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Characterization of Human Antibodies to *Salmonella typhi* by Gel-Filtration and Antigenic Analysis

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Summary. A normal adult human male was immunized with a heat-killed typhoid vaccine. The appearance of antibodies to 'H' and 'O' antigens was subsequently studied. Antibodies were identified as belonging to specific immunoglobulin classes by gel-filtration on G-200 'Sephadex', followed by specific immunochemical identification using a two-stage agglutination test. Serum antibodies to 'H' antigen were initially of γ_{1M} class; later the 7S γ class became predominant. γ_{1A} antibodies were transient in appearance. Serum antibodies to 'O' antigen were of γ_{1M} and γ_{1A} classes. Urinary antibodies to 'H' antigen were of γ_{1A} and 7S γ classes. No antibody activity could be detected in the low molecular weight γ-globulins of urine.

INTRODUCTION

At the present time four major classes of immunoglobulins are recognized in man (Fahey, 1963). These comprise 7S γ-globulins, γ_{1M}-globulins (19S γ-globulins), γ_{1A} (β_{2A})-globulins and γ_u(γ_L,γ_μ)-globulins. (This latter class comprises a heterogeneous collection of low molecular weight immunoglobulins recently identified in normal urine and also present in serum (Berggård, 1961).) Proteins of all these classes possess common antigenic determinants admitting them to the immunoglobulin group, and all, except perhaps γ_u-globulins, possess specific determinants by which they may be individually identified. Antibody activity associated with γ_{1M}- and 7S γ-globulins has been extensively described. In the case of γ_{1A}-globulin antibody activity is less well established, partly on account of the more recent recognition of this protein, and also on account of the difficulty of adequately separating it from 7S γ-globulin. Antibody function of γ_u-globulin is at present uncertain; Merler, Remington, Finland and Gitlin (1963) have, however, reported antibody activity in proteins of low molecular weight in normal human urine.

We report here an investigation of antibody activity of these four immunoglobulins following immunization of a normal adult male with a typhoid vaccine. Serum antibodies to both flagellar and somatic antigens and urine antibodies to the flagellar antigen were studied, and the sequence of appearance of serum antibodies of different immunoglobulin classes was investigated.

MATERIALS AND METHODS

Vaccine and Immunization

'Wellcome' brand, Typhoid–Para-typhoid A and B vaccine (Wellcome Research Laboratories, Beckenham, England) was used for the immunization course. The vaccine contained 10^9 heat killed *Salmonella typhi* organisms per ml.

A healthy male (M. W. T.) was injected subcutaneously with 0·5 ml. and 1·0 ml. of vaccine with a 27 day interval between injections.

Blood Samples

Samples of blood (20 ml.) were taken by venepuncture at weekly intervals (after the first injection) for 5 weeks and at monthly intervals thereafter. These samples were defibrinated and the sera separated by centrifugation. After the addition of sodium azide (0·1 g. per 100 ml.) the sera were deep frozen (−20°). Five weeks from the first immunization a larger sample of blood was withdrawn and allowed to clot. The serum obtained in this manner was freeze dried.

Urine Collections and Methods of Concentrating Urine

Urine samples were collected at the time of highest serum titres of *S. typhi* 'H' and 'O' agglutinins. The urine was collected into a vessel containing sodium azide as preservative and stored at 4° until concentrated. Two procedures for concentration were adopted. Urine sample (i) was a 48 hour sample concentrated ×300 by ultrafiltration through $\frac{8}{32}$ in. Visking dialysis tubing using a negative pressure of 60 cm. Hg and maintaining the apparatus at approximately 12°. The technique followed was essentially similar to that described by Grant (1957).

In order to reduce a possible selective loss of low molecular weight antibodies, an alternative approach applied to urine concentrate (ii) was to adsorb the urinary protein on to an ion exchanger at an alkaline pH and then specifically elute the γ-globulins at a more acid pH. Two litres of urine were dialysed at room temperature for 20 hours against running cold tap water using several feet of $\frac{24}{32}$ in. Visking dialysis tubing. Ten grams (dry weight) of DEAE–cellulose (Whatman DE-50, H. Reeve Angel & Co. Ltd., London) were washed with 5 l. of distilled water and then added to the urine. Sodium carbonate (10 per cent) was then added to the mixture (whilst stirring in the cold) to bring it to pH 9·5. After mixing for 15 minutes, the bulk of the urine was removed by filtration on a Buchner funnel. The moist exchanger was taken up in a small volume of saline and the slurry dialysed against pH 7·0, 0·1 M phosphate buffer overnight. This removed γ-globulins from the exchanger and the liquid in the dialysis sac was finally separated from the resin by filtration. In this manner urine (ii) was concentrated by a factor of 520.

Sephadex Gel-Filtration

Samples of serum and concentrated urine were fractionated by gel-filtration following a method similar to that of Flodin and Killander (1962) using a column of Sephadex G-200 (Pharmacia, Uppsala, Sweden). Serum or urine concentrate (3 ml.) were applied to a column measuring 2·8×57 cm. and elution was performed at room temperature using 0·85 per cent saline, previously degassed and containing 0·01 per cent sodium azide. Fractions were collected in 3 ml. amounts and examined for absorbence at 280 mμ in a Unicam spectrophotometer.

Assay of S. typhi *'H' Agglutinins*

Doubling dilutions (0·2 ml.) of fractions were prepared and mixed in Dreyer agglutination tubes with equal volumes of *S. typhi* 'H' suspension (The Standards Laboratory, Central Public Health Laboratory, London). The tubes were incubated at 37° for 2 hours

and then stood at room temperature overnight before assessing agglutination macroscopically.

Assay of S. typhi *'O' Agglutinins*

Doubling dilutions (0·5 ml.) of fractions were prepared in $3 \times \frac{1}{2}$ in. round-bottomed tubes and to each was added 1/30 ml. volume of concentrated *S. typhi* 'O' suspension (Standards Laboratory). After mixing, the tubes were incubated at 37° for 4 hours, stood overnight at 4° and then at room temperature for 30 minutes before assessing agglutination macroscopically.

Antisera

All the antisera used (with the exception of GP4 Anti-F) were raised in rabbits following courses of immunization which included intramuscular injections of emulsified antigen in complete Freund's adjuvant and intravenous injections of 1 per cent aqueous solutions of the antigens. GP4 anti-F was raised in a guinea-pig following foot-pad and intraperitoneal injections of antigen in Freund's adjuvant and intradermal injections of aqueous antigen.

Anti-7S γ-Globulin

A Cohn fraction II 1 : 2 preparation (free of γ_{1M}-globulin) was used as antigen. On immunoelectrophoresis with normal human serum the antiserum gave a strong 7S γ line only.

Anti-γ_{1A}-Globulin

A urinary γ_{1A} (Type II)-myeloma protein was used as antigen and the antiserum obtained was absorbed with both albumin and 7S γ-globulin. Two preparations were used; both were shown to be specific for γ_{1A}-globulin by immunoelectrophoresis with normal serum.

Anti-γ_{1M}-Globulin

This antiserum was raised against the washed cryoglobulin from the serum of a patient with macroglobulinaemia and absorbed with 7S γ and a selected hypogammaglobulinaemia serum. It has been described previously (Ratcliff, Soothill and Stanworth, 1963). Immunoelectrophoresis with normal human serum showed only a γ_{1M} precipitin line.

Anti-Fast (F) Component of 7S γ-Globulin

(a) *Absorbed rabbit anti-7S γ-globulin.* This antiserum to the fast (F) migrating component of papain digested 7S γ-globulin was prepared by absorbing anti-7S γ-globulin serum with slow (S) component of 7S γ-globulin to slight antigenic excess.

(b) *GP4 anti-F.* This antiserum was prepared by direct immunization of a guinea-pig with the purified F component of papain digested γ-globulin prepared by chromatography on ion exchange cellulose by a method similar to that of Franklin (1960). Immunoelectrophoretic analysis showed the antiserum to be specific.

Anti-Slow (S) Component of 7S γ-Globulin

This antiserum was raised in a rabbit using purified S component prepared by the method of Rowe (1961) and freed from contaminating 7S γ-globulin by gel-filtration on

Sephadex G-100. Immunoelectrophoretic analysis, with papain digested 7S γ-globulin, showed the single precipitin line of S component.

Anti-Bence Jones Protein

This antiserum was raised to an unfractionated urinary preparation from an individual with Bence Jones proteinuria.

Antigenic Studies

Double diffusion in agar was carried out by the method of Ouchterlony (1958). One per cent Oxoid Ionagar No. 2 (Oxoid Division, Oxo Ltd.) in phosphate buffer pH 7·0 was used and diffusion was allowed to proceed at room temperature for 12–24 hours or longer. The plates were then photographed by dark ground illumination or fixed in 5 per cent formalin in pH 7·4 phosphate buffered saline, dried and stained with a 0·15 per cent solution of Ponceau S in 3 per cent trichloro-acetic acid.

Immunoelectrophoretic analysis was by a method similar to that of Grabar and Williams (1953) using 0·8 per cent Oxoid Ionagar No. 2 in barbitone buffer pH 8·6 (I = 0·05).

Antigenic Features of Antibodies

Studies on the antigenic features of antibodies to the 'H' antigen in the different Sephadex regions were made by 'indirect' agglutination tests. An appropriate volume of *S. typhi* 'H' suspension was treated with one half of the minimal agglutinating dose of each fraction and incubated at 37° for 2 hours. After leaving overnight at 4°, the bacilli were spun down and the supernatant withdrawn. The packed bacilli, now coated with antibody but not agglutinated, were resuspended in their original volume of saline and agglutination was then attempted using the rabbit antisera to the immunoglobulins (diluted to 1:100) in the manner of the Coombs test (Coombs, Mourant and Race, 1946). The resuspended bacilli were incubated at 37° for 2 hours and then stood at room temperature overnight before agglutination was assessed. The possibility of direct agglutination, resulting from inaccurate estimation of the subagglutinating dose, was controlled by mixing each batch of resuspended bacilli with saline as well as with antisera. Before performing the agglutination tests all antisera were absorbed with *S. typhi* 'H' suspension to remove rabbit typhoid agglutinins, and a control of untreated bacilli in diluted antiserum was included in each experiment.

Similar studies were made on the antigenic features of antibodies to the 'O' antigen. An appropriate volume of concentrated *S. typhi* 'O' suspension was treated with one half of the minimal agglutinating dose of each fraction and incubated at 37° for 4 hours. After leaving overnight at 4° the supernatants were withdrawn from the bacilli which were then resuspended in the original volume of saline. Each fraction was then divided into aliquots of 0·5 ml. in 3 × ½ in. tubes and 0·005 ml. of appropriate antiserum added to each, thus achieving a final concentration of 1:100 for the antiserum. GP4 anti-F component and anti-γ_{1A}-globulin were the only antisera to be used in this test. Both were absorbed with *S. typhi* 'O' suspension prior to the agglutination tests. Appropriate saline and antiserum controls were again included. After mixing, the tubes were incubated at 37° for 4 hours, stood overnight at 4° and then at room temperature for 30 minutes before assessing agglutination.

RESULTS

SERUM STUDIES

Gel-filtration of serum on Sephadex G-200 was found to give very reproducible elution patterns. Three main protein peaks were consistently obtained (Fig. 1) and shown to correspond broadly to the '19S', '7S' and '4·5S' components of normal serum as resolved by the analytical ultracentrifuge (cf. Flodin and Killander, 1962).

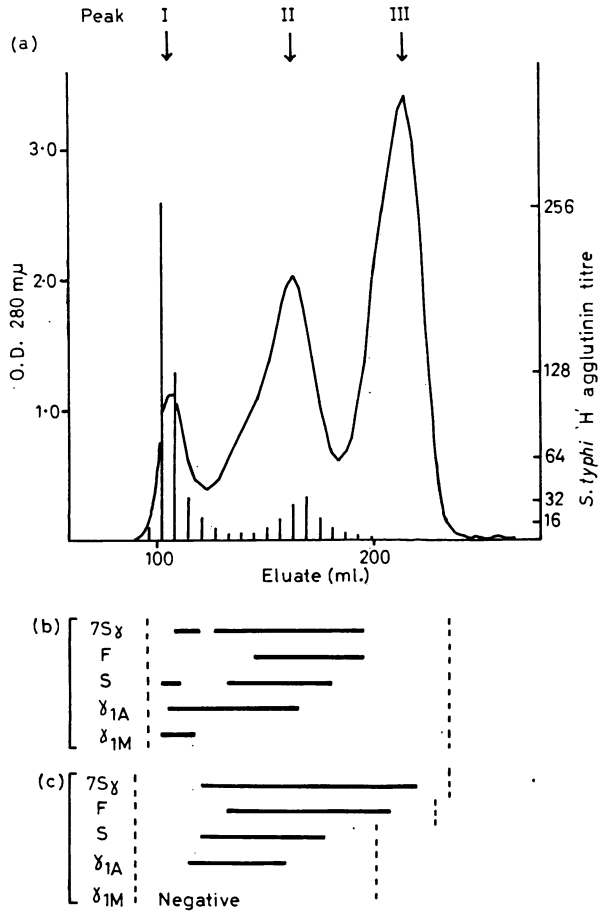

FIG. 1. Sephadex G-200 separation of serum (iv) of Fig. 5 showing: (a) distribution of agglutinins to *S. typhi* 'H'; (b) ranges of reactivity of the fractions with antisera to various immunoglobulins and the fast (F) and slow (S) components of 7S γ-globulin as determined by Ouchterlony analysis; (c) distribution of antibody activity associated with specific immunoglobulins and S and F components detected serologically as described in the text. Vertical dashed lines indicate ranges over which tests were performed.

None the less, to avoid confusion of nomenclature the first peak ('19S' for serum) will be called Peak I, the second peak ('7S') will be referred to as Peak II, and the third peak ('4·5S') as Peak III. Compared with the 19S component of ultracentrifugal analysis, Peak I appeared disproportionately large in many of these separations. It should be noted, however, that the protein concentrations were expressed as optical densities at 280 mμ and the values obtained were therefore considerably elevated by the turbidity which was an almost

constant feature of the fractions eluted in this region. Some sera were submitted to high-speed centrifugation before gel-filtration (to remove supranatant lipid) since this was found to be effective in reducing this turbidity.

The eluted fractions had pH values ranging from 5·0 to 7·3.

One serum sample was studied in greater detail. This was obtained 5 weeks after the primary immunization and is serum (iv) in Fig. 5. The distribution of several plasma proteins was established by both immunoelectrophoretic and Ouchterlony analyses using specific antisera. Peak I was shown to contain γ_{1M}-globulin and β-lipoprotein; γ_{1A}-globulin and 7S γ-globulin were identified in Peak II and albumin and siderophilin in Peak III. The precise distribution of the immunoglobulins is illustrated in Fig. 1(b) which is based on Ouchterlony analyses.

The distribution of reactivity of the antiserum to 7S γ-globulin with the fractions from the Sephadex column was rather broad and extended from Peak II into Peak I. However, the antiserum to F component, which is specific for 7S γ-globulin, reacted only in the Peak II region. γ_{1A}-globulin was present in highest concentration in the Peak II region and could be detected in fractions extending to Peak I. An antiserum to γ_{1M}-globulin reacted only with fractions in Peak I.

The typhoid 'H' agglutinin titres of the different fractions show two main peaks of activity (Fig. 1), corresponding to Peaks I and II, with some activity in the intermediate region. Thirty-six per cent of the antibody activity was recovered in this fractionation.

The nature of the antibodies in the different regions was defined by their antigenic features using the indirect agglutination test (see Materials and Methods). Bacilli were sensitized by incubation with subagglutinating doses of antibodies present in the appropriate fractions from the Sephadex column and then agglutination attempted with various specific antisera (Fig. 1c). The antisera to 7S γ-globulin, to the F and S components of 7S γ-globulin, and to γ_{1A}-globulin, agglutinated bacilli which had previously been incubated with certain fractions, thereby indicating that the adherent antibodies possessed determinants characteristic of 7S γ- and γ_{1A}-globulins. The antiserum to 7S γ-globulin again showed a broad range of reactivity which did not, however, extend into Peak I. Antisera to F and S components and γ_{1A}-globulin were also reactive, but again this reactivity did not extend into Peak I. The antiserum to γ_{1M}-globulin caused no agglutination in this test. The antibody nature of the reactivities of the various proteins was confirmed by similar experiments with a non-immune serum. Although the same distribution of immunoglobulins was observed on analysis on Ouchterlony plates, no sensitization of typhoid bacilli for agglutination by the specific antisera could be demonstrated.

Thus in these experiments the presence of 7S γ-globulin antibodies was inferred both from the appearance of an agglutination peak in the Peak II region and more specifically by the indirect agglutination test using the antiserum to F component. γ_{1A}-globulin antibodies did not appear as a separate peak and could only be demonstrated by indirect agglutination using the anti-γ_{1A} antiserum. Conversely, the presence of γ_{1M} antibodies could be inferred only from the agglutinating activity of Peak I since the antiserum to γ_{1M}-globulin was inactive in the indirect agglutination test.

URINE STUDIES

The elution pattern obtained when urine concentrate (i) (concurrent with serum (iv)) was subjected to gel-filtration on Sephadex G-200 again showed three absorption peaks at 280 mμ eluted in identical regions to Peaks I, II and III of the serum (Fig. 2).

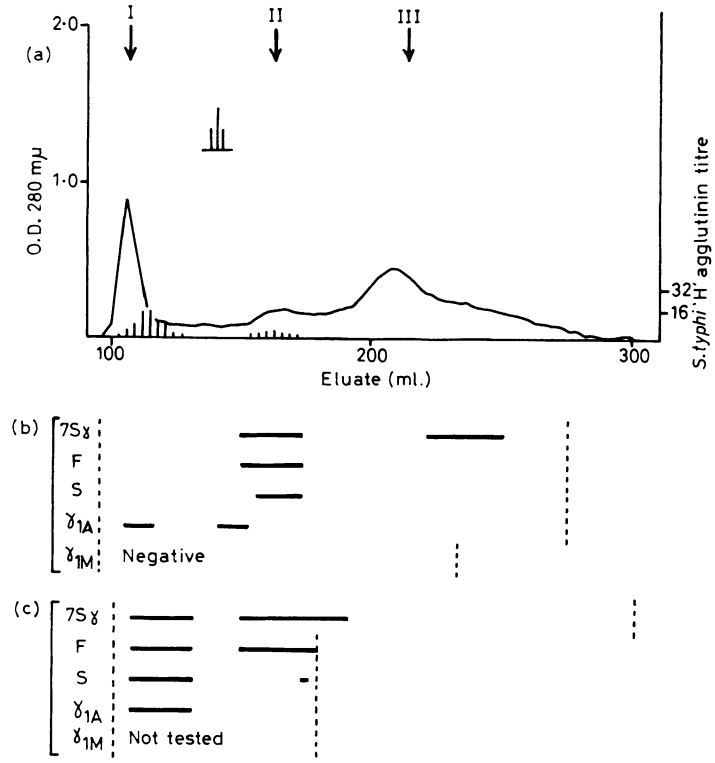

FIG. 2. Sephadex G-200 separation of urine concentrate (i) showing: (a) the distribution of agglutinins to *S. typhi* 'H' suspension; (b) ranges of reactivity of the fractions with antisera to the various immunoglobulins and the fast (F) and slow (S) components of 7S γ-globulin as determined by Ouchterlony analysis; (c) distribution of antibody activity associated with specific immunoglobulins and S and F components detected serologically as described in the text. Vertical dashed lines indicate ranges over which the tests were performed. Vertical arrows I, II and III indicate the positions of Peaks I, II and III in the elution pattern of serum on the same column.

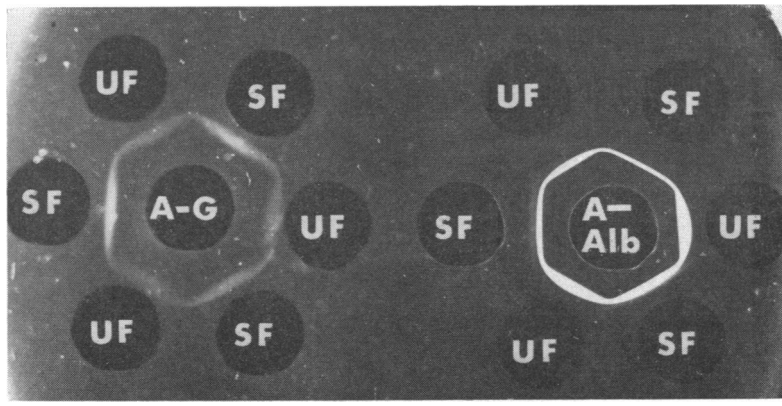

FIG. 3. Ouchterlony analysis showing (at left) reactions of complete identity between Peak II fractions of the urine separation (UF) and Peak II fractions from the separation of the concurrent serum (SF) using a specific anti-7S γ-globulin antiserum (A-G). Also showing (at right) reactions of complete identity between Peak III fractions of the urine separation (UF) and Peak III fractions from the separation of the concurrent serum (SF) using a specific anti-albumin antiserum (A-Alb). (Serum Peak II fractions diluted 1:6 and Peak III fractions diluted 1:10.)

Peak I was discrete but Peaks II and III were much less well defined than the corresponding serum peaks. Peak III was shown to contain antigenically complete albumin (Fig. 3).

The distribution of the immunoglobulins in the fractions from the Sephadex column is also shown in Fig. 2. The antiserum to 7S γ-globulin reacted in two well-separated regions—the Peak II (7S) region itself and a range of fractions appearing after Peak III. The γ-globulin in Peak II appeared to be intact protein since reactions of complete identity were obtained between Peak II fractions of the urine separation and Peak II fractions from the separation of the concurrent serum, using a specific anti-7S γ-globulin antiserum (Fig. 3).

Analysis by Ouchterlony's method of the material reacting with the anti-γ-globulin antiserum and appearing in the fractions eluted after Peak III is shown in Fig. 4. 7S

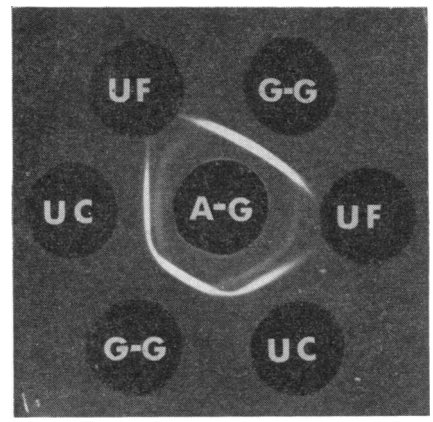

FIG. 4. Ouchterlony analysis using anti-7S γ-globulin antiserum (A-G) showing that protein in urine (i) fractions (UF) eluted after Peak III (Fig. 2) is antigenically deficient compared with 7S γ-globulin (G-G) but is identical with a γ-globulin component present in the original urine concentrate (UC). (The urine fractions were not concentrated prior to analysis.)

γ-globulin (a Cohn Fraction II 1:2 preparation) forms a spur with the urine fractions, indicating that the γ-globulin in them is antigenically incomplete with respect to the intact protein. The line given by the urine fractions fuses with the inner of the two lines given by the whole urine concentrate. Both of these latter lines are related to 7S γ-globulin since they in turn fuse with the single line given by this protein. (Immunoelectrophoresis of the urine concentrate against anti-7S γ-globulin antiserum also showed two main lines extending through the γ region).

Thus in the double diffusion analysis the inner of the two lines given by the urine concentrate appears to be similar to that of the antigenically incomplete γ_L-globulin of normal urine described by Berggård (1961) and also termed γ_u by Stevenson (1962). Furthermore its position of elution from the Sephadex column is in conformity with the observation by Stevenson (1962) that this protein has a lower molecular weight than that of 7S γ-globulin. This protein will subsequently be referred to as γ_u-globulin.

γ_{1A}-globulin was detected in two well-separated regions. The first of these was eluted slightly after Peak I and the other slightly ahead of Peak II. These findings were con-

firmed by Ouchterlony analyses which showed reactions of complete identity between γ_{1A} containing fractions and the γ_{1A} myeloma protein to which the antiserum had been raised. Both of the regions of reactivity fall within the range of γ_{1A} distribution of the corresponding serum separation. γ_{1M}-globulin could not be detected in any region and Ouchterlony analysis of the whole urine concentrate also failed to reveal this protein.

The typhoid 'H' agglutinin titres of the eluted fractions are also shown in Fig. 2. All fractions were tested from Peak I to fractions eluted when the column had been perfused with its own volume of saline. Two distinct regions of activity were found, one corresponding to Peak II of serum, the other being eluted close to Peak I, but appearing in slightly later fractions than the urine optical density peak and the Peak I agglutinin peak of the corresponding serum. This region contained approximately three-quarters of the eluted antibody. The antibody recovery from the column was 34 per cent.

Fig. 2 also illustrates the antigenic features of the antibodies in different regions detected in the same way as in the serum fractions. Antisera to 7S γ-globulin and the F component of this protein were reactive in the indirect agglutination test with fractions from both agglutinin regions. The antiserum to the S component was also active in both regions although its range of activity in the '7S' region was very limited. The detection of antibodies of 7S γ type in the first agglutinating peak was an unexpected finding, since this protein could not be detected in these fractions by Ouchterlony analysis. The likely explanation is that the Ouchterlony test is less sensitive than the indirect agglutination test on this material. This is also suggested by the broader spread of reactivity in the indirect agglutination tests compared with Ouchterlony tests in the serum analysis shown in Fig. 1.

The antiserum to γ_{1A}-globulin was reactive in the first agglutinin region only, and agglutinated the sensitized bacilli much more strongly than did the antiserum to 7S γ-globulin indicating that perhaps the bulk of the activity in the first agglutinin peak was due to γ_{1A} antibody.

A striking feature of these analyses was the failure of any fraction after Peak II, including those from the γ_u region, either to agglutinate or to sensitize bacilli for agglutination by anti-7S γ-globulin antiserum or by an anti-Bence Jones protein antiserum (which was also capable of precipitating γ_u protein from these fractions). We have also separated on Sephadex G-200 another sample of urine concentrated by adsorption on DEAE–cellulose (see Materials and Methods). This sample was concurrent with serum (v). Two distinct agglutinating regions were again observed in the same positions. Ouchterlony analyses showed 7S γ-globulin, albumin and γ_u protein also to be present in the same regions as in Fig. 2, but no antibody could be detected in any fraction after Peak II by direct agglutination or by the 'indirect' agglutination test using antisera to 7S γ-globulin and Bence Jones protein. Fractions were tested as far as those eluted with one column volume of saline.

SEQUENCE OF ANTIBODY PRODUCTION TO 'H' AND 'O' ANTIGENS IN IMMUNOGLOBULINS OF γ_{1M}, γ_{1A} AND 7S γ CLASSES

A normal adult male (M. W. T.) received two injections of TAB vaccine at an interval of 27 days. He had never previously been immunized against typhoid, and had no history of the disease. Pre-immunization serum was not tested, but serum obtained immediately after immunization showed zero titres of agglutinins to *S. typhi* 'H' and 'O' suspensions.

Fig. 5 shows the titres of post-immunization sera to 'H' and 'O' suspensions. 'O' titres were measured within a few days or after a longer period of storage at −20°. Repeat determinations on the same serum showed that storage in this way did not appear to affect the serum titre. 'H' titres were always measured within a few days.

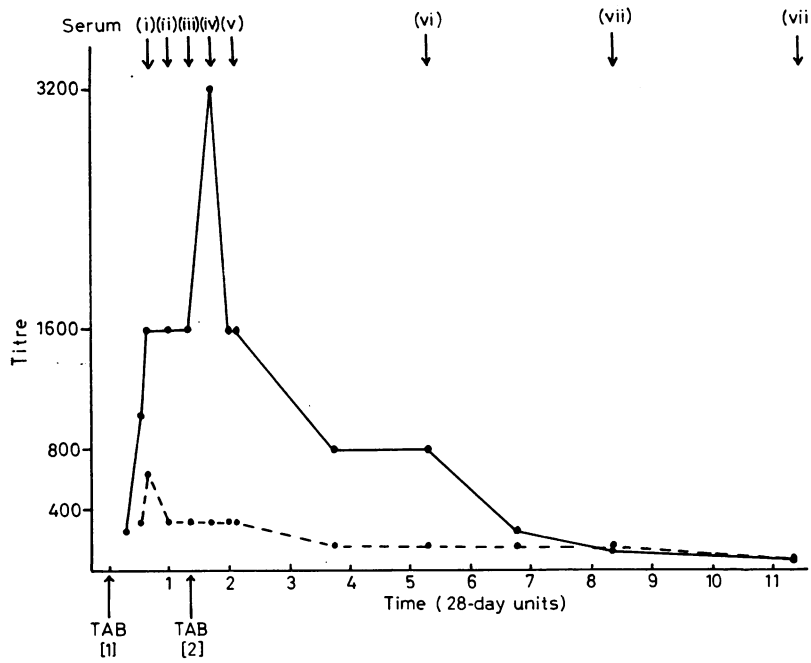

FIG. 5. Titres of antibodies to *S. typhi* 'H' (—) and 'O' (- - -) suspensions following TAB immunization.

'H' agglutinins were first detected on the sixth day and 'O' agglutinins on the eleventh day after immunization, these being the first sera to be tested. There was insufficient serum to test for 'O' agglutinins at any earlier date. The highest 'O' titre was observed on the thirteenth day and no rise in the titre followed the second immunization. In contrast the highest 'H' titre was found after the second immunization. Subsequently the titres of both antibodies declined, 'H' more rapidly than 'O'.

Sera (i) to (viii) as indicated in Fig. 5 were fractionated on G-200 Sephadex columns after periods of storage ranging from 1 to 8 months. Although, as noted previously, there was little evidence of change in 'O' titres during that time, there was a decline in 'H' titres which in some cases fell to a quarter of their original value.

Agglutinin recoveries following Sephadex fractionation, when compared with the serum titre at the time of separation, ranged from 40 to 60 per cent. This has been a consistent finding for antibodies of various types, and is in contrast to the higher recoveries reported by Killander and Högman (1963) who fractionated human blood group antibodies on columns on G-200 Sephadex under somewhat different experimental conditions. In consideration of the sequence of antibody production we cannot therefore eliminate the possibility of error due to selective losses of antibodies of a particular immunoglobulin class, either on storage or following separation on Sephadex. However, observations on serum (iv) suggest that a serious distortion of apparent antibody distribution did not

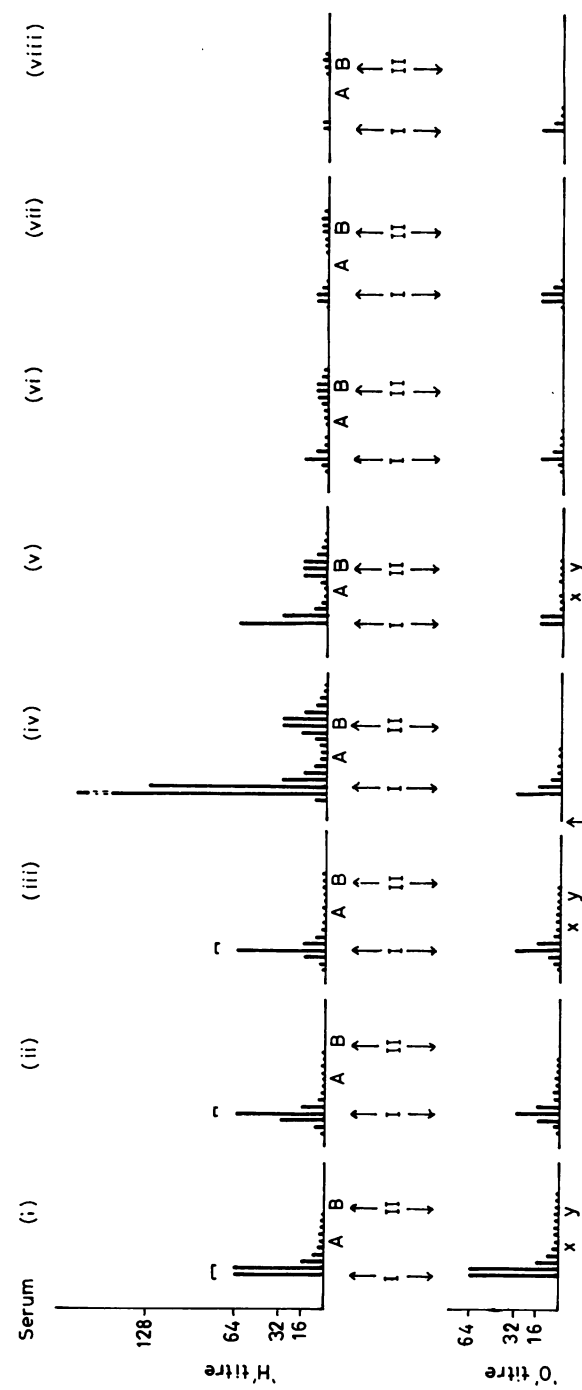

FIG. 6. Serial Sephadex fractionations of antibodies to *S. typhi* 'H' and 'O' suspensions. (i)–(viii) are sera as indicated in Fig. 5. I and II represent the positions of Peaks I and II on the O.D. curves. Horizontal brackets represent fractions which were concentrated for refractionation on Sephadex G-200 (see Fig. 7). X and Y are fractions from the 'intermediate' and Peak II regions respectively which were used in sensitization tests with 'O' suspensions (see Table 1). A and B are fractions from the 'intermediate' and Peak II regions respectively which were used in sensitization tests with 'H' suspensions (see Table 2). (The peak of the second fractionation of serum (iv) represents an 'H' titre of 256.)

occur on storage. This serum was fractionated twice on Sephadex, at an interval of 3 months, during which time the serum titre to 'H' suspension had fallen to half of the initial value. None the less, the distribution of antibody activity to the 'H' antigen in the Sephadex fractions was very similar on both occasions.

SEPHADEX FRACTIONATION OF ANTIBODIES TO *S. typhi* 'O'

As Fig. 6 shows, these antibodies were predominantly in the Peak I region of column eluates. However, all the separations showed a low titre of activity extending towards or into the Peak II region. This suggested either that antibodies of γ_{1A} or 7S γ classes might be present, or that the γ_{1M} protein trailed from the Peak I region across into fractions eluted later from the column. Although Ouchterlony analysis results shown in Fig. 1 indicated that γ_{1M}-globulin was confined to Peak I, it is probable that this test would be insufficiently sensitive to detect the small amount of trailing γ_{1M}-globulin necessary to account for the broad distribution of antibody activity. Hence antibody eluted in several Peak I fractions was pooled, concentrated and re-run on a Sephadex column, using normal serum as a 'carrier'. The fractions used were those indicated by horizontal brackets in Fig. 6. The pooled material was concentrated sevenfold by ultrafiltration and 1·2 ml. of concentrate added to 1·2 ml. of carrier serum from a non-immunized individual. This material was then applied to the Sephadex column, and the resulting elution diagram is shown in Fig. 7. (Similar results were obtained with '19S' fractions of antibodies to tetanus toxoid, detected by a passive haemagglutination technique.)

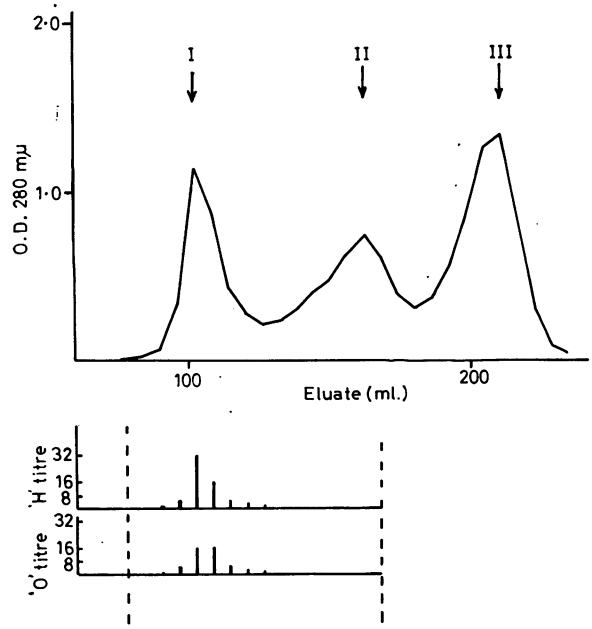

FIG. 7. Refractionation on Sephadex G-200 of concentrated pooled Peak I material from three serum separations plus non-immune carrier serum. The fractions used are indicated by horizontal brackets in FIG. 6. The vertical dashed lines indicate the range over which fractions were tested for presence of agglutinins.

The re-run 19S antibodies gave a slightly asymmetric peak, indicative either of an imperfection in the column separation or of antibody heterogeneity. However, the results showed that the distribution of titres as far as the '7S' region (as shown for example by serum (i), Fig. 6) is not likely to be accounted for by trailing of antibodies of γ_{1M} class. This conclusion was supported by the results of indirect agglutination experiments. *S. typhi* 'O' suspensions were incubated with subagglutinating doses of fractions from the 'intermediate' region and from the '7S' region of the eluates of sera (i), (iii) and (v). These fractions are indicated on Fig. 6 as X and Y respectively. Subsequently, agglutination was attempted using antisera to γ_{1A}-globulin and the F component of 7S γ-globulin (GP4 anti-F was used in this instance). Table 1 shows that in every case the antiserum to

TABLE 1

SENSITIZATION OF *S. typhi* 'O' SUSPENSIONS TO AGGLUTINATION BY ANTI-γ_{1A} AND ANTI-F ANTISERA BY FRACTIONS FROM 'INTERMEDIATE' AND 7S REGIONS OF SEPHADEX FRACTIONATIONS

Serum	*Resuspension medium*	*Fraction X ('intermediate' region)*	*Fraction Y (Peak II region)*
Serum (i)	Anti-γ_{1A}-globulin	++	+
	Anti-F component	−	−
	Saline control	−	−
Serum (iii)	Anti-γ_{1A}-globulin	+	++
	Anti-F component	−	−
	Saline control	−	−
Serum (v)	Anti-γ_{1A}-globulin	+	++
	Anti-F component	−	−
	Saline control	−	−

See Fig. 6 for exact location of Fractions X and Y.
+ shows the presence and degree of agglutination; − shows the absence of agglutination.

γ_{1A}-globulin caused agglutination, whereas the 'sensitized' bacilli suspended in antiserum to F component and also in saline as a control were not agglutinated. Particular care was taken to ensure that the anti-γ_{1A} antiserum had been completely absorbed; agglutination tests of this absorbed antiserum with *S. typhi* 'O' suspensions were negative.

It thus appears that production of antibody of γ_{1A} class was an early and continued response to the somatic antigen by this individual, and these antibodies may account for the spread of agglutinin activity in the eluates from the Sephadex column. However, they can represent no more than a small proportion of the total agglutinating activity as compared with agglutinins eluted in the '19S' region which were presumably of γ_{1M} type.

SEPHADEX FRACTIONATION OF ANTIBODIES TO *S. typhi* 'H'

The distribution of these agglutinins in fractions from sera (i), (ii) and (iii) were similar to the 'O' agglutinin distribution in showing activity in the Peak I region but with agglutination extending towards and into the Peak II region. In later sera an agglutination peak also appeared in the Peak II region which increased in proportion to that in Peak I until from serum (vi) onwards approximately equal amounts of agglutinating activity were found in each peak.

Indirect agglutination tests on fractions indicated as 'A' (intermediate) and 'B' (Peak II or 7S) in Fig. 6 were performed for all sera. Table 2 shows the results obtained using anti-γ_{1A} antiserum and anti-F component antiserum—both absorbed with *S. typhi* 'H' suspension.

TABLE 2

SENSITIZATION OF *S. typhi* 'H' SUSPENSIONS TO AGGLUTINATION BY ANTI-γ_{1A} AND ANTI-F ANTISERA BY FRACTIONS FROM 'INTERMEDIATE' AND 7S REGIONS OF SEPHADEX FRACTIONATIONS

Serum	*Resuspension medium*	*Fraction A ('intermediate' region)*	*Fraction B (Peak II region)*
Serum (i)	Anti-γ_{1A}-globulin	−	−
	Anti-F component	−	−
	Saline control	−	−
Serum (ii)	Anti-γ_{1A}-globulin	−	−
	Anti-F component	−	+
	Saline control	−	−
Serum (iii)	Anti-γ_{1A}-globulin	−	−
	Anti-F component	Trace	++
	Saline control	−	−
Serum (iv)	Anti-γ_{1A}-globulin	+	−
	Anti-F component	+	+
	Saline control	−	−
Serum (v)	Anti-γ_{1A}-globulin	+	−
	Anti-F component	+	+
	Saline control	−	−
Serum (vi)	Anti-γ_{1A}-globulin	+	−
	Anti-F component	+	+
	Saline control	−	−
Serum (vii)	Anti-γ_{1A}-globulin	−	−
	Anti-F component	+	+
	Saline control	−	−
Serum (viii)	Anti-γ_{1A}-globulin	−	−
	Anti-F component	−	+
	Saline control	−	−

See Fig. 6 for exact location of Fractions A and B.
+ shows the presence and degree of agglutination; − shows the absence of agglutination.

It will be noted that these tests (using the anti-F antiserum which is specific for 7S γ-globulin) provided the earliest conclusive indication of 7S γ class antibodies, which were present in fraction B from serum (ii). Antibodies of γ_{1A} class were detected in sera (iv), (v) and (vi), at the time of peak antibody response. This antibody was only detected in the intermediate region. γ_{1A} antibody production is therefore apparently more transient to the flagellar than to the somatic antigens, although it may be that its presence in some of the sera is masked by a preponderance of immunoglobulins of other classes.

DISCUSSION

Repeated serum fractionations on Sephadex G-200 columns yielded highly reproducible optical density patterns having three main peaks, as in Fig. 1. Ouchterlony studies of the segregation of certain plasma proteins in these peaks agreed with the findings of Flodin

and Killander (1962). Thus Peak I contained γ_{1M}- and α_2-macroglobulins, and β-lipoprotein. Peak II included 7S γ-globulin; siderophilin and albumin were detected in Peak III.

More detailed analysis for immunoglobulins revealed γ_{1M}-globulin sharply confined to Peak I, and an antiserum to the specific F papain fragment of 7S γ-globulin showed this protein to be confined to Peak II. On this evidence gel-filtration is superior to preparative ultracentrifugation in the separation of these two proteins, since it has been our experience in the fractionation of Cohn Fraction III-1 of plasma that three sequential runs are necessary in a sucrose density gradient to free γ_{1M}-globulin of 7S γ-globulin (Walton, Rowe, Soothill and Stanworth, 1963).

γ_{1A}-globulin was broadly distributed, extending from Peak I to Peak II, and the intensity of the precipitin lines suggested its presence in highest concentration in the fractions immediately before the Peak II maximum. This distribution is in accordance with the findings of Heremans, Heremans and Schultze (1959) that γ_{1A}-globulin is heterogeneous in the ultracentrifuge, with a major component of approximately 7S and minor components which sediment more rapidly. A similar distribution following Sephadex G-200 fractionation has been reported by Fireman, Vannier and Goodman (1963). Asymmetry of the leading edge of Peak II, which is a region of high γ_{1A}-globulin concentration, was a constant finding in our separations. This was not due to changes consequent upon storage, since it appeared even when serum was applied to the column within a few minutes of venepuncture. Analytical ultracentrifugation of fractions from this region concentrated by ultrafiltration showed peaks intermediate between 19S and 7S, as well as a preponderance of 7S material. There is, however, no direct evidence that the asymmetry is due to γ_{1A}-globulins.

Antisera to the S component of papain digests of 7S γ-globulin and to 7S γ-globulin itself, yielded precipitin lines on Ouchterlony analyses with fractions extending from Peak I to Peak II. This is in conformity with the current concept that antigenic determinants of S component are common to immunoglobulins of all classes. Precipitin lines with Peak I fractions were slow to appear compared with those of Peak II, and were concave toward the antigen well, suggesting that they did indeed represent reactions of γ_{1M}- and not of possible 7S γ-globulin contaminants.

In the serum analysis shown in Fig. 1 agglutinin activity to *S. typhi* 'H' was associated with Peaks I and II. It was noticeable that activity associated with Peak II was eluted slightly after (to the right of) the optical density peak, so that fractions of relatively high optical density in the leading edge of the peak possessed only low agglutinin titre. This finding could be explained by the presence of γ_{1A}-globulin in relatively high concentration but of low agglutinin activity in these fractions (see below).

The activity of Peak I can almost certainly be ascribed to the content of γ_{1M}-globulin, since the only other immunoglobulin found in this region was γ_{1A}-globulin, present only in the later fractions of the peak. However, activity in Peak II and the intermediate zone was less readily associated with a specific immunoglobulin, and could possibly be related to either γ_{1A}- or 7S γ-globulin, or both. This was elucidated by the indirect agglutination test, a method previously used in the antigenic analysis of rheumatoid factor (Rowe, 1962). To avoid any ambiguity in the interpretation of this test mono-specific antisera were employed throughout. An additional check on the specificity was provided by a broadly similar distribution of immunoglobulin as antigen, revealed by the Ouchterlony test, and as antibody, shown by the indirect agglutination test. The exception to this was the

region of Peak I. Here all the antisera employed, including that to γ_{1M}-globulin, were inactive in the indirect agglutination test. A possible explanation for this finding is that the antibodies of γ_{1M} type were of high agglutinating efficiency, so that the amount of antibody on the surface of the bacilli necessary to produce agglutination would be similar to, or less than, the amount necessary to sensitize the bacilli in the indirect test. In addition, in fractions of high agglutinating titre a small amount of γ_{1A} or other antibody could go undetected by the indirect test, since this antibody would be diluted to below a sensitizing level in the high dilution necessarily employed in the first sensitizing stage of the test.

In the analysis of urine (Fig. 2) the optical density curve again shows three main peaks, corresponding fairly closely in position to Peaks I, II and III of serum. Peak I comprised material unable to penetrate the Sephadex gel; γ_{1M}-globulin was not detected in it, and its optical density was due, at least in part, to turbidity. This peak has no counterpart in ultracentrifugal analysis of concentrates of normal urine (Webb, Rose and Sehon, 1958; Rowe and Soothill, 1961). Peak II contained 7S γ-globulin; both the position of elution from the column and Ouchterlony analysis (Fig. 3) indicated that this was intact, unsplit material. In a previous report from this laboratory (Rowe and Soothill, 1961) 7S material was not detected in urine concentrates by analytical ultracentrifugation, but Franklin (1959) and recently Merler *et al.* (1963) have demonstrated γ-globulin sedimenting at 7S which was apparently antigenically complete.

Peak III of the urine separation contained albumin, also apparently antigenically complete (Fig. 3). A feature of the separation was the extension of optically dense fractions beyond Peak III. Some of these fractions contained material antigenically deficient compared with 7S γ-globulin, which was also precipitated by an antiserum to Bence Jones protein. Surprisingly this material did not precipitate with the anti-S component antiserum; however, this antiserum did not produce strong precipitin lines with any of the immunoglobulins, and was the weakest of those used in this study. The evidence indicates that this protein can be regarded as similar to the γ_u protein of Stevenson (1962).

γ_{1A}-globulin was not associated with a particular optical density peak, but appeared in two distinct regions, both within the range of distribution of γ_{1A}-globulin in the serum. This distribution cannot be accounted for by selective molecular sieving of plasma proteins by the glomerulus. It suggests either the possibility of aggregation of the protein in the urine, or that some specific mechanism may exist for its secretion, perhaps analogous to that responsible for the relatively high concentration of the protein in milk and saliva.

Agglutinin activity to *S. typhi* 'H' appeared in two regions. Indirect agglutination tests defined the activity in Peak II as that of 7S γ-globulin and activity close to Peak I was associated with γ_{1A}- and also 7S γ-globulin, but with γ_{1A} activity probably predominant. Although 7S γ-globulin was not detected in this region by Ouchterlony tests, there is evidence in the serum analyses of Fig. 1 that the indirect agglutination test may be more sensitive in detecting this immunoglobulin as antibody than is the Ouchterlony test in detecting it as antigen, since the former test has the wider range of distribution about Peak II. Aggregation of 7S γ-globulin in the urine possibly with γ_{1A}-globulin, could provide an explanation of its activity close to Peak I.

Despite careful search no agglutinating or incomplete antibody was found in any fraction eluted after Peak II.

We have, in addition, studied the antibodies to tetanus toxoid in the urine of a normal adult and antibodies to thyroglobulin in the urine of a patient with myxoedema. Urine from these individuals was concentrated by lyophilization and by adsorption and elution

from DEAE–cellulose respectively. The urine concentrates were fractionated by gel-filtration through Sephadex G-200 and the distribution of antibodies in the eluted fractions determined by appropriate passive haemagglutination techniques. There was a notable similarity between the distribution of agglutinins to tetanus toxoid and the distribution of agglutinins to *S. typhi* 'H' shown in Fig. 2. Thus, two regions of agglutinating activity (comprising three-quarters and one-quarter of the total) were eluted in exactly comparable positions and no agglutinating antibody could be detected in any fraction eluted after Peak II. Antibody activity in the case of the fractionated anti-thyroglobulin urine was confined to fractions in the Peak II region.

These results contrast with the findings of Merler *et al.* (1963), who studied antibodies to tetanus toxoid and poliomyelitis virus in normal urine. Following urine concentration by dialysis and lyophilization, by pervaporation and dialysis, or using G-25 Sephadex, they separated the urinary γ-globulin by block electrophoresis or chromatography on DEAE–cellulose. These γ-globulin preparations contained two components in the ultracentrifuge; a minor component of approximately 7S, antigenically complete on Ouchterlony analysis using an antiserum to serum 7S γ-globulin, and a major component of approximately 1S which was antigenically deficient. Antibody activity was associated with the major component only.

Apart from possible variability of individual responses to particular antigens it is difficult to reconcile our findings with this report. It appears that the urine may have been concentrated by a greater factor in the work of Merler *et al.* than in our studies, and it is also possible that the method of concentration by ultrafiltration through stretched dialysis tubing used in some of our experiments had a greater likelihood of loss of low molecular weight materials. Thus the low molecular weight antigenically deficient component of Merler *et al.*, may not be the same as that found by us. However, these differences in technique do not offer an explanation for the lack of activity in the 7S fraction of the preparation of Merler *et al.* Further attempts to obtain low molecular weight urinary antibodies are in progress, since such material could be of importance in elucidation of the structural basis of antibody activity.

Previous studies of the sequence of antibody production to antigens of *S. typhi* have been made by LoSpalluto, Miller, Dorward and Fink (1962) in humans, and Bauer, Mathies and Stavitsky (1963) in rabbits. Our findings with regard to 7S γ and γ_{1M} antibodies are in agreement with these reports. In addition we report the transient appearance of γ_{1A}-globulin as antibody to the flagellar antigen, at a time of peak titre following the second immunization, and as antibody to the somatic antigen appearing early and persisting for a prolonged period. There is, however, only suggestive evidence from these studies that γ_{1A}-globulin is active as agglutinin, and certainly such activity appeared insignificant compared with the activities of γ_{1M}- and 7S γ-globulins.

These studies raise again the question of the significance of γ_{1A} antibody in the immune response. Evidence for the antibody activity of this protein is quickly mounting. Schultze (1959) described a range of naturally acquired antibodies, including antibodies to *S. typhi* 'O' and *S. paratyphi* B in a γ_{1A} preparation, a finding which was interpreted with caution since the preparation was known to contain γ_{1M}-globulin. Heremans and Vaerman (1962) suggested that skin sensitizing activity of human allergic sera is a function of γ_{1A}-globulin, and this was substantiated by Fireman *et al.* (1963) studying skin sensitizing activity in ragweed sensitive individuals. Whether γ_{1A} antibody is invariably and exclusively capable of skin sensitization is uncertain, but it appears from the findings reported here that γ_{1A}

antibodies can be produced to bacterial antigens in a normal individual with no history of allergy, and it will be of interest to discover whether such antibody can produce skin sensitization. The findings do suggest that the chemical nature of the antigen is of importance in determining the γ_{1A} antibody response, as well as the the response in 7S γ- and γ_{1M}-globulins. They thus describe another parameter of variability of antibody response, the significance of which in health and disease remains to be assessed.

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