Studies on the Characterization and Structure of the Immobilization Antigens of *Paramecium aurelia*

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1. Methods for the extraction and purification of the immobilization antigens of *Paramecium aurelia* have been developed. 2. The immobilization antigen was shown to be a protein of molecular weight 250 000. The amino acid content of two allelic antigens and one controlled by a separate genetic locus was determined and many differences were observed. 3. The properties of antigen denatured by reduction and alkylation were examined. The molecular weight fell to 80 000 or perhaps less. 4. Peptide maps of allelic types were more similar than those of types controlled by different genetic loci. 5. It is suggested that the antigen consists of two identical half-molecules held together by disulphide bonds. These half-molecules may contain further sub-units.

Extracts of *Paramecium aurelia* contain a large number of substances capable of eliciting antibodies in rabbits, but it has been shown that the specific immobilization effect produced on living animals by antisera is due to only one of these (Preer, 1959a). The genetic control of this antigen, the immobilization antigen, has been studied by T. M. Sonneborn, G. H. Beale, J. R. Preer and others and it is now known to be specified by a series of multiple alleles at several genetic loci (for review see Beale, 1957). In any single animal only one locus may be expressed at any time except for a brief period during transformation from one 'type' to another. The particular locus which is expressed is determined by the reaction of the environment on the cytoplasm, and by suitable manipulation of the conditions, particularly temperature, it is possible to obtain large cultures in which practically all the organisms are of the same serotype and which will maintain this state almost indefinitely.

In variety 1 of *P. aurelia* three types, S, G and D, have been studied carefully (Beale, 1954) and several others are known. The loci determining these types do not appear to be linked. Many different alleles at each locus have been found in stocks isolated from the wild and immunological comparisons amongst these have been made (Bishop, 1963). Some allelic types show almost no serological relationship, but there are others which cross-react so strongly as to be practically indistinguishable although their very diverse origins suggest that they are unlikely to be identical.

Preer (1959b) and Bishop & Beale (1960) isolated the immobilization antigen in a fairly pure state

and showed it to be a protein of molecular weight about 2.5×10^5 . Steers (1961) has studied the electrophoretic mobility of types A, B and D in stock 51 of variety 4 and shown them to have isoelectric points of 4.0, 3.9 and 4.3 respectively. Bishop & Beale (1960) showed mobility differences on starch gel between types G, D and T in stock 60 of variety 1 and also found some differences in chromatographic behaviour. Steers (1962) found that different types gave very distinct peptide maps, but failed to show any difference between two allelic subtypes at one of the loci. The only report to date of a chemical difference between allelic types is by Bishop & Beale (1960), who showed a separation of antigens 60D and 90D in hydroxyapatite columns.

The present work describes the purification and characterization of antigens of variety 1 and includes some observations on the molecular structure.

EXPERIMENTAL

Materials. The sources of stocks of *P. aurelia* variety 1 were as follows: stocks 33, 60, 90, 103, 145, 178 and 181 were originally provided by Professor T. M. Sonneborn and stocks 521 and 535 were collected by Professor G. H. Beale. Trypsin was either Light's $2 \times$ recrystallized, salt-free, or Worthington's trichloroacetic acid-treated. No significant difference was found between these. Other reagents were of analytical reagent grade and no further purification was found necessary.

Culture methods. Dried grass was extracted with water (108 g./l.) by boiling for 15 min. The extract was filtered through muslin and sterilized by autoclaving at 20 lb./in.² for 15 min. Working strength medium was prepared by diluting this extract 1:20 and adjusted to pH 6.8 with Na₂HPO₄. This medium was dispensed in 11. portions in

Thompson bottles and autoclaved at 20 lb./in.² for 15 min. Growth was started by adding 50 ml. of a culture of *P. aurelia* and a small inoculum of *Aerobacter aerogenes*. The bottles were stacked flat to give a culture depth of 3–4 cm. For G type organisms the temperature was maintained at 23–25° and for D types at 28–30°. After 3 days the cultures were harvested.

Collections. After filtering through muslin the fluid was passed through an Alfa-Laval cream separator at a flow rate of 700 ml./min. The concentrate was removed from the rotor at 30 min. intervals to avoid death of the animals and further concentrated by centrifuging in pear-shaped bottles for 2 min. at 1000g in an oil-testing centrifuge. From 2.5–3 l. of culture 1 ml. of packed animals, corresponding to about 10⁷ cells, was usually obtained.

Extraction. The antigen was extracted by treatment of the concentrated living animals with 4 vol. of 15% ethanol in 0.045% NaCl at 2–3° according to the method of Preer (1959b). After centrifugation at 20000g for 5 min. the clear extract was stored frozen at –15° until sufficient had been accumulated.

Purification. Solid (NH₄)₂SO₄ was added to the extract to give 75% saturation and, after stirring at 2–3° for 3 hr., the precipitate was collected by centrifugation at 10000g for 15 min. It was redispersed in a minimum volume of water, dialysed against several changes of distilled water and finally freeze-dried. Any precipitate which appeared during dialysis was removed by centrifugation.

The final step in purification was chromatography on SE-Sephadex; for treatment of 0.5 g. of protein a column 1.5 cm. × 20 cm. was used. The SE-Sephadex C-50 (Pharmacia, Uppsala), after preliminary swelling and washing, was equilibrated with 0.05 M-sodium acetate, pH 4.2. After packing the column was washed with this buffer overnight at 2–3°. The protein was applied as a 2–3% solution in the same buffer and elution performed with a gradient of pH in 0.05 M-sodium acetate between pH 4.2 and 5.2 in a total volume of 800 ml. The extinction at 280 mμ of the fractions was measured and antigenic activity assayed by inhibition of the immobilization reaction. The pooled fractions containing antigen were dialysed against distilled water and freeze-dried.

Assay of immobilization antigen (Bishop, 1963). A portion (0.05–0.10 ml.) of the solution to be assayed was mixed with equal volumes of diluted antiserum and fluid containing about 100 homologous paramecia in a cavity slide. The time taken to immobilize the organisms completely was measured (*t*). The immobilization time in a similar mixture in which the antigen was replaced by dilute saline was determined (*t*₀). Then the activity of the antigen solution, in arbitrary units/ml. (*g*), is given by

$$g = 3(1/t_0 - 1/t)$$

This method is valid only if the immobilization time lies between 3 and 10 min. and if *t* is less than 2*t*₀. The necessary dilution of antiserum usually was between 1:10 and 1:100 and solutions to be assayed were diluted serially to obtain the correct concentration.

Reduction and alkylation. Antigen was reduced with 2-mercaptoethanol in 8 M-urea and alkylated with iodoacetic acid, the precautions recommended by Crestfield, Moore & Stein (1963) being observed. The reduced carboxymethylated antigen was recovered by dialysis against four changes of 5 mM-ammonium carbonate and freeze-dried.

Succinylation. Reduced carboxymethylated antigen was treated with solid succinic anhydride at pH 7.5 in an autotitrator as described by Klotz & Keresztes-Nagy (1963).

Amino acid analyses. About 2 mg. of protein was weighed into a thick-walled glass bulb (20 mm. diam.) with a long stem (10 mm. × 60 mm.). Constant-boiling HCl (1 ml.) was added and the air removed from the solution by alternately freezing and thawing carefully (Crestfield *et al.* 1963). The vial was then sealed under vacuum and treated at 110–114° for 36 hr. The vial was opened and the HCl removed rapidly by rotating the bulb in vacuum with gentle heating from an infrared lamp. The sample was then dissolved in 1–2 ml. of 0.1 N-HCl and 0.4 μmole of norleucine was added as an internal standard. Approximately half of the sample was applied to the column of the Technicon Amino Acid Analyser and the chromatogram developed under the standard conditions with 10% (v/v) methanol in the first buffer to improve the separation of serine and threonine. The values obtained by Moore & Stein (1954) were used to correct for varying ninhydrin colour yield and the results expressed in molar terms by reference to the internal standard (Walsh & Brown, 1962).

Estimation of tryptophan. Tryptophan was estimated by reaction with *p*-dimethylaminobenzaldehyde according to Spies & Chambers (1949). Antigen (2–4 mg. in 0.5 ml. of water) was mixed with 4 ml. of 23.7 N-H₂SO₄ and 0.5 ml. of 2 N-H₂SO₄ containing 15 mg. of *p*-dimethylaminobenzaldehyde. After 15 hr. at 28° 0.05 ml. of 0.045% NaNO₂ was added and 30 min. later the blue colour was read at 590 mμ. A standard curve was prepared with pure tryptophan.

Estimation of free thiol groups. (1) Titration of free thiol groups with *p*-chloromercuribenzoate was done according to the method of Boyer (1954). The method was checked against 2-mercaptoethanol. (2) Antigen 90D (3 mg.) was dissolved in 2 ml. of 8 M-urea containing 2 mg. of iodoacetic acid at pH 9.0. After 1 hr. at room temperature the solution was dialysed against running water for 24 hr. A portion (1 ml.) of the solution was hydrolysed and an amino acid analysis made.

Sedimentation and diffusion. Sedimentation coefficients were determined in the Spinco model E ultracentrifuge. An apparent diffusion coefficient was determined from the spreading of the boundary in the boundary-forming cell of the ultracentrifuge. Unless otherwise stated the solvent was 0.2 M-NaCl in 5 mM-sodium phosphate, pH 6.8.

Equilibrium ultracentrifugation. Molecular weights were measured by equilibrium ultracentrifugation in short columns (Yphantis, 1960). The standard 12 mm. double-sector cells were used and a layer 0.7 mm. deep was applied over a 2 mm. layer of carbon tetrachloride. The other channel held buffer to a slightly greater depth. For native antigen centrifugation at 7000 rev./min. for 3 hr. set up an equilibrium, and for reduced forms 1 hr. at 12000 rev./min. was usually sufficient. The concentration of the protein solution was determined in a comparison experiment in the boundary-forming cell.

Peptide maps. Two-dimensional separations of tryptic digests of reduced carboxymethylated antigens were made by a method similar to that of Katz, Dreyer & Anfinsen (1959). Approximately 3 mg. of reduced carboxymethylated antigen was dissolved in 0.05 ml. of 2% ammonium carbonate at pH 8.0–8.5, and 0.05 ml. of 0.07% trypsin in 5 mM-HCl was added. A trace of phenol red was added to act as a marker during chromatography. The digest was

incubated at 37° for 1.5–2 hr. It was then applied directly to one corner of a sheet of Whatman 3MM paper (46 cm. × 57 cm.) as a spot 1–1.5 cm. diam. A stream of cool air was directed on to the spot for 30 min. to remove ammonium carbonate. The paper was equilibrated in the chromatography tank for 12 hr. before starting irrigation with the solvent, butan-1-ol-acetic acid-water (17:4:9, by vol.). After development for 20 hr. the paper was dried thoroughly and electrophoresis performed in the second dimension for 75 min. at 2500 v. The buffer was pyridine-acetic acid-water (3:7:290, by vol.), pH 4.2. The paper was cooled by immersion in White Spirit 100 (Esso Petroleum Co.). After drying, the papers were sprayed or dipped in 0.25% ninhydrin in acetone-water (9:1, v/v) and heated at 75° for 90 min. Papers were then stained for tryptophan by dipping in 1% *p*-dimethylaminobenzaldehyde in acetone-conc. HCl (9:1, v/v) (Baldridge & Lewis, 1953).

RESULTS

Culture methods. The method used here is based on that developed by Connolly, Reisner & Sobey (1961), in which *P. aurelia* and *A. aerogenes* are simultaneously inoculated into a Vegemite medium (a commercial vegetable and yeast extract) and grown in vessels with a depth of 3–4 cm. High densities of 3000–4000 paramecia/ml. were obtained. However, Vegemite cultures smell very strongly and we have found it more satisfactory to use a strong grass medium in this technique. The same depth of 3–4 cm. was found to be optimum. Population density was proportional to the concentration of grass extract over a wide range and a maximum of 8000–10000/ml. could be attained. However, these cultures were not reproducible and were critically dependent on the condition of the animals. At the concentration used here densities of 3000–4000/ml. were regularly produced. To maintain this density it was necessary occasionally to restart the culture in lettuce medium (Sonneborn, 1950) and allow the paramecia to pass through autogamy. It is probable that under the conditions of mass culture autogamy is rather rare and it is known that the regular occurrence of autogamy is essential to the maintenance of a healthy stock (Jennings & Sonneborn, 1935).

Extraction and purification. Table 1 describes the

course of purification of an antigen 60D sample. The immobilization antigen amounts to some 1% of the dry weight of the cell and according to Preer (1959b) is extracted almost completely by treatment of the living cells with 15% ethanol in sodium chloride solution. The cells are not lysed by this process and contamination with cytoplasmic protein is kept to a minimum. Immobilization antigen accounts for 12–15% of the extracted protein. Following Preer (1959b), ammonium sulphate fractionation of this extract was attempted but it was not possible to obtain a high degree of purification. Antigens of the D series precipitate near 50% ammonium sulphate saturation and were always contaminated with a red protein. With other serotypes better purification was possible but it was considered preferable to use ammonium sulphate precipitation merely to concentrate the antigen and to achieve a slight initial purification.

Fig. 1 shows the fractionation of the ammonium sulphate precipitate on SE-Sephadex. The elution was stopped after the appearance of immobilization antigen and some 50% of the applied protein was retained on the column. The overall yield, based on titration of the initial extract, was 60%. Other batches have given the same or greater yields. Other cation-exchangers, e.g. CM-cellulose and cellulose phosphate, were also used and gave very similar separations, but SE-Sephadex was more re-

Fig. 1. Elution diagram for antigen 60D on a column of SE-Sephadex. Gradient: 800 ml. in 0.05M-sodium acetate between pH 4.2 and 5.2. The yield was 90 mg. after dialysis and freeze-drying of the fraction indicated.

Table 1. *Extraction and purification of antigen 60D*

Vol. of culture	500 l.
Vol. of packed paramecia	150 ml.
Vol. of ethanol-NaCl extract	630 ml.
Antigenic activity of ethanol-NaCl extract	1950 units
Wt. of freeze-dried (NH ₄) ₂ SO ₄ fraction	935 mg.
Total wt. of resolvable protein	320 mg.
Wt. of antigen from SE-Sephadex column	87 mg.
Activity of final fraction	1190 units
Overall yield	60%

Fig. 2. Ultracentrifugal analysis of antigen 60D (0.3% in 0.2M-NaCl-0.01M-sodium phosphate, pH 6.8) at 59780 rev./min.: (a) 12 min. after reaching speed (phase-plate angle 75°); (b) 36 min. after reaching speed (phase-plate angle 65°).

producible and yielded more compact elution peaks. A gradient of pH at constant molarity was necessary with this material to avoid changes in the bed volume which occurred when a gradient of salt concentration was used. The anion-exchanger DEAE-cellulose bound antigen only weakly at pH 7.0 and poor purification resulted.

Fig. 2 is the result of an ultracentrifugal analysis and indicates the absence of contaminants of differing sedimentation properties. Paper electrophoresis at pH 9.0 showed a single component. No contaminating antigens could be detected by immunodiffusion against antisera either homologous or heterologous. Starch-gel electrophoresis at pH 9.5 of the ethanol-sodium chloride soln. extract showed six or seven major contaminants. Examination of the purified material under the same conditions failed to reveal any of these components. (Immobilization antigen itself was difficult to resolve in starch-gel electrophoresis because of its very low mobility; Bishop & Beale, 1960.) Very occasionally a sample contained a contaminant moving more rapidly towards the anode in paper electrophoresis at pH 9.0 and sedimenting more rapidly in the ultracentrifuge. This never amounted to more than 10% and was removed by a second passage through SE-Sephadex.

No significant difference in the behaviour of different antigenic types on these columns has been observed.

Molecular weight. $S_{20,w}$ for antigen 145D was 8.3s and the diffusion coefficient of a 0.5% solution was 2.8×10^{-7} c.g.s. units. A partial specific volume of 0.71 was assumed (Preer, 1959c). The molecular weight M_{SD} was 250 000.

The same sample by equilibrium ultracentrifugation gave M_{eq} 240 000.

Table 2. Amino acid compositions of autogens 90D, 178D and 90G

Results are expressed as the number of residues/molecule of molecular weight 250 000.

Amino acid	Antigen type ... 90D	178D	90G
Aspartic acid	289	290	271
Threonine	297	313	369
Serine	185	176	169
Glutamic acid	154	161	138
Proline	60	50	46
Glycine	246	224	231
Alanine	237	212	306
Valine	93	82	105
Methionine	7	9	12
Cystine (half)	252	244	253
Isoleucine	59	60	58
Leucine	104	105	115
Tyrosine	98	98	76
Phenylalanine	70	59	37
Lysine	207	191	138
Histidine	11	9	21
Arginine	35	39	35
Ammonia	140	128	146
Tryptophan	16	16	26

Similar values were obtained for other antigens, both D types and G types. This is in agreement with the data of Preer (1959c) for antigens of variety 2 of *P. aurelia*. Antigen 178D centrifuged in 8M-urea gave M_{eq} 260 000.

Amino acid composition. Table 2 shows results for antigens 90D, 178D and 90G, expressed as moles of residue/250 000g. of protein. The hydrolysates were prepared by the method of Crestfield *et al.* (1963) and in agreement with their findings no significant loss of threonine and serine was detected when antigen 90D was treated for 12, 24 and 36 hr. A standard time of 36 hr. was therefore used and no correction for loss applied.

It is apparent that the two D antigens are more closely similar to each other than to the G antigen and there are very significant differences particularly in the content of charged amino acids. Since the G antigen contains some 50 fewer lysine residues this must yield many fewer peptides on digestion with trypsin.

The nitrogen content calculated from these compositions is 15.9%. Determination by semi-micro-Kjeldahl gave 15.4%. This agreement lies within the probable accuracy of the methods.

All three antigens have an unusually high content of cystine, approximately 10% by wt., and it is important to determine whether these form disulphide bonds in the native protein or exist as free thiol.

Estimation of free thiol groups. Titration of the

native antigen with *p*-chloromercuribenzoate failed to reveal any free thiol groups. Neither could carboxymethylcysteine be detected in a hydrolysate of antigen treated with iodoacetate in 8M-urea. These results suggest that all the cysteine is involved in disulphide bridges, although whether these are inter- or intra-molecular has not been determined.

Reduction and alkylation. To prepare the antigen for digestion by trypsin it was denatured by reduction with 2-mercaptoethanol in 8M-urea and alkylated by reaction with iodoacetate according to Crestfield *et al.* (1963). The properties of the antigen were drastically altered by this treatment. It was no longer soluble below pH 7 and only slowly soluble in alkaline solution. Precipitation occurred below pH 7. It could, however, be maintained in neutral solution in the presence of 4M-urea or sodium lauryl sulphate. These properties are very similar to those reported for proteins modified by the introduction of other negatively charged groups (Pechère, Dixon, Maybury & Neurath, 1958). When alkylated with iodoacetamide instead of iodoacetate the product was completely insoluble even in alkaline solvents. If the charge were further modified by the reaction with succinic anhydride of the ϵ -amino groups of lysine residues (Klotz & Keresztes-Nagy, 1963) the product became once more freely soluble.

No simple solvent proved satisfactory for ultracentrifugal analysis. Even at pH 9–10 aggregation occurred. Solutions in 6M-urea yielded symmetrical peaks in the ultracentrifuge and reproducible molecular weights were obtained by the equilibrium method. Since, however, the interpretation of measurements in strong urea solution is uncertain because of the possibility of selective solvation and errors of the order of +100–50% are possible (Schachman, 1960), the same samples were caused to react with succinic anhydride and examined in 0.2M-sodium chloride. For reduced carboxymethylated 90D in 6M-urea: $M_{eq.} = 3.0 \times 10^4$; for reduced carboxymethylated 90D succinate: $M_{eq.} = 3.45 \times 10^4$.

The agreement is good if allowance for the added weight of succinyl groups is made. Thus measurements in 6M-urea are valid for these products.

However, different preparations of reduced carboxymethylated antigen gave values ranging from 1.6×10^4 to 8.0×10^4 .

To decrease the possibility of degradation during alkylation and subsequent isolation an equilibrium ultracentrifugation was made directly on the reduced antigen 178D in 8M-urea containing 2-mercaptoethanol (4mg./ml.): $M_{eq.} = 8.7 \times 10^4$.

To check the completeness of reduction a sample of reduced carboxymethylated 178D was hydrolysed and an amino acid analysis made. Carboxymethylcysteine was clearly separated from aspartic acid at the beginning of the chromatogram and the

expected 240 residues/molecule were found. No cystine could be detected on the chromatogram.

A sample of antigen 90D was reduced with sodium borohydride in 8M-urea according to Crestfield *et al.* (1963): $M_{eq.} = 7 \times 10^3$.

These results do not permit a definite conclusion about the size of the reduced antigen. The value determined in the reduction mixture would seem to set a maximum of 8×10^4 – 9×10^4 for the molecular weight. However, since there are some 120 disulphide bonds/molecule it is highly probable that complete reduction will not always be achieved, and if only one or two bonds remained intact these would not be detectable by amino acid analysis but could give a false value for the molecular weight of the chains.

Peptide maps. A preliminary digestion with trypsin of reduced carboxymethylated antigen in unbuffered solution in an autotitrator showed virtual completion of the reaction in 15–20 min. Since this was a cumbersome technique all subsequent work was done with digests buffered with ammonium carbonate to pH 8.0–8.5.

The chromatographic solvent contains a higher proportion of water (30%) than is usual. This was necessary to separate clearly the more slowly moving peptides. With the more common solvent, the upper phase of the system butan-1-ol-acetic acid-water (4:1:5, by vol.) (approx. 25% of water), much material was unresolved at the origin and formed streaks in electrophoresis. These slowly moving peptides probably constitute the core which Steers (1962) found it necessary to remove by Sephadex filtration.

Fig. 3 shows tracings of peptide maps of 90D, 178D, 60G and 90G antigens. All four differ considerably but there is a closer similarity between the members of each pair of alleles than between the different serotypes. The D antigens produced 90–100 spots and only 65–70 spots were observed in the patterns of the G antigens. Seven spots stained for tryptophan in 90D antigen and 14 in 90G antigen. Both 90D and 90G antigens contained 17 peptides staining specifically for arginine.

DISCUSSION

The immobilization antigen of *P. aurelia* provides an interesting system for the study of variation in protein structure. There is in fact an embarrassingly large amount of variation. No two stocks isolated from different localities have proved to have antigens of identical structure, even though they might be indistinguishable by immunological criteria (Jones & Beale, 1963). Some stocks can produce up to 12 different immobilization antigens, each under the control of a separate genetic locus, in response to environmental change (Beale, 1957).

Fig. 3. Tracings of peptide maps of tryptic digests of reduced carboxymethylated antigens: (a) 178D; (b) 90D; (c) 60G; (d) 90G. Chromatography was from left to right in butan-1-ol-acetic acid-water (17:4:9, by vol.). Electrophoresis was carried out vertically for 75 min. at 2500 v in pyridine-acetic acid-water (3:7:290, by vol.), pH 4.2 (cathode at top of diagram).

This work has concentrated on the best characterized loci in variety 1, the D and G loci.

The amino acid data and peptide maps show clearly that the proteins produced by the D and G loci are quite distinct and differ in primary structure, not merely in folding of the polypeptide chains as has been suggested by Beale (1954). In view of the large differences in the content of charged amino acids between 90D and 90G, it is perhaps surprising that their behaviour on ion-exchange columns should be almost identical. They can, however, be separated very slightly by starch-gel electrophoresis at high potential gradient (Bishop & Beale, 1960), but their mobilities are very low at pH 9.6 even though the isoelectric point is near pH 4. These features may possibly be explained if the charged amino acids are buried within the molecule and effectively masked. Separation of several types is possible on columns of hydroxyapatite (Bishop & Beale, 1960) but here behaviour is dependent on more complex adsorption phenomena and not simply on charge.

Although the actual function performed by these

antigens is obscure it would seem that the different serotypes must each serve the same purpose. This may be inferred from the mutually exclusive synthesis and the fact that the different proteins occupy the same sites on the cilia and pellicle of the animal (Mott, 1963). Two types can co-exist only for a brief period during adaptation to a new environment, and therefore it would seem that the animal must somehow function more efficiently when expressing the antigen appropriate to its environment. Since no evidence of this has been obtained, however, it is possible that the array of different antigens has arisen through a series of genetic accidents and that no special advantage results from the expression of one serotype rather than another. In any case it is clear that we have here a very primitive form of cell differentiation which could lead to an insight into the mechanism of differentiation in metazoan cells.

Considerable variation exists within each set of allelic types. There are some 20 points of difference between the two members of each of the allelic pairs shown in Fig. 3. A previous report on nine allelic D types (Jones & Beale, 1963) showed that no two were identical although five were completely indistinguishable by immunological criteria. However, it was clear that, in general, a close correlation could be established between similarity of peptide maps and degree of serological cross-reaction.

The data for the molecular weight of the reduced antigen show that the molecule contains sub-units, which, since no change in molecular weight takes place in 8M-urea, are most probably held together by disulphide bonds. No definite number of sub-units can be assigned on this basis alone, but examination of the peptide maps can lead to an answer.

The amino acid analysis of 90D antigen showed about 240 lysine and arginine residues, and that of 90G, 170 residues. Since trypsin breaks only those bonds involving the carboxyl groups of lysine and arginine one would expect some 240 and 170 peptides from 90D and 90G respectively. The observed numbers of 90–100 and 65–70 suggest the presence of two or three identical chains within the molecule. Specific staining permits a decision between two or three chains. There are 16 tryptophan residues in 90D and 26 in 90G. The number of peptides staining for tryptophan are seven and 14 respectively. The corresponding figures for arginine are 35 residues in both 90D and 90G and 17 specifically staining spots. Taken together these results point to the presence of two identical chains. The numbers of peptides produced should therefore be 120 in 90D and 85 in 90G. Several factors can lead to this discrepancy. Some spots are very much more intense than the rest and these probably consist of small peptides produced from several different parts of the sequence. Again, the two-dimensional system may

not resolve peptides containing a similar proportion of acidic and basic, polar and non-polar residues.

The maximum molecular weight indicated for the reduced antigen is 8×10^4 – 9×10^4 , which is too small for a half-molecule. It is therefore possible that further non-identical sub-units exist within the half-molecule. The obvious way to approach this problem would be by determination of end group. However, although a radioactive labelling method which could readily detect one *N*-terminal group in a molecular weight of 250 000 was used (Beale & Whitehead, 1962) no trace of *N*-terminal amino acid could be found in either native or reduced forms. There are some indications that this is due to blocking of the terminal amino group by some small substituent, perhaps acetyl, as has been found for several other proteins recently, but this needs further investigation.

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