparison with the situation found in Paramecium aurelia. In this study, cell fractionation experiments have yielded preparations which on chemical analysis and electron microscopic examination have corresponded to separated membrane-bound ribosomes and unattached ribosomes. This demonstration of the two ribosomal forms has been confirmed to some extent by electron microscope studies on cell sections (Sinden, unpublished). Both types of preparation have been shown to contain labelled protein after labelling living cells, and also to be active in synthesising protein in cell-free systems. The synthetic activity as a function of ribosomal numbers, is greater in the membrane preparations. A higher efficiency, on an RNA basis, of protein synthesis in microsomes over free ribosomes has been noted in other systems (e.g. Siekevitz and Palade, 1960; Moore and Umbreit, 1965).

The degree of interaction between membrane and ribosome in the living cell is not known, but there does appear to be a tendency for some ribosomes, active in synthesis, to adsorb on to the membrane material (c.f. Aronson, 1966).

Unfortunately, the preparations of membrane were not homogenous in appearance (see Figure 10) and contained in addition to rough membranes: smooth vesicles, microtubules, possibly some fragmented cilia and pellicle, and large numbers of glycogen granules. The most apparent effect of the heterogeneity of constituents in such preparations was the inhibition of synthesis encountered when this material, above a certain concentration, was incubated. This was probably due to ribonuclease activity associated with the preparation.

Free ribosome preparations, derived from paramecium homogenates, appear to have little or no membranous elements associated with them; electron micrographs of pelleted preparations appear homogenous. Cell sections also show ribosomes with seemingly no attachment to membranes, so the unattached state is a 'native' form. If extracted carefully with gentle homogenization and maintainance of a low temperature, most free ribosomes are found as aggregates which in some preparations, are distributed in the sucrose gradient as peaks of periodic size (see Figure 8). These are polysomes which range in size from aggregates of two, to aggregates of

thirty or more - calculated on the basis of proportionality between sedimentation coefficient and polysome size (see Figure 35). Incubation has the effect of reducing the size and number of polysomal aggregates with a corresponding increase in monosomal material. This is believed to be the effect of ribonuclease. Polysomal breakdown is concurrent with loss of synthetic ability. The breakdown, or unit, component has a sedimentation value not detectably different from that of reticulocyte monosomes, that is 80S, and appears to be more like the mammalian component than the bacterial component in this respect.

The association of radioactivity with ribosomal monosomes appears to be largely an artefact due to breakdown of polysomes in the process of synthesising polypeptides. Isolated monosomes are far less effective in incorporating labelled amino acids than are isolated polysomes. The peak of incorporating activity is around 120S (2 to 4 ribosomal units) in most preparations whereas the highest specific activity in terms of incorporation per A260 mμ. unit is found in material sedimenting at about 300S (20 to 30 ribosomal units). These results emphasize the importance of fairly large aggregates of ribosomes in protein synthesis in Paramecium.

It appears that also the membrane-bound ribosomes are present in an aggregated form (i.e. two or more ribosomes linked by messenger-RNA). On deoxycholate treatment, the

membrane fraction releases not only an 80S peak of absorbing material but also peaks of heavier material. These heavier peaks, in distribution of A260 and associated labelled protein, strongly suggest ribosomal aggregates. Also, it has been shown that this material is capable of protein synthesis. Thus the evidence favours the view of ribosomes linked by messenger-RNA rather than ribosomes linked by virtue of their associated nascent i-antigen . However, both means of aggregation are possible. For instance, Zipser and Perrin (1963) have shown that sub-unit aggregation of β-galactosidase can occur while one of the sub-units is still attached to the ribosome, possibly in a nascent form. It is possible that aggregation can occur while both polypeptides are in a nascent ribosome-attached form. One group of workers (Manner et al., 1965), who found γ-globulin synthesis occurring on single ribosomes as well as small aggregates, concluded that these aggregates were held together by protein or polypeptide which did not appear to be nascent polypeptide.

Thus both ribosome forms, free polysomes and membrane-bound polysomes, capable of synthesising proteins are to be found in Paramecium aurelia: a situation which might be considered to lie between the entirely free polysomal synthesis that occurs in reticulocyte cells and the predominantly membrane-bound synthesis that occurs in liver and tumour cells.

II The Nature, Location and Function of i-Antigens.

Before considering how the synthesis of immobilization antigen fits into the picture of protein synthesis in Paramecium it might be useful to briefly consider the location, nature and function of i-antigen, particularly with reference to the protein synthetic mechanisms already discussed.

Since practically all of the i-antigen is detected on the pellicle and cilia, it might seem to be a protein 'secreted' out to occupy this surface position. This view would suggest a globular protein and is supported by the readily soluble state of the extracted i-antigen. On the basis of the rather high frictional ratio (1.8) and high intrinsic viscosity (0.11) Preer (1959c) suggested that the i-antigen molecule was fibrous in nature. Also the sedimentation coefficient of 8.2S - 8.3S for a protein of molecular weight of 240-310,000 (Preer, 1959c; Bishop, 1961; Jones, 1965a; Steers, 1965) is a bit low for a globular protein. However, these results do not clearly indicate a fibrous protein and might be explained by the degree of hydration of the purified i-antigen. The only direct evidence that the i-antigens are, in fact, globular in nature is that on electron microscope examination purified material appears as roughly hexagonal in outline, made up of subunits arranged with the symmetry of a regular solid, and approximately 200A^{o} in diameter (Mott, 1964). These

observations fit the physical data well, assuming the molecule to be globular.

Since the purified i-antigen has identical immunological properties to the i-antigen *in situ*, it has been assumed that they are of a similar form. However, on the surface of the cell, the i-antigen may be more spread out in sheets, perhaps folding to assume a more spherical shape on release.

The function of the i-antigens is not known but they do appear to be essential for the existance of the cell. The indispensibility of the i-antigens is demonstrated by the facts that natural selection has preserved a large number of genes all concerned with the production of i-antigens and that no paramecium has been found to lack an i-antigen. The fact that the i-antigens coat the surface of the paramecia suggests that they function in regulating the cell to its environment, perhaps by controlling membrane permeability or membrane transport. The only difficulty here, is to imagine how so many different i-antigens with different structures, apparently function equally well.

The function of the i-antigens remains unknown and their properties and location give little information as to their probable mode of synthesis, although the suggested globular protein, secreted to the cell-surface, might be considered to resemble the situation, already discussed, in liver and tumour cells.

III Paramecium Protein Synthesising Systems.

In first considering the problem of obtaining a system suitable for the study of i-antigen synthesis in Paramecium aurelia, two possibilities were apparent; following the appearance of the new type of i-antigen resulting from a serotype transformation (this could easily be obtained by changing the culture temperature), or studying the incorporation of a radioactive precursor into i-antigen by the process of synthesis. Both types of study present problems but those of the former are far greater, particularly when considering that transformation is a physiological phenomenon in its own right, undoubtably involving parameters extra to those of normal synthesis and requiring time (often variable) for its response to the changed environment.

The time elapsing between environmental change and the detection of change in immobilization properties by treating living paramecia with antiserum is at least 7-8 hr.. Balbinder and Preer (1959) studied serotype transformation, using gel diffusion techniques, and did not detect newly synthesised i-antigen until 15-18 hr. after change in temperature. However, Beale and Mott (1961) and Mott (1965) used the transformation method and detected new-type i-antigen on the pellicle and cilia between 3 and 5 hr., using fluorescein-labelled antiserum in conjunction with electron microscopy. Even a few hours is long for a

biochemical study of protein synthesis and it is not known whether the delay experienced in the detection of new-type i-antigen results form a delay in response or limitations in the detection techniques. The study of i-antigen synthesis, using radioactive precursors as the marker seemed to offer better possibilities.

The ideal situation would be to add a radioactive amino acid to a chemically defined medium containing growing paramecia, but since such a medium was not obtained for variety I Paramecium in preliminary studies, an alternative means of labelling had to be found.

One method involved the culture of paramecia in bacteria-containing medium, and labelling the paramecia after thorough washing, in a simple salts solution containing the labelled amino acid and a bacteriostatic drug (100 µg./ml. chloramphenicol). Such experiments were not always successful, perhaps due to the fact that the paramecia were not actively feeding under these experimental conditions thereby reducing the uptake of endogenous label, but paramecium protein synthesis seemed to be chloramphenicol resistant (see Figure 12).

It was found far more convenient to supply the labelled amino acid to paramecia by way of bacteria. This technique has been reported (Berger and Kimball, 1964) as an efficient and specific method for the incorporation of labelled precursors into the DNA of Paramecium aurelia. The authors point out the

obvious advantages of this method in that Paramecium normally derives its metabolites from the digestion of bacteria.

The bacteria were labelled by methods suggested by Roberts et al.,(1955). The amino acid leucine was selected since, when supplied in trace amounts (1 µg./ml.), it is totally incorporated into bacterial protein as leucine and is not metabolized. It was assumed that the Aerobacter aerogenes used in this study did not differ markedly in this respect from the Escherichia coli used by Roberts et al.. Also leucine is a fairly well represented amino acid in the i-antigen molecule (about 105 residues/molecule).

By using this technique, the efficiency of incorporation into paramecium protein was 20% and into i-antigen 0.1% to 0.2% (see Table 2). This would indicate that approximately 0.5% to 1% of the leucine incorporated into paramecium protein was incorporated into i-antigen. However, there is no reason to assume that i-antigen synthesis is always proportional to total protein system (indeed, variable results were encountered). To best ensure that i-antigen was synthesised in the experimental material, cells were harvested which were actively growing and dividing. This factor was equally important for the experiments based on cell-free protein synthesis.

For information on the components of the cell-free amino acid incorporation mix and their probable effective concentrations, I am indebted to Dr J.O. Bishop and Dr J. Preer. Since the

conclusion of this work, a publication has appeared on cell-free protein synthesis on Paramecium aurelia (Reisner and Macindoe, 1967) which largely agrees with the system used in this study. Other studies on protein synthesis in protozoa have been few, synthesis by way of ribosomes being restricted to reports on the ciliate Tetrahymena pyriformis (Magar and Lipmann, 1958), on the trypanosome Crithidia oncopelti (Chesters, 1966) and on the phytoflagellates Euglena gracilis (Eisenstadt and Brawerman, 1964) and Chlamydomonas reinhardi (Sagar et al., 1963).

The Paramecium system containing membrane fraction as well as free ribosomes regularly gave an incorporation of ~700 counts/min./mg. protein while the system containing free ribosomes alone regularly gave an incorporation of ~500 counts/min./mg. protein. Since much of the protein in these supernatant preparations is extraneous and counts incorporated are dependent on the specific activity of the label and the efficiency in counting it, the more meaningful value of 120 µµg. leucine/mg. RNA was derived for the ribosome preparation. This value compares favourably with values obtained from other cell-free incorporating systems.

That the incorporation was genuine protein synthesis was strongly indicated from the studies involving the omission of various components of the system and the addition of various drugs to the system.

According to current concepts (see Schweet and Heintz, 1966),

the biosynthesis of proteins from free amino acids proceeds through the following steps:

a) activation of the amino acids by specific enzymes (aminoacyl synthetases) in the presence of an energy source (ATP).

b) transfer of the activated amino acids by specific transfer ribonucleic acids (S-RNA's) to ribosomes where messenger-RNA-directed assembly of polypeptides takes place. This step requires GTP and a reducing agent - GSH or mercaptoethanol.

It was assumed that the dialysed Paramecium supernatant contained aminoacyl synthetases, S-RNA's and the enzymes involved in the formation of polypeptides in addition to messenger-RNA containing ribosomes. The findings from this study demonstrate requirements for cell sap, GTP, 2-mercaptoehtanol, an energy generating system (ATP-creatine phosphate - creatine phosphokinase) and amino acids, in incorporating a labelled amino acid into acid insoluble material. There is also an absolute dependence on Mg^{++}, the optimum concentration of 5-6 mM being similar to that found for mammalian cell-free systems rather than bacterial systems.

The time-course of incorporation by the paramecium cell-free system showed that most of the activity was lost after 10 min. and that little increase in incorporated material occurred after 30 min.. An unusual feature of the system was that even after 30 min. there was little release of soluble labelled material i.e. more than 80% of the acid insoluble radioactivity remained

in association with the ribosomes. This result was confirmed by Reisner and Macindoe (1967), and suggests that some component required for polypeptide chain termination was absent from the system. An alternative suggestion (Reisner and Macindoe, 1967) is that there was little completion of polypeptides during the life of thessystem.

Total inhibition by 20 µg./ml. ribonuclease indicates that the paramecium messenger-RNA is highly susceptible to breakage by this enzyme. It also supports the view that none of the incorporation is due to contaminating bacteria, which should be resistent to the action of this enzyme.

Amino acid incorporating activity of paramecium supernatants was not inhibited by actinomycin D. This is as expected, since actinomycin D is known to be a specific inhibitor of DNA dependent RNA polymerase (reviewed, Reich, 1966). Any remaining amino acid incorporation is a function of the stability of the already formed messenger-RNA. Since the paramecium supernatants incorporated for up to 30 min. (half-life 10 min.) a fairly stable messenger-RNA is indicated for this system.

As in the treatment of living cells, the paramecium cell-free system was found to be resistant to chloramphenicol, which in some systems inhibits protein biosynthesis. Since all organisms and systems sensitive to low concentrations of chloramphenicol (bacteria, blue-green algae, chloroplasts and mitochondria) have 70S ribosomes whereas all chloramphenicol

resistant systems (yeast, mammalian cells and pea seedlings) contain 80S ribosomes (reviewed, Vazquez, 1966), this finding agrees with the value of 80S already determined for paramecium ribosomes.

The inhibitory effect of puromycin does not differentiate between the protein synthesising systems of bacteria and other cells but, as with chloramphenicol, acts at the ribosome level and can also be used to test whether a given synthesis is ribosomal or not. This drug is believed to act by substituting for soluble-RNA and causing a break in the growing polypeptide chain (reviewed, Franklin, 1966). Inhibition of the paramecium system by this drug and also release of protein radioactivity from the ribosomes after treatment with this drug, suggest that puromycin is effective in breaking growing polypeptide chain in Paramecium.

In conclusion, these results indicate that the amino acid incorporation witnessed with the Paramecium cell-free system is indeed genuine protein synthesis which proceeds by the conventional pathway of polypeptide biosynthesis. However, the results concerning paramecium proteins as a whole do not necessarily refer to the biosynthesis of one particular protein - the immobilization antigen. But protein synthesis was considered as the first indication of the success of the system and deemed likely the concurrent synthesis of i-antigen.

IV The Separation and Detection of Labelled i-Antigen.

In order to detect and assay labelled i-antigen in protein synthesising material, it is necessary to employ techniques whereby the labelled i-antigen can be separated in fairly pure form from other labelled proteins.

Jones, (1965a) showed that paramecium i-antigen could be purified by column chromatography using the cation-exchanger, SE-Sephadex. This was the final purification step after extracting cell surface protein with salt/alcohol solution and concentrating the protein by ammonium sulphate precipitation as suggested by Preer (1959b). Another possible technique for the physical separation of i-antigen is electrophoresis. However, it has been found that the i-antigen has only a low electrophoretic mobility (Preer, 1959c; Beale and Bishop, 1960).

The basic mechanism used, in this study, for i-antigen separation was that of specific precipitation by antiserum. This technique could not be employed successfully until antiserum was prepared in reasonably high titre against purified i-antigen. Such an antiserum should not contain antibodies against any other paramecium protein; the measure of the unspecific reaction being the effect of antiserum prepared against a heterologous purified i-antigen (or, in some instances, normal serum).

Immobilization antigen, purified by column chromatography

and incorporated into a multiple emulsion system suggested by Herbert (1966) served the purpose of stimulating antibody production, in rabbits, specifically directed against i-antigen.

Direct precipitation by antiserum of antigen in solution followed by measuring the amount of precipitated complex is a commonly used immunological assay method (Glick et al., 1958). This method has been lately adapted in the precipitation of radioactively labelled antigen proteins, particularly γ-globulin (Scharff and Uhr, 1965; Williamson and Askonas, 1967). One drawback in using this method is to devise a suitable control since some material in the preparation may precipitate spontaneously or be brought down with the antigen-antibody complex. Small amounts of radioactive contaminent are critical when measuring a minor labelled component, such as i-antigen. The technique of precipitating all antiserum, specific and non-specific (control) for the experimental material, with anti-rabbit serum, removes most of the variables between experimental and control tubes, and these tubes can be quite easily adjusted to give the same amount of non-specific precipitation of radioactive constituents. This technique also increases the probability of sedimenting 'soluble' antigen-antibody complexes, particularly when antiserum against reduced carboxymethylated i-antigen was used. The degree of non-specific precipitation was variable according to the type of preparation (see Table I) and at least 50% specific precipitation

of total radioactive precipitate was normally aimed at. In general, precipitates formed from soluble protein or salt/alcohol extracts were more specific than more particulate and unstable preparations such as ribosomes.

An elaboration of the precipitin reaction is the technique of immunoelectrophoresis (Graber and Williams, 1955). Here, preliminary physical separation of proteins in an electrical field allows a highly specific reaction with antiserum. The i-antigen appears as a discrete arc when allowed to diffuse through the agar to meet diffusing antibodies, and in all experiments of this type, no more than one precipitin arc was found in any one sample - antiserum diffusion, when the whole gel was tested along its length. This one arc always corresponded, in distance of migration, from the well,with runs of 'carrier' purified i-antigen. Usually, both heterologous and homologous 'carrier' i-antigens were added to each preparation and, after electrophoresis, each preparation was allowed to diffuse against both heterologous and homologous antisera. This gave control precipitin arcs, which were useful when the arcs were assayed for radioactivity. The actual position of the precipitin arc was never removed far from the sample well, always towards the cathode. The factors causing this particular migration are not known. Since, in this technique, the precipitate is suspended in agar, other non-complexed proteins can easily be washed out and good

SEPARATION	ASSAY	ADVANTAGES	DISADVANTAGES	USES
Ammonium sulphate precipitation and column chromatography	Direct counting of radioactive protein precipitates	Quantitative method independent of immunological reaction	Large amounts of labelled material required	Batch preparation Quantitative method for estimating total radioactive i-antigen
Antiserum precipitation in solution	as above	Results obtained quickly. Low levels of i-antigen activity detectable	High levels of cross-reaction	Analysis of large numbers of samples e.g. sucrose gradient fractions.
Immuno-electrophoresis	Autoradiography	Highly specific. Small amounts of material required	Not quantitative. Take 6-7 weeks to prepare and develop.	Analysis of preparations e.g. subcellular fractions for the prescence or absence of labelled i-antigen.

TABLE 8 Summary of methods used in the separation and assay of labelled i-antigen.

Facing p.84

preparations, stained for protein, only showed i-antigen precipitin arcs in the agar.

Radioactivity in separated i-antigen was assayed by liquid scintillation counting methods, if the material had been precipitated from solution, or by autoradiography, if the material had been precipitated in agar. Autoradiography of immunoelectrophoresis patterns has been used in other systems, particularly to detect synthesis of serum proteins in cell-free extracts (von der Decken, 1963; Ganoza et al., 1965) and tissue slices (Williams et al.,1961). Scintillation counting gave quantitative results which were counted with fairly high efficiency while autoradiography gave qualitative results which, nevertheless, often demonstrated a more specific reaction.

In conclusion, Table 8 summarizes the advantages and disadvantages of each separation and assay technique and the types of preparation best assayed by each are shown.

V The Mechanism of i-Antigen Synthesis.

In the early stages of experimentation with radioactively labelled cells and cell-free supernatants, it became apparent that an important factor in i-antigen synthesis was the early appearance of labelled i-antigen in the membrane fraction. Such membrane preparations contained labelled i-antigen at times

(30 min.-45 min.) before labelled i-antigen was detectable on surface structures or in the surface protein extracted from living labelled cells (60 min.). These results suggested that the components responsible for i-antigen synthesis might be contained in the membrane fraction rather than in the structures - the pellicle and cilia - on which the newly synthesised i-antigen is finally located. This view was supported by the ability of isolated membrane fraction to synthesise i-antigen in the cell-free amino acid incorporating system.

It has been seen, in the earlier discussion, (Part I) that ribosomes are essential for protein synthesis. Ribosome-like structures were apparent in electron micrographs of the membrane fraction (see Figure 10), and when this fraction was treated with the detergent, sodium deoxycholate, some of the i-antigen activity sedimented with ribosome-like components (see Figure 22). Also, in sections of Paramecium, electron micrographs have revealed ribosomes in association with membranes in the cell cytoplasm but there is no evidence for ribosomes attached to the pellicle and cilia.

It has been argued (Mott, 1964) that from the point of view of transport, it would be more convenient for the i-antigen to be synthesised at its final site. However, it is difficult to envisage how the components required for protein synthesis - particularly ribosomes, messenger-RNA and enzymes - could be

maintained and function at these sites which are quite separate from the cell cytoplasm.

The evidence from the present study strongly suggests that i-antigen is synthesised on membrane-bound ribosomes which are located in the cell cytoplasm. Whether or not these synthetic sites occupy a special position within the cell (e.g. near the perimeter of the cytoplasm) is not known. Nor is it known how the synthesised i-antigen is transported to its final site. It is possible that this protein is secreted across the endoplasmic reticulum membrane in the manner already discussed for mammalian systems. However, such problems cannot be solved by the type of experiment used in this study, since cellular organization is always disrupted by homogenization.

That the ribosomes involved in i-antigen synthesis are strongly attached to membranous elements in the membrane fraction was indicated by the failure, on prolonged centrifugation, to sediment the i-antigen synthesising components through 2M sucrose (see Table 7). The possibility that the i-antigen synthesising components were very large polysomes (in the order of 100 ribosomes) which happened to sediment on sucrose gradient analysis with the membrane fraction, was dismissed because, if this were the case, they should have sedimented through the dense sucrose. Strong membrane attachment of i-antigen synthesising ribosomes was also supported by the fact that they were only released after detergent

treatment and were then found to be relatively small polysomes (less than clusters of 30 ribosomes).

In most gradient analyses, of supernatants prepared from labelled paramecia and of cell-free labelled supernatants, some labelled i-antigen was detected in association with free ribosomes, Since the i-antigen activity in these fractions was always less than that detected in association with the membrane fraction, the free ribosome activity was considered to represent a breakdown product resulting from homogenization or enzymatic degradation. However, free ribosome fractions isolated from sucrose gradients had activity in incorporating radioactively labelled amino acids into i-antigen (see Figures 32a and 33) which means that they retained some activity after homogenization. Also, there appeared to be an optimal size for i-antigen synthesising polysomes which sedimented at about 220S to 320S (8 to 30 ribosomes). The activity in these fractions was increased after deoxycholate treatment of the supernatant including the membrane fraction, prior to gradient separation (see Figure 32b), indicating that the membrane-bound polysomes and free polysomes, both synthesising i-antigen, were of similar size.

A further degree of refinement in the analysis of i-antigen synthesis was made possible by the use of antiserum directed against reduced carboxymethylated i-antigen (Jones, 1965a). The use of chemically reduced protein (Scharff and Uhr, 1965) or

enzymatically fragmented protein (Williamson and Askonas, 1967) have been used in other studies to stimulate antibodies which would detect nascent polypeptides or protein sub-units.

The effect of anti-RCM-i-antigen serum appears to be specific but it reacts with some material not detected by anti-i-antigen serum. Both types of serum precipitate about equal amounts of radioactivity from incubated membrane fraction, where most of the synthesised material precipitable by both, occurs (see Figures 32a and 33). However, with the incubated free ribosomes, the two types of sera precipitate activity from different fractions. It appears that the free polysomes synthesising protein precipitable by anti-RCM-i-antigen are of smaller size - 140S-240S (or 4-12 ribosomes) - than the free polysomes capable of synthesising protein precipitated by anti-i-antigen - 220S-320S (or 8-30 ribosomes). Since the smaller size polysome is involved in synthesising a protein recognisable by the antiserum against the 'sub-unit' form, while the larger size polysome can synthesise protein recognisable by the antiserum against the 'native' protein, it is possible that the former represents an earlier step in the synthesis of the latter. This view of the relationship between polysome size and the stage of synthesis (or state of aggregation of 'sub-units') of i-antigen, rather than that of the free ribosomes representing breakdown products with different limitations of synthesis, is supported

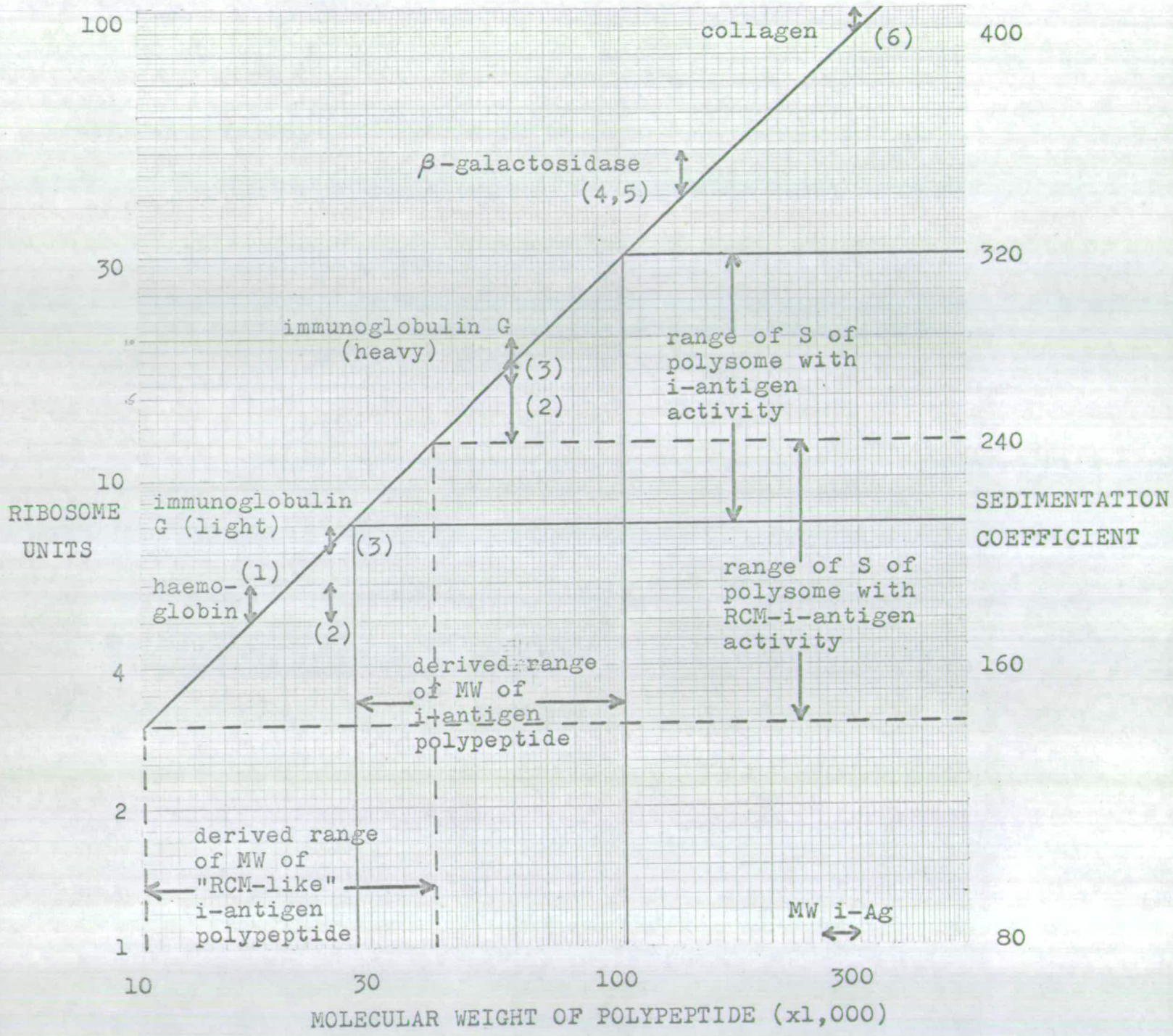


FIGURE 35. The relation between the size of polysome and the size of polypeptide being synthesised. The data were found to approximate to a straight line. Known sedimentation coefficients of Paramecium polysomes with associated i-antigen activity were extrapolated from the straight line to give an approximate range of molecular weight of polypeptide being synthesised.

References:

(1) Gierer (1963) (2) Williamson and Askonas (1967)
(3) Becker and Rich (1967) (4) Zipser (1963)
(5) Kiho and Rich (1964) (6) Manner et al. (1967)

by the relatively narrow size-range of each type of polysome: a result not expected from a random breakdown process.

It is also interesting to note that the puromycin released labelled protein precipitated by antiserum against RCM-i-antigen sedimented at a slower rate than did the released labelled protein precipitated by antiserum against native i-antigen (see Figure 34). This result indicates that the former protein is a small fragment, perhaps a 'sub-unit' polypeptide.

At this stage it is perhaps worthwhile to consider the theoretical relationship between polysome size and the expected size of polypeptide synthesised. Figure 35 summarizes the results obtained in other studies and relates polysome size, and the corresponding sedimentation coefficient, to the molecular weight of the polypeptide considered to be synthesised. A straight line can be drawn to fit the data and the values of polysome size, derived from sedimentation coefficients obtained in this study, can be calibrated to give an approximate value for the molecular weight of polypeptide they would be expected to be capable of synthesising. The upper values for sedimentation coefficient might be a truer estimate, since there was always a variable degree of polysome breakdown in the paramecium preparations. On the basis of this relationship, none of the polysomes with associated i-antigen activity would be large enough to synthesise a complete i-antigen molecule. However, the range of obtained

polysomal size includes polysomes large enough to be capable of synthesising a polypeptide of molecular weight 100,000 i.e. one third of the total molecule. Since, this has already been favoured as the size of the i-antigen identical sub-unit (Steers, 1965) these results are consistent with the hypothesis that the identical sub-units are synthesised on separate polysomes. The probable size of polypeptide synthesised by polysomes reacting with anti-serum against RCM-i-antigen was of molecular weight 10-40,000. This may represent the smaller non-identical sub-units, of which there may be three per identical sub-unit (Steers, 1965).

Some indication as to the relation between i-antigen synthesising polysomes and the membrane fraction is given by the experiments where labelled ribosome-containing supernatants were mixed with unlabelled isolated membrane fraction and subjected to sucrose gradient centrifugation (see Figure 24). Here, a substantial amount of the labelled i-antigen activity was found in the membrane fraction. However, when the ribosomes were removed from such a labelled preparation, soluble labelled i-antigen failed to sediment when mixed with unlabelled membrane material (see Figure 19), indicating that the adsorption effect may be due to the presence of the nascent i-antigen polypeptides rather than to the completed soluble i-antigen. Such an effect might represent a possible mechanism in the cell whereby nascent i-antigen poly-peptides are brought together and aggregate to form the completed

molecules on the membranes through which they can be secreted and finally transported to occupy their sites on the cell surface. This situation would be similar to other cases of membrane synthesis (Sabatini et al., 1966; Aronson, 1966) already discussed.

In conclusion, the following mechanism for the biosynthesis of i-antigen is proposed. Immobilization antigen is synthesised on membrane-bound polysomes. This synthesis may be initiated by the synthesis of 'sub-unit' polypeptides on free ribosomes which adsorb, by virtue of their growing nascent polypeptides, on to the membranes. The synthesis and aggregation of sub-units is completed on the membranes and the complete i-antigen is then transported to the surface of the cell.

VI General Conclusions, Criticism and Prospects.

The protein synthetic system in Paramecium aurelia has proved useful, not only in the elucidation of the mechanism of immobilization antigen synthesis, but also as a model system for the synthesis of cell-surface proteins. Of particular significance has been the correspondance between results obtained from experiments with living material and results obtained from in vitro studies: it is felt that a good approximation to the natural process of i-antigen synthesis has been achieved.

The main restriction of this particular study has been the

disruption of cellular organization by homogenization prior to analysis of the system. As a result, it has been difficult to draw conclusions as to the location of various processes within the cell. However, experiments along the lines of those described in this study, but using electron microscopy to follow the appearance of newly synthesised i-antigen, should clarify this issue.

Another unknown factor was the stability of the messenger-RNA for i-antigen. While the messenger-RNA of bacterial systems has been shown to be very unstable (Gros et al., 1961; Tissieres and Hopkins, 1961; Levinthal et al., 1962), stable messenger-RNA has been reported in mammalian systems, e.g. reticulocytes (Marks et al., 1962), rat-liver cytoplasm (Revel and Hiatt, 1964) and sheep thyroid (Singh et al., 1965). The kinetics of cell-free protein synthesis in Paramecium were more akin to mammalian systems than bacterial systems, indicating that the messenger-RNA was not short-lived, but the effect of nucleases and the possible difference of the i-antigen 'messenger' from other paramecium 'messengers' makes an estimate of half-life difficult.

As already mentioned, the association of polysomes with membranes may be an important factor in influencing messenger-RNA stability (Schlessinger, 1963; de Petris et al., 1963; Aronson, 1965). It is also interesting to note that Bach and Johnson, (1966) have presented evidence for an association of polysomes

with nuclear membranes in HeLa cells. These authors suggest that the movement of the polysomes from the nucleus to the cytoplasm may take place on the membranes of the nucleus and endoplasmic reticulum, perhaps by movement of the membranes themselves. Such a system could be relevent to the transport of i-antigen polysomes, although evidence has been presented in this study for free i-antigen synthesising polysomes. Nevertheless, the importance of membranes in the organization and transport of protein synthetic machinary should be stressed.

Messenger-RNA stability is especially important from the point of view of serotype transformation. Preliminary studies involving the treatment of living paramecia during transformation with actinomycin D, have indicated that the half-life of the 'messenger' for the old-type i-antigen is 30 to 45 min.. The results also indicate that the transformation process occurs at the gene level, since the blockage of DNA-RNA-transcription prevents the formation of new-type antigen.

Since the transformation process is an interesting and, as yet, little understood phenomenon, it is hoped to explore the possibilities of this aspect of i-antigen formation using some of the techniques and results of this study.

ACKNOWLEDGEMENTS

I wish first to acknowledge gratefully the continued advice and encouragement offered by Professor G.H. Beale, F.R.S..

I would also like to thank Dr J.O. Bishop and Dr I.G. Jones for helpful discussion and Mr R.E. Sinden for taking the electronmicrographs.

The work was made possible by a grant from the Science Research Council.

REFERENCES

ABRAMS, A., L. NIELSON & J. THAEMERT, (1964). Biochim. Biophys. Acta, 80, 325.

ARONSON, A. (1965). J. Mol. Biol. 13, 92.

ARONSON, A. (1966). J. Mol. Biol. 15, 505.

BACH, M.K. & H.G. JOHNSON, (1966). Nature 209, 893.

BALBINDER, E. & J.R. PREER, Jr. (1959). J. Gen. Microbiol. 21, 156.

BEALE, G.H. (1957). Intern. Rev. Cytol. 6, 1.

BEALE, G.H. & H. KACSER, (1957). J. Gen. Microbiol. 17, 68.

BEALE, G.H. & M.R. MOTT, (1962). J. Gen. Microbiol. 28, 617.

BECKER, M.J. & A. RICH, (1967). Nature, 212, 142.

BERGER, J.D. & R.F. KIMBALL, (1964). J. Protozool. 11, 534.

BISHOP, J.O. (1961). Biochim. Biophys. Acta, 50, 471.

BISHOP, J.O. (1963). J. Gen. Microbiol. 30, 271.

BISHOP, J.O. (1965). Nature, 208, 361.

BISHOP, J.O. & G.H. BEALE, (1960). Nature, 186, 734.

BLOOR, W.R. (1947). J. Biol. Chem. 170, 67.

BRENNER, S., F. JACOB, & M. MESELSON, (1961). Nature, 190, 576.

BRITTEN, R.J. & R.B. ROBERTS, (1960). Science, 131, 32.

BROCK, T.D. (1961). Bact. Rev. 25, 32.

CAMPBELL, P.N. (1965). Progress in Biophysics and Molecular Biology, 15, 3.

CHESTERS, J.K. (1966). Biochim. Biophys. Acta, 114, 385.

CRICK, F.H.C., L. BARNETT, S. BRENNER & R.J. WATTS-TOBIN, (1961). Nature, 192, 1227.

DAVIS, B.D. (1950). J. Bacteriol. 60, 17.

DECKEN, A. von der, (1963). Biochem. J. 88, 385.

EISENSTADT, J.M. & G. BRAWERMAN, (1964). J. Mol. Biol. 10, 392.

FINGER, I. (1956). Biol. Bull. 111, 358.

FINGER, I.& C. HELLER, (1963). J. Mol. Biol. 6, 190.

FINGER, I.& C. HELLER, (1964). Genetics, 49, 485.

FINGER, I., F. ONORATO, C. HELLER & H.B. WILCOX, (1965). 2nd. Intern. Conf. Protozool., Excerpta Medica, 91, 244.

FOLCH, J., M. LEES & G.H. SLOANE STANLEY, (1957). J. Biol. Chem. 226, 497.

FRANKLIN, T.J. (1966). Symp. Soc. Gen. Microbiol. 16, 204.

GANOZA, M.C., C.A. WILLIAMS & F. LIPMANN, (1965). Proc. Natl. Acad. Sci. U.S. 53, 619.

GIERER, A. (1963). J. Mol. Biol. 6, 148.

GLICK, D., R.A. GOOD, L.J. GREENBERG, J.J. EDDY & N.K. DAY, (1958). Science, 128, 1625.

GRABAR, P. & C.A. WILLIAMS, (1955). Biochim. Biophys. Acta, 17, 67.

GROS, F., W. GILBERT, H.H. HIATT, G. ATTARDI, P.F. SPAHR & J.D. WATSON, (1961). Cold Spring Harbor Symp. Quant. Biol. 26, 111.

HALBERG, P.A. & J.B. HAUGE, (1965). Biochim. Biophys. Acta, 95, 80.

HAUGE, J.B. & H.O. HALVORSON, (1962). Biochim. Biophys. Acta, 61, 101.

HENDLER, R.W. & J. TANI, (1964). Biochim. Biophys. Acta, 80, 294.

HENDLER, R.W., W.G. BANFIELD, J. TANI & E.L. KUFF, (1964). Biochim. Biophys. Acta, 80, 307.

HENSHAW, E.C., T.B. BOJARSKI & H.H. HIATT, (1963). J. Mol. Biol. 7, 122.

HERBERT, W.J. (1965). The Lancet, October 16, 771.

JONES, I.G. (1965a). Biochem. J. 96, 17.

JONES, I.G. (1965b). Nature, 207, 769.

JONES, I.G. & G.H. BEALE, (1963). Nature, 197, 205.

KIHO, Y. & A. RICH, (1964). Proc. Natl. Acad. Sci. U.S. 51, 111.

KIHO, Y. & A. RICH, (1965). Proc. Natl. Acad. Sci. U.S. 54, 1751.

KRETSINGER, R.H., G. MANNER, B.S. GOULD & A. RICH, (1964). Nature, 202, 438.

KUFF, E.L. & R.F. ZEIGEL, (1960). J. Biophysic. & Biochem. Cytol, 7, 465.

KUFF, E.L., W.C. HYMER, E. SHELTON & N.E. ROBERTS, (1966). J. Cell. Biol. 29, 63.

LEVINTHAL, C., A. KEYNAN & A. HIGA, (1962). Proc. Natl. Acad. Sci. U.S. 48, 1631.

LOWRY, O.H., N.J. ROSEBROUGH, A.L. FARR & R.J. RANDALL, (1951). J. Biol. Chem. 193, 265.

MACH, B. (1963). Cold Spring Harbor Symp. Quant. Biol. 28, 263.

MAGAR, J. & F. LIPMANN, (1958). Proc. Natl. Acad. Sci. U.S. 44, 305.

MALT, R. & P. SPEAKMAN,)1964). Life Sci. 3, 81.

MANNER, G., B.S. GOULD & H.S. SLAYTER, (1965). Biochim. Biophys. Acta, 108, 659.

MANNER, G., R.H. KRETSINGER, B.S. GOULD & A. RICH, (1967). Biochim. Biophys. Acta, 134, 411.

MARKS, P.A., E.R. BURKA & D. SCHLESSINGER, (1962). Proc. Natl. Acad. Sci. U.S. 48, 2163.

MARR, A.G. (1960). Ann. Rev. Microbiol. 14, 241.

MARTIN, R.G. & B.N. AMES, (1961). J. Biol. Chem. 236, 1372.

MOORE, L.D. & W.W. UMBREIT, (1965). Biochim. Biophys. Acta, 103, 466.

MOTT, M.R. (1963). J. Roy. Microscop. Soc. 81, 159.

MOTT, M.R. (1964). Ph.D. Thesis. Edinburgh University.

MOTT, M.R. (1965). J. Gen. Microbiol. 41, 251.

NANNEY, D.L. & J.M. DUBERT, (1960). Genetics, 45, 1335.

NANNEY, D.L., M.J. NAGEL & R.W. TOUCHBERRY, (1964). J. Exptl. Zool. 155, 25.

NANNEY, D.L., S.J. REEVE, M.J. NAGEL & S.de PINTO, (1963). Genetics, 48, 803.

NEU, H.C. & L.A. HEPPEL, (1964). Biochem. Biophys. Res. Comm. 17, 215.

NIRENBERG, M.W. & H. MATTHAEI, (1961). Proc. Natl. Acad. Sci. U.S. 47, 1588.

NORTON, W.L., D. LEWIS & M. ZIFF, (1965). Proc. Natl. Acad. Sci. U.S. 54, 851.

PALADE, G.E. (1958). In Microsomal Particles and Protein Synthesis, ed. R.B. Roberts, p.36. Pergamon Press.

PALADE, G.E. & P. SIEKEVITZ, (1956). J. Biophys. Biochem. Cytol. 2, 171.

PETERMAN, M.L.)1964). The Physical and Chemical Properties of Ribosomes. Elsevier.

PETRIS, S.de. (1967). J. Mol. Biol. 23, 215.

PETRIS, S.de. & G. KARLSBAD, (1965). J. Cell. Biol. 26, 759.

PETRIS, S.de, G. KARLSBAD & B. PERNIS, (1963). J. Exp. Med.117, 849.

PORTER, K.R. (1961). In The Cell, ed. J. Brâhet & A. Mirsky, 2, 621. Academic Press.

PREER, J.R., Jr. (1956). J. Immunol. 77, 52.

PREER, J.R., Jr. (1959a). J. Immunol. 83, 276.

PREER, J.R., Jr. (1959b). J. Immunol. 83, 378.

PREER, J.R., Jr. (1959c). J. Immunol. 83, 385.

PREER, J.R., Jr. (1959d). Genetics, 44, 803.

PREER, J.R., Jr. & L.B. PREER, (1959). J. Protozool, 6, 88.

REDMAN, C. & D.D. SABATINI, (1966). Fed. Proc. 25, 216.

REDMAN, C., P. SIEKEVITZ & G.E. PALADE, (1966). J. Biol. Chem. 241, 1150.

REICH, E. (1966). Symp. Soc. Gen. Microbiol. 16, 266.

REISNER, A.H. & H. MACINDOE, (1967). J. Gen.Microbiol. 47, 1.

REVEL, M. & H.H. HIATT, (1964). Proc. Natl. Acad. Sci. U.S. 51, 810.

ROBERTS, R., D.B. COWIE, P.H. ABELSON, E.T. BOLTON & R.J. BRITTEN, (1955). Carnegie Inst. Wash. Publ. No. 607, 3.

SABATINI, D.D., Y. TASHIRO & G.E. PALADE, (1966). J. Mol. Biol. 19, 503.

SAGER, R., I.B. WEINSTEIN & Y. ASHKENAZI, (1963). Science, 140, 304.

SALTON, M.R.J. (1964). The Bacterial Cell Wall p.188. Elsevier.

SCHAPIRO, A.L., M.D. SCHARFF, J.V. MAIZEL & J.W. UHR, (1966). Proc. Natl. Acad. Sci. U.S. 56, 216.

SCHARFF, M.D. & J.W. UHR, (1965). Science, 148, 646.

SCHARFF, M.D., H.J. SHATKIN & L. LEVINTOW, (1963). Proc. Natl. Acad. Sci. U.S. 50, 686.

SCHLESSINGER, D. (1963). J. Mol. Biol. 7, 569.

SCHLESSINGER, D., V.T. MARCHESI & B.C.K. KWAN, (1965). J. Bact. 90, 456.

SCHWEET, R. & R. HEINTZ, (1966). Ann. Rev. Biochem. 35, 723.

SIEKEVITZ, P. & G.E. PALADE, (1960). J. Biophys. Biochem. Cytol. 7, 619.

SINGH, V.N., E. RAGHUPATHY & I.L. CHAIKOFF, (1965). Biochim. Biophys. Acta, 103, 623.

STEERS, E., Jr. (1962). Proc. Natl. Acad. Sci. U.S. 48, 867.

STEERS, E., Jr. (1965). Biochemistry, 4, 1896.

TISSIERES, A. & J.W. HOPKINS, (1961). Proc. Natl. Acad. Sci. U.S. 47, 2015.

VAZQUEZ, D. (1966). Symp. Soc. Gen. Microbiol. 16, 169.

WAAGTENDONK, W.J. van, (1955). In Biochemistry and Physiology of Protozoa, ed. S.H. Hutner & A. Lwoff, p.66. Academic Press.

WARNER, J.R., P.M. KNOPF & A. RICH, (1963). Proc. Natl. Acad. Sci. U.S. 49, 122.

WARNER, J.R., A. RICH & C. HALL, (1962). Science, 138, 1399.

WILLIAMS, C.A., R. ASOFSKY & G.J. THORBECKE, (1961). J. Exptl. Med. 114, 459.

WILLIAMSON, A.R. & B.A. ASKONAS, (1967). J. Mol. Biol. 23, 201.

WISSEMAN, C.L., Jr., J.E. SMADEL, F.E. HAHN, H.E. HOPPS, (1954). J. Bacteriol. 67, 662.

ZIPSER, D.J. (1963). J. Mol. Biol. 7, 739.

ZIPSER, D.J. & D. PERRIN, (1963). Cold Spring Harbor Symp. Quant. Biol. 28, 533.