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The Significance of Multiple Antibody Components in Serum of Immunized Rabbits

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Summary. The electrophoretic patterns of six sera from rabbits immunized by two or more courses of intravenous injections of killed pneumococci type III showed multiple peaks in the γ-globulin region. Such sera contained large amounts of antibody (up to 85 per cent of the total γ globulin) against the capsular polysaccharide. One serum contained a cryoglobulin, which contained almost as great a proportion of specific antibody as did the remaining γ globulin.

The electrophoretic patterns and antibody contents were similar in the water-soluble and water-insoluble fractions of γ globulin.

The sedimentation constant and diffusion coefficient of a water-soluble fraction of γ globulin, containing 85 per cent specific antibody, were measured. The values, at 0.4 per cent protein concentration, were $S_{20.w} = 6.97 \times 10^{-13}$ and $D_{20.w} = 4.16 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, corresponding to molecular weight 159,000.

The antibody-containing globulin from one serum was separated by zone electrophoresis into three fractions with different electrophoretic mobilities. These contained 53–71 per cent of antibody precipitable by type III pneumococcus capsular polysaccharide. Only doubtfully significant differences were found in respect of amino-acid composition, hexose and hexosamine contents, or antigenic characteristics.

A method was devised for detecting small amounts of antibody against capsular polysaccharide by means of red cells sensitized with culture filtrates of capsulated pneumococci.

The antibody was also fractionated by chromatography on anion-exchange cellulose, and numerous fractions with antibody activity were obtained. It was shown by labelling the γ globulin with ^{131}I that similar fractionation occurred both in the presence and absence of other serum components. All the chromatographic fractions of γ globulin were found to contain approximately similar proportions of antibody. By electrophoresis in starch gel the fractions were found to differ from one another and to be heterogeneous.

The implications are discussed of the finding that antibody against type III pneumococcus capsular polysaccharide can occur over the entire range of γ-globulin molecules.

INTRODUCTION

When normal rabbit serum is examined electrophoretically, whether by free electrophoresis, by zone electrophoresis, or by immunoelectrophoresis, the γ globulin is found to consist of a single rather broadly spread out component. In the course of hyperimmunizing rabbits by multiple intravenous injections of formalin-killed type III pneumococci we observed that several sera developed two or three electrophoretic components

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in the γ-globulin region, and in one instance a cryoglobulin component also appeared. The sera of some of these immunized rabbits contained very large amounts of antibody (40–60 mg./ml.) specifically precipitable with type III pneumococcus capsular polysaccharide, and in one serum this single antibody comprised 82 per cent of the total γ globulin. We thought that further investigation of the multiple γ globulins might shed light on their origin, and on the nature of the differences between them, and we chose one serum especially for closer examination by several techniques, including chromatography on anion exchange cellulose. In order to measure conveniently the small amounts of antibody present in some of the chromatographic fractions a method was devised for detecting antibody by a haemagglutination technique. Since the γ globulin from one of the rabbits consisted largely of a single antibody it was possible to obtain almost pure antibody by simple salt fractionation, instead of the usual method of dissociation of antigen-antibody complexes by high salt concentration or low pH – procedures open to the objection that only part of the antibody is normally recovered. The opportunity was therefore taken of measuring the sedimentation constant and molecular weight of this rabbit antibody.

MATERIALS AND METHODS

RABBITS

The appearance of multiple γ globulins during immunization was observed in rabbits of the sandylop strain bred at this Institute, and in Dutch and New Zealand strains. The rabbits were maintained on pelleted diet No. 18 (Bruce and Parkes, 1940).

IMMUNIZATION PROCEDURE

Intravenous injections of formalin-killed pneumococci type III prepared as described by Kauffmann, Bjørneboe and Vammen (1938) were given three times weekly in doses increasing from 5×10^8 to 10^{10} organisms during the course of 3–4 weeks. A second course of injections was given 3–4 months later, and sometimes a third course after a similar period of time. Some of the rabbits began to lose weight rapidly towards the end of the 2nd or 3rd courses — this change being accompanied by a marked rise in serum antibody levels. Multiple antibody components were never observed after a single course of injections, but were found in the majority of sera examined at the end of 2nd or 3rd courses, when antibody levels had reached 25 mg./ml. or over

TYPE III PNEUMOCOCCUS CAPSULAR POLYSACCHARIDE

Prepared according to Heidelberger, Kendall and Scherp (1936). It contained no detectable C-polysaccharide, and gave no precipitate with a rabbit antiserum containing 15 mg./ml. of antibody against type I pneumococcus capsular polysaccharide.

ESTIMATION OF ANTIBODY AGAINST TYPE III PNEUMOCOCCUS CAPSULAR POLYSACCHARIDE

(*a*) *By precipitation*

Antibody was precipitated by adding antigen in slight excess of the equivalence ratio, and was washed three times at 2° with 0.9 per cent (w/v) NaCl solution. The amount of antibody was measured by dry weight, after washing with ethanol and ether, or by the absorption at 280 mμ, after dissolving the washed specific precipitate in 0.1 N NaOH. The ratio of antigen to antibody in the precipitate was 1 : 20–25.

(b) By haemagglutination

For reasons of convenience, and because sometimes only small amounts of antibody were available, a sensitive method for estimating antibody was required which did not involve isolation of a specific precipitate. We attempted unsuccessfully to sensitize red cells (human, sheep and rabbit) with purified pneumococcus type III polysaccharide, both before and after treatment with dilute alkali (cf. sensitization with gram-negative somatic antigens, as described by Davies, Crumpton, Macpherson and Hutchison, 1958). Keogh, North and Warburton (1948) have described sensitization of human or fowl red cells with a phenol extract of pneumococci and Hayes (1951) sensitized sheep red cells with a purified aqueous extract. These methods, although successful, gave a rather disappointing degree of sensitization. However, it was found that the supernatant fluid from 8–12 hour cultures of type III pneumococci in the medium of O'Meara and Brown (1936), kept at pH about 7 by addition of $NaHCO_3$, produced excellent sensitization of red cells. For convenience the cultures were killed by addition of 1.6 per cent formaldehyde, followed by further incubation at 37° for 18 hours. The organisms were removed by high speed centrifugation, and formaldehyde was removed by dialysis against saline buffered at pH 7.0. The supernatant fluid could be stored frozen for at least 1 year without noticeable deterioration.

Haemagglutination

Conditions were similar to those described for bacterial antigens by Davies *et al.* (1958). 0.2 ml. packed sheep red blood cells (washed three times with saline) were suspended in 10 ml. saline-phosphate (1 volume of 0.9 per cent (w/v) NaCl plus 1 volume of 0.15 M – Na phosphate buffer pH 7.2) and sensitized with 0.2 ml. pneumococcal culture fluid (for preparation see above) by incubating at 37° for 45 minutes. After the incubation, the cells were washed three times with 1 per cent (v/v) normal rabbit serum in 0.9 per cent (w/v) NaCl and then suspended in 20 ml. of this solution to make a 1 per cent red cell suspension. The rabbit serum had been absorbed with 0.15 ml. of packed sheep red blood cells per ml. serum for 30 minutes at room temperature in the presence of 0.1 per cent neutral Na-ethylenediamine tetraacetate (used to inactivate complement). The antibody fractions were similarly absorbed with red blood cells and serial dilutions were made with 1 per cent (v/v) rabbit serum. 0.1 ml. of 1 per cent red blood cell suspension was added to 0.2 ml. of diluted antiserum and the agglutination plates left at room temperature overnight in a polythene bag. An antiserum containing 0.45 mg./ml. of antibody against type III pneumococcal polysaccharide gave an haemagglutination titre of 1/2048. The sensitivity of the method is increased 2–3 fold by decreasing the red cell concentration. Haemagglutination was specifically inhibited by addition of 1 μg./ml. type III pneumococcus capsular polysaccharide, and the haemagglutination titres of several sera correlated well with the contents of precipitating antibody.

Free electrophoresis

Carried out in veronal buffer at pH 8.6, I = 0.1 in a Perkin Elmer apparatus, a gift from Eli Lilly & Co.

Zone electrophoresis

In a column of treated cellulose was carried out in borate-phosphate buffer pH 8.4, I = 0.05 (Gedin and Porath, 1957) or in 0.075 M tris(hydroxymethyl)aminomethane

(tris)-maleate buffer (Gomori, 1955). Electrophoresis was continued for 40 hours, with a current of 4.5 mA/cm.^{2}, in order to obtain good separation of the γ globulins. The protein content of the eluted fractions (3 ml.) was determined by measurement of absorption density at 280 mμ. Since tris-maleate buffer has considerable absorption at this wavelength, known dilutions of the fractions were compared with blanks of buffer diluted to the same extent.

Agar-gel diffusion analyses

Were performed in 2.0 per cent agar (Difco) made up in 0.1 M Na phosphate buffer pH 7.8. The antisera were prepared in goats, guinea pigs and fowls by injection of partially purified normal rabbit γ globulin in solution or as an oil-in-water emulsion with acid-fast bacilli (Freund and McDermott, 1942).

Chromatography on DEAE cellulose

Whole serum or globulin fractions were chromatographed on diethylaminoethyl (DEAE) cellulose as described by Peterson and Sober (1956). The buffer solutions used for stepwise elution were 0.01 M Na phosphate, pH 7.5; followed by 0.02 M Na phosphate, pH 6.2; and 0.5 M NaH_2PO_4, pH 4.6. Three ml. serum, or the equivalent amount of γ globulin, were loaded on to 2.5 g-columns. The protein content of the eluted fractions was measured in terms of light absorption at 280 mμ. or, in some experiments described below, by measurement of ^{131}I radioactivity. Amino-acid analyses were kindly made by Dr. S. Jacobs, using the method of Moore and Stein (1954) on protein samples hydrolysed in 6N HCl at 105° for 30 hours.

Carbohydrate analyses

Were kindly made by Dr. H. R. Perkins. Total carbohydrate was determined as hexose by the method of Mokrash (1954), and hexosamine by Boas's (1953) modification of the Elson-Morgan reaction.

Radioactive iodine

Iodination with ^{131}I was performed by the ICl method of McFarlane (1958), the amount of iodine introduced being sufficient to give an average of 0.5–1 atom per mol. Radioactivity in samples was measured at a standard volume in a well-type scintillation counter. The efficiency of the counter was about 16 per cent, and no sample had less than twice the background radioactivity.

Sedimentation constants

Were determined in a Spinco analytical ultracentrifuge in 12 mm. cells in rotor head An 'A'. The temperatures during the runs were between 17° and 20.6°, and during any single run the maximum temperature variation was 2.2°. The rotor speed was 59,780 rev./min. Three runs were made at concentrations 0.39, 0.78 and 1.3 per cent in K phosphate buffer pH 7.8, I = 0.1 with NaCl added to give I = 0.2. Corrections for the effect of temperature on density and of the buffer salts were taken from Svedberg and Pedersen (1940), and the sedimentation constants were calculated by the method of Cecil and Ogston (1948).

Diffusion constants

Were measured by Dr. E. A. Caspary, the Lister Institute of Preventive Medicine. The Gouy interferometric method was used, as modified by Kegeles and Gosting (1947). The apparatus was that described by Gosting, Hanson, Kegeles and Morris (1949).

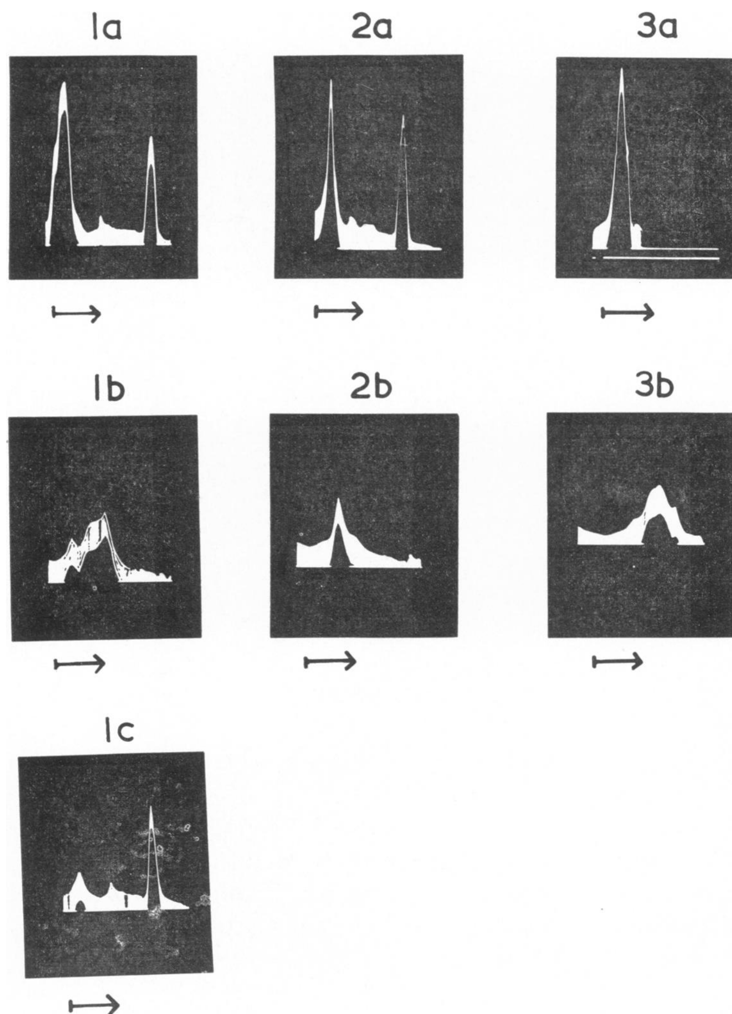

Fig. 1. Electrophoretic patterns of antisera against type III pneumococci.

Electrophoresis was carried out in a Perkin Elmer apparatus in veronal buffer, I = 0.1, pH 8.4 Arrows indicate position of starting boundary and direction of migration.

Column 1: Serum R 49/57 containing 50 mg. antibody per ml. 1(*a*) 2 hours; 1(*b*) 6 hours; 1(*c*) R 49 serum after removal of specific antibody by precipitation with type III pneumococcal polysaccharide run 1½ hours.

Column 2: Serum R 42/56 containing 32 mg. antibody/ml. 2(*a*) 1.5 hours; 2(*b*) 5 hours.

Column 3: γ-globulin concentrate (prepared from serum of R 109/55 by Na_2SO_4 fractionation, consisting of 70 per cent antibody precipitable with type III polysaccharide. 3(*a*) 1.8 hours; 3(*b*) 6.5 hours.

RESULTS

HETEROGENEITY OF γ GLOBULIN IN HYPERIMMUNE RABBIT SERA

Electrophoretic patterns and mobilities

In Fig. 1 are shown some of the pictures obtained by free electrophoresis of sera from hyperimmunized rabbits. By contrast with the single γ-globulin peak of normal serum these sera show 2 or 3 γ-globulin peaks. After removal of specifically precipitable antibody by addition of a small excess of pneumococcal capsular polysaccharides the γ-globulin peaks were much smaller (Fig. 1), but in some sera at the end of the run they were still obviously complex.

In Table 1 are listed the specific antibody contents of the sera examined, and the electrophoretic mobilities of the main γ-globulin peaks. Certain of these sera were selected for further examination of the physical and chemical properties of the γ globulins in them.

TABLE 1

MOBILITIES IN FREE ELECTROPHORESIS OF MAIN γ-GLOBULIN PEAKS IN SERA OF RABBITS HYPERIMMUNIZED AGAINST PNEUMOCOCCUS TYPE III

Rabbit No.	*Antibody content (mg./ml.)*	*Mobility in veronal buffer pH 8.6 $I = 0.1$ ($\times 10^{-5}$ cm.^{2}/v./sec.)*
R 49/57	50	1.03, 0.78, 0.42
R 49/57 (after removal of antibody by precipitation at equivalence)	—	1.14 (approx.) (heterogeneous)
R 42/56	32	1.17, 0.73, 0.54
R 46/56	27	0.92, 0.76, (0.58)
R 109/55	67	1.57, 1.37, 102
R 110/51*	19	1.46, 0.95
R 110/51* (after removal of antibody)	—	2.04 (approx.) (2 peaks)

* Run in phosphate buffer I = 0.2, pH 7.78 at room temperature, since cryoglobulin was also present.

Solubility in water

It was of interest to ascertain whether the fractions differed in water solubility. Serum 109/52 containing 82 per cent precipitable antibody, was fractionated with Na_2SO_4 by the method of Kekwick (1940) to obtain γ globulin. A portion of the γ globulin was dialysed at 4° for 3 days against three changes of 100 volumes distilled water saturated from time to time with CO_2. The sticky precipitate of water-insoluble 'euglobulin' was collected by centrifuging and was dissolved in 0.2 M phosphate buffer, pH 7.8, to give a slightly opalescent solution. The water-soluble 'pseudoglobulin' was perfectly clear. The water-soluble fraction comprised 54 per cent of the total protein, and of it 85 per cent was precipitable antibody. The electrophoretic pattern of each fraction resembled that of the parent material in having similar shouldered γ-globulin peaks, but the water-insoluble fraction (which contained 78 per cent precipitable antibody) showed in addition a small β-globulin component. There was no evidence to suggest that water solubility was related to the electrophoretic behaviour of the γ-globulin fraction or to its antibody content.

Properties of a cryoglobulin

The serum of R 110/51, taken 3 days after the last injection of pneumococci, contained 1.2 mg./ml. of a protein which precipitated at 2–4° and redissolved on warming to 37°.

Although the rabbit had received 500 i.u. heparin before bleeding, and the serum was obtained by allowing the plasma gradually to clot on standing, the cold precipitable protein was not a complex of heparin and altered fibrinogen of the type described by Thomas, Smith and von Korff (1954) as occurring in rabbit plasmas after administration of bacterial endotoxin. The protein was collected, and washed with 0.9 per cent (w/v) NaCl at 2°. It had the properties of a cryoglobulin, and contained antibody, which was precipitable at 37° by type III pneumococcus capsular polysaccharide (added in small amounts until no more precipitate was formed). In Table 2 the proportion of antibody in the cryoglobulin is compared with that in other globulin fractions obtained by precipitation with Na_2SO_4. It will be seen that the proportion was almost as great as that in other γ-globulin fractions, and the cryoglobulin must presumably have been formed in cells stimulated by the same process as other antibody-forming cells.

TABLE 2

ANTIBODY CONTENT OF CRYOGLOBULIN AND OTHER FRACTIONS OF SERUM 110/51

Fraction	*Total protein in fraction (mg./ml. original serum)*	*Percentage of protein precipitated by antigen*
Cryoglobulin	1.2	31
8–12% (w/v) Na_2SO_4 precipitate	23	42
12–13% ,, ,, ,,	5	43
13–16% ,, ,, ,,	11	50
16–18% ,, ,, ,,	2	39

Sedimentation constant and molecular weight

Examination of whole serum from R 49/57 and R 109/52 in the ultra-centrifuge at a protein concentration of 10 mg./ml. revealed no component sedimenting faster than the main γ-globulin peak. The water-soluble γ-globulin fraction from the serum of R 109/52 described above, of which 85 per cent was precipitable antibody, was examined in more detail. Although it contained two electrophoretically distinct γ globulins, it sedimented as a single sharp peak. The values for $S_{20.w}$ were 6.97, 6.69 and 6.35×10^{-13} at protein concentrations 0.39 per cent, 0.78 per cent and 1.3 per cent respectively.

The diffusion coefficient, measured by Dr. E. A. Caspary using the first interference minimum, was $D_{20.w} = 4.16 \times 10^{-7}$ cm. ^{2}sec. $^{-1}$ at protein concentration 0.42 per cent (w/v). Some deviation from ideal conditions was evident in the diffusion test, indicating a spread of molecular size. When the value for $D_{20.w}$ is combined with $S_{20.w} = 6.97 \times 10^{-13}$, using the value of 0.745 for the partial specific volume (Svedberg and Pedersen, 1940), the molecular weight is calculated at 159,000, the frictional ratio 1.418, and the axial ratio about 8 (assuming no hydration).

Chemical properties of γ-globulin fractions

Serum from R 49/57 was chosen for extended study. The serum was subjected to zone electrophoresis on a treated cellulose column as described under Methods, and the γ-globulin components were isolated in amounts sufficient for amino-acid and carbohydrate analyses, and for estimation of the proportion specifically precipitable with pneumococcus type III capsular polysaccharide. Fig. 2 shows the elution diagram of the γ globulin, and

the fractions selected are indicated. Table 3 summarizes the findings relating to antibody and carbohydrate contents of different fractions. The proportion of antibody was somewhat greater in the faster main fraction, but the difference between the main fractions was small. All had very similar antigen-combining ratios in the antibody precipitated at

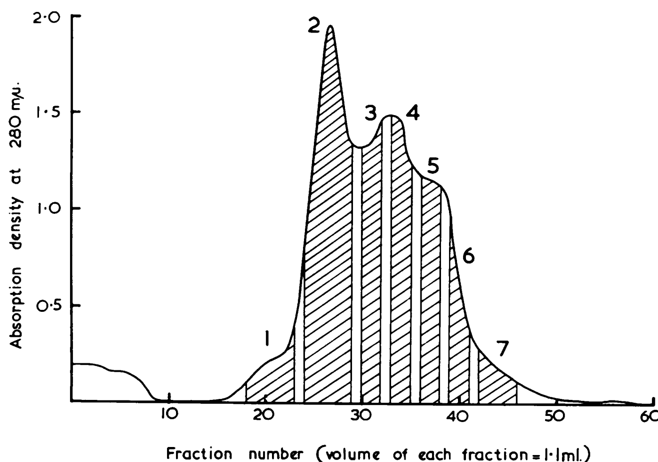

Fig. 2. Elution diagram of γ-globulin of rabbit serum containing 50 mg. antibody against type III capsular polysaccharide.
Zone electrophoresis was carried out on treated cellulose (Gedin and Porath, 1956) for 40 hours, using 0.075 M tris-maleate buffer pH 8.6 (see Methods section). The albumin, α and β globulins had been permitted to run off the column. 1.1 ml. fractions were collected. Shaded areas represent the samples pooled for fractions 1–7.

Table 3

CARBOHYDRATE CONTENTS AND PERCENTAGES OF TOTAL PROTEIN PRECIPITATE WITH PNEUMOCOCCUS TYPE III CAPSULAR POLYSACCHARIDE IN γ-GLOBULIN FRACTIONS OF SERUM OF R 49/57

Fraction No.§	*mg. protein/ml. serum*	*Percentage precipitable Ab.*	*Ab./Ag. ratio*	*Percentage hexose**	*Percentage glucosamine*†
1	3	n.t.‡	n.t.	2.3	n.t.
2	24	71	23	0.80	0.29
3	13·3	57	18	0.78	} 0·33
4	11	55	18	0.75	
5	7·3	53	21	0.85	n.t.
6	4·3	n.t.	n.t.	1.02	n.t.
7	1	n.t.	n.t.	4·5	n.t.

* Percentage hexose in another preparation of rabbit γ globulin, of which 27 per cent was *Ab.* = 0.75.
† Percentage glucosamine in another preparation of rabbit γ globulin, of which 27 per cent was *Ab.* = 0.18.
‡ n.t. = not tested.
§ Fractions prepared by zone electrophoresis on treated cellulose (see Fig. 2 for elution diagram).

the equivalence point. The four main protein fractions 2–5, each contained essentially the same percentages (about 0.8 per cent) of hexose and hexosamine. There was more hexose in the first and last fractions, but since these fractions were obtained by pressure dialysis of large volumes of column eluate, and contained only little protein, not too much reliance

can be placed on the values. The percentages of hexose and hexosamine reported by Smith, McFadden, Stockell and Buettner-Janusch (1955) for pooled rabbit antibody were 1.03 and 1.26 g./100 g. respectively. The value for hexosamine is four times that which we found, but this may be attributable to the fact that they used a method of estimation which does not involve prior removal of other substances which give a colour with the reagent.

TABLE 4

AMINO-ACID COMPOSITION OF γ-GLOBULIN FRACTIONS FROM R 49/57

Amino acid	Percentage of total nitrogen		
	*Rabbit antibody**	*Fraction 2*	*Fraction 5*
Aspartic	6.38	5.6	6.5
Threonine	9.69	9.1	11.0
Serine	9.13	8.5	7.55
Glutamic	7.0	6.5	6.6
Proline	6.5	6.0	5.1
Glycine	6.7	5.8	5.1
Alanine	5.56	4.7	4.7
Cystine	2.25	1.95	1.1
Valine	7.6	6.6	6.9
Methionine	0.81	0.74	0.6
Isoleucine	2.94	2.5	2.4
Leucine	5.25	5.7	4.75
Tyrosine	3.19	2.9	2.65
Phenylalanine	2.94	2.6	2.4
Hydroxylysine		0.4	
Lysine	7.75	8.31	9.8
Ammonia	6.7	8.36	9.8
Histidine	2.56	3.1	2.6
Arginine	10.13	10.1	9.3

* Average figures for rabbit antibody against pneumococcus types I, VII, VIII and XIV, taken from Smith *et al.* (1955).

Each set of figures is the average of two complete analyses.

Fractions 2 and 5 were prepared by zone electrophoresis (see Fig. 2).

Portions of two well separated fractions (2 and 5) were hydrolysed in 6 N HCl for 30 hours and their amino-acid composition was analysed. The results are given in Table 4, in which are included for comparison the figures obtained by Smith *et al.* (1955) on a pool of rabbit antibody against various pneumococcal types. The differences between the amounts of amino acids, with the exception of cystine (whose recovery after hydrolysis is unreliable), in the two fractions are less than 20 per cent. The significance of differences of this order in amino-acid composition is difficult to assess from our data, since the duplicate columns showed 10 per cent differences for some of the amino acids. To account for the considerable difference in electrophoretic mobility it might have been expected that the sums of the dicarboxylic or the basic amino acids would vary between the fractions but no such variation was evident. The agreement with the earlier analyses of Smith *et al.* (1955) is close.

Chromatographic examination

Chromatography on DEAE cellulose provides a distinctly different method of fractionating γ globulin, and the serum of R 49/57 was studied by this technique. The amount

of antibody and γ globulin in some of the eluted fractions was too small to be measured by quantitative precipitation, and by absorption of the protein at 280 mμ. Antibody was therefore determined by the less accurate but more sensitive haemagglutination method

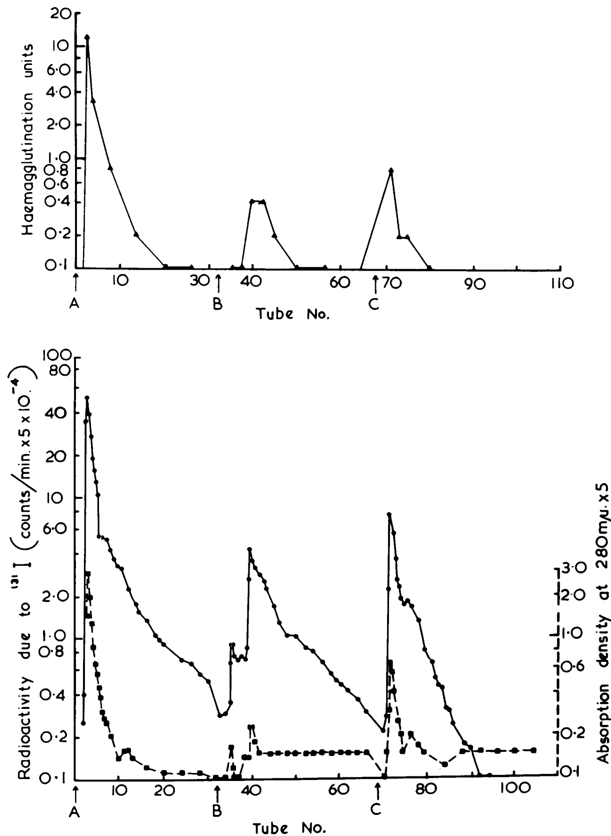

FIG. 3. Distribution of γ globulin and antibody in chromatographic fractions of γ globulin of serum R 49/57.

γ globulin, prepared by zone electrophoresis, was labelled with ^{131}I. A 15-mg. portion was applied to a DEAE cellulose column, and eluted with (A) 0.01 M phosphate buffer, pH 7.6; (B) 0.02 M phosphate buffer, pH 6.4; (C) 0.5 M NaH_2PO_4, pH 4.6.

In each 3-ml. fraction the protein concentration was measured by absorption at 280 mμ., and the total ^{131}I counts measured in a scintillation counter. Selected fractions were titrated by haemagglutination for antibody against type III pneumococcus capsular polysaccharide, and the antibody content expressed as 'units'/ml. (1 unit/ml. = concentration giving complete haemagglutination at 1/10, but not at 1/20, dilution).

N.B. The ordinate scales are logarithmic, for convenience of presentation.

▲——▲ Haemagglutination units.
●——● Radio activity due to ^{131}I.
■ - - - - ■ Absorption density of protein at 280 mμ.

and the presence of γ globulin was measured in the following way. Five ml. whole serum was fractionated by zone electrophoresis on a column of treated cellulose and the protein, eluted after the β-globulin peak was combined and concentrated by dialysis under pressure. The material was next iodinated with ^{131}I, as described above, and remaining traces of

free iodide removed by dialysis. The specific activity was about 25 μC/mg. Only γ globulin was present, as judged by paper electrophoresis, and radioactivity was confined to the γ-globulin area. This radioactive γ globulin was now subjected to chromatography on DEAE cellulose (see Methods); (*a*) 15 mg. alone; (*b*) after mixing 26 mg. with 2.5 ml. of normal unlabelled rabbit serum. The absorption at 280 mμ. and radioactivity of the eluted

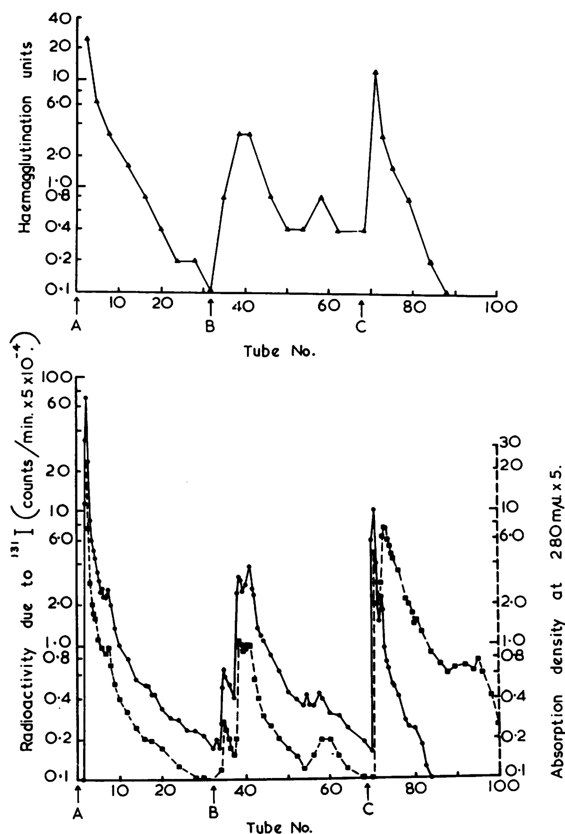

FIG. 4. Distribution of γ globulin and antibody in chromatographic fractions of total γ globulin from serum R 49/57 mixed with normal rabbit serum.

Details as for Fig. 3, except that 26 mg. ^{131}I-labelled total γ globulin was mixed with 2.5 ml. unlabelled normal rabbit serum before chromatography. The ^{131}I radioactivity has diminished by a factor of 0.6, owing to radioactive decay.

▲——▲ Haemagglutination units.
●——● Radioactivity due to ^{131}I.
■ - - - ■ Absorption density of protein at 280 mμ.

material in each tube were measured, and selected tubes titrated for antibody content. At the end of the experiment the radioactivity remaining on the column was also estimated, and in each case 97–98 per cent of the radioactivity had been recovered in the eluted fractions. On the assumptions that only γ globulin had been iodinated, and that the specific radioactivity of all the γ globulin was uniform, the radioactivity measurements should indicate radioactive γ globulin present in the fractions.

The results of the two experiments are shown in Figs. 3 and 4. After the first main peak several further γ-globulin peaks emerged under conditions of pH and ionic strength which elute also albumin and α globulin if whole serum is present. Antibody against pneumococcus capsular polysaccharide was present in all fractions containing radioactive protein. Furthermore the distribution of radioactivity and of antibody activity was closely similar in each experiment. The separations achieved must therefore depend upon properties of the γ-globulin molecules themselves, and not upon interaction with other plasma proteins or upon partial denaturation of γ globulin on the column, in the absence of other proteins.

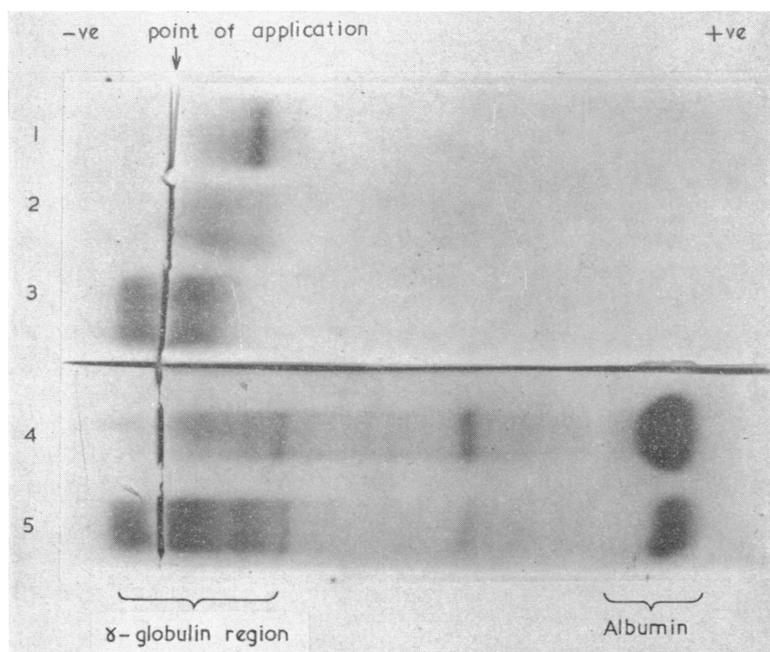

FIG. 5. Starch-gel electrophoresis.
1, 2 and 3 represent the three main antibody fractions obtained by chromatography of serum from R 49/57 on DEAE cellulose (see Fig. 3).
4 = Normal rabbit serum.
5 = Serum R 49/57 containing 50 mg. of antibody against type III pneumococcal polysaccharide.

As an additional safeguard to ensure that the process of separation and iodination had not significantly altered the properties of the γ globulins, a further experiment was carried out in which 26 mg. [^{131}I] γ globulins were added back to 2.5 ml. of unlabelled serum from R 49/57, and the chromatography repeated. The overall distribution of antibody was similar to that in the other experiments, and furthermore the antibody content of all the fractions bore an approximately constant relationship to their radioactivity. This indicates that radioactive and unlabelled antibodies were fractionated together.

When whole serum (R 49/59) and three main antibody fractions from the DEAE column (see Fig. 3) were examined by electrophoresis in starch gel (Smithies, 1955) in 0.03 M tris-borate buffer (Aronsson and Grönwald, 1957) it was found that the whole antiserum showed at least three distinct bands in the γ-globulin region. The antibody-containing fractions were not homogeneous and contained several bands (Fig. 5). The fact that the

behaviour of DEAE cellulose fractions differed on starch gel confirms that the DEAE cellulose column fractionates molecules which have different properties.

Gel diffusion studies

The different γ-globulin fractions were examined by the double diffusion technique in agar gel (Ouchterlony, 1948) against various antisera, using several different concentrations of antigen. With hen antisera all fractions gave single lines, which fused and showed reactions of identity. With goat antiserum the electrophoretic fractions 2, 3+4, 5+6 (see Fig. 2) gave lines which were usually single, although under conditions of antigen excess they had a complex structure, and were identical. With guinea-pig antiserum all the rabbit

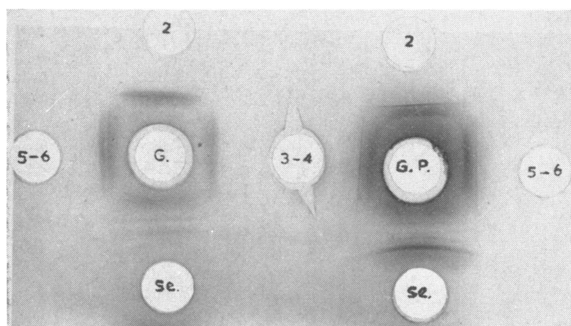

FIG. 6. Double diffusion analysis in agar of electrophoretic fractions from R 49/57.
G = goat antiserum with approximately 1 mg./ml. anti-rabbit γ globulin.
G.P. = guinea pig antiserum with approximately 1 mg./ml. anti-rabbit γ globulin.
Se = whole rabbit serum diluted 1:10.
2, 3–4, 5–6 = electrophoretic γ-globulin fractions (see Fig. 2) containing 1.5 mg. protein/ml.
The agar was washed and the precipitin lines were stained before photography.

γ-globulin fractions gave three precipitation lines all of which were common to each fraction but appeared to be present in different proportions (Fig. 6). Although it is not impossible that such apparent antigenic heterogeneity is due to the presence of traces of material unrelated to γ globulin, such as β globulin, it must be stated that we found no other evidence of such material. The fractions obtained by chromatography on DEAE cellulose behaved similarly to those obtained by zone electrophoresis.

It appears, therefore, that the rabbit γ globulin was immunologically heterogeneous, but the heterogeneity was not obviously related to the electrophoretic or chromatographic properties of the γ globulin. A similar situation has been observed with comparable fractions of human γ globulin prepared by chromatography on DEAE cellulose (Humphrey and Porter, unpublished).

DISCUSSION

By 'γ globulin' we refer in this paper to proteins which migrate more slowly than the visible β-globulin peak on free electrophoresis. Although immunoelectrophoresis of rabbit

serum showed traces of antigenic γ globulin to extend into the region of β globulins, the bulk of the antibody, although heterogeneous, fell into the definition given above.

We examined the sera of rabbits immunized with protein antigens, with antibody contents up to 20 mg./ml. On free electrophoresis the γ-globulin boundaries were broad but no definite peaks with different mobilities were observed. This may mean that distinct peaks occur only in the sera of rabbits whose γ globulin has been elevated by immunization above a certain level, not reached with protein antigens, or that pneumococcal antigens are peculiar in stimulating synthesis of unusual γ globulins. Since rabbit γ globulin is, in any event, heterogeneous, and the γ globulins which we studied, apart from the cryoglobulin, were not abnormal in their physical, chemical, or immunological properties, it seems probable that the distinct peaks represent exaggerations of normal rather than the consequences of abnormal antibody or γ-globulin production mechanisms.

The possible ways in which the chromatographic and electrophoretic complexity of γ globulin may come about have been discussed by Askonas, Humphrey and Porter (1956). From studies of synthesis by tissue slices, and fractionation of the γ globulin by partition chromatography, they concluded that the most likely explanation was that the population of cells synthesizing γ globulin was heterogeneous, and that the proportions of different cells might vary from time to time and from tissue to tissue as a result of antigenic stimulation. Such an explanation is consistent with our present observations, although these in no way prove its correctness. It is interesting that the proportion of specific antibody in the different γ-globulin fractions of the serum which was examined in detail was much the same, even when the fractions were obtained by chromatography on DEAE cellulose. Similar results have been obtained with antiserum from a rabbit hyperimmunized with alum-precipitated crystallized ovalbumin (Farthing, unpublished). These observations contrast with those of Fahey (1960), who examined the distribution of different antibodies in human sera fractionated in DEAE by similar methods and found that different antibodies occurred predominantly in different chromatographic fractions. Stelos and Talmage (1957) working with rabbits also found that different antibodies evoked by immunization with sheep erythrocytes were separable by zone electrophoresis in a starch block. The explanation probably lies in the fact that the rabbits whose sera we studied had been immunized with two or more courses of repeated injections of antigen, and consequently all potential γ-globulin forming cells had been stimulated maximally by the antigen, and a similar proportion of all the hypothetical cell types had responded by antibody formation.

Müller-Eberhardt and Kunkel (1956) found that the electrophoretic mobilities of normal and abnormal globulins, from sera of patients with multiple myeloma, were correlated with their carbohydrate content – those globulins with a higher proportion of carbohydrate (hexose and sialic acid) having the greater mobilities. Fahey and Horbett (1959) have found a similar correlation between carbohydrate content and electrophoretic mobility of normal human serum γ-globulin fractions obtained by chromatography on DEAE cellulose. We expected to find significant differences in the carbohydrate contents of the rabbit γ-globulin fractions, but no such differences were detected, except in the extreme fractions which contained very little protein. Furthermore, there were only very minor differences in the overall amino-acid compositions of the two fractions examined. Despite their markedly different electrophoretic mobilities, it is important to remember that even these fractions were still heterogeneous. The electro-

phoretic differences must presumably depend on differences in order and/or folding of the amino-acid chains, or linkage of some other substance like sialic acid to the amino acids. Such differences do not seem to be connected with the antigen-binding sites, since both the proportion of specific antibody to other γ globulin, and the immunological character of the antibody was similar in each fraction. A fuller understanding of this problem probably depends upon enzymic degradation of the γ globulin to smaller sub-units, on the lines indicated by Porter (1959).

The appearance in one rabbit of a cryoglobulin, part of which was identifiable as specific antibody, is of considerable theoretical interest. Cryoglobulins have been described as occurring in sera of patients suffering from a variety of diseases associated with high plasma γ-globulin levels, but it has not been possible to show that they were antibodies – even when known antigens have been administered to such patients. The fact that antibody cryoglobulin was formed in an animal during hyperimmunization suggests that cryo-globulins also may be the products of exaggeration of a normal synthetic mechanism, rather than new and abnormal proteins.

The capsular polysaccharide of type III pneumococci is a polymer of [3β-O-glycosyl-4β-O-glucuronosyl]-units, with little or no branching (Reeves and Goebel, 1941). It is thus antigenically rather simple, in contrast to proteins, which have a wide variety of potential antigenic determinants. Consequently, all, or most, of the antibody molecules identified by their ability to combine with the capsular polysaccharide probably do so by virtue of similar specific groupings.* For this reason, our finding that antibody against this antigen can be present throughout all the population of γ-globulin molecules excludes the possibility that the heterogeneity of normal γ globulin is due to folding of identical molecules in different ways, complementary to different specific antigenic groupings. Furthermore the observation that as much as 85 per cent of the γ globulin may be composed of antibody molecules with similar specificities, and that these are distributed throughout the whole range of γ globulins, has implications for theories of antibody formation. If, as is postulated by clonal selection theories (Burnet, 1959; Lederberg, 1959) globulin-forming cells arising from a given clone are genetically determined to make at the most two unique forms of globulin molecule, then the great majority of such cells must have been making a single form, namely specific antibody against the repeating unit in the type III pneumococcus capsular polysaccharide. Furthermore, these cells must have been originally present, or have become represented by mutation, in the cell populations which make all the physico-chemically different kinds of γ globulin discussed above. 'Instructive', as opposed to 'selective', theories make less specific predictions about the number of types of molecule which a cell synthesizing γ globulin may make, and for them the implications are less clear. Thus if the instruction to make antibody is passed from one type of cell to another – say from macrophage A to plasma cell precursor B — cell B could presumably belong to any of the families of cells making any of the physico-chemically different γ globulins. Alternatively, if antibody formation results from a change in the globulin-forming cell (or its precursor) induced directly or indirectly by ingestion of the antigen there is no reason, in our present state of knowledge, to suppose that any one kind of potential γ-globulin-forming cell would be stimulated in preference to another. Finally, there is nothing in 'instructive' theories to prevent a cell from making several physico-chemically distinct γ globulins, all or any of which might be specific antibody.

* When tested, for example, for cross-reactions with type VIII capsular polysaccharide (Squibb 194, kindly provided by Dr. Michael Heidelberger) less than 1% of the antibody was precipitated after 7 days at 2°.

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