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Immunochemical Studies of Human Serum Proteins

ANTIGENIC INHOMOGENEITIES OF THE GAMMA GLOBULINS, THEIR SUBUNITS AND NUMBER OF DETERMINANTS AND THEIR RELATION TO A WALDENSTRÖM MACROGLOBULIN

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Summary. Human γ globulins are shown by means of special antisera and papain digestion to consist of three to five different species which share only some of their determinants. A minimum total number of eight determinants is suggested. Only four of these determinants are possessed by the bulk of the serum globulins and one of these four determinants is shown to be shared with the cryoglobulin of a Waldenström serum. This cryoglobulin is shown to have a further minimum of two determinants shared with one of the subsidiary γ globulins occurring in normal human sera, as well as at least one specific determinant not found in normal sera. Other macroglobulinaemic sera are shown to have variable amounts of globulins antigenically related to this cryoglobulin and even normal sera appear to have traces of such macroglobulins. All γ globulins appear to be split more readily than other serum proteins, but the Waldenström macroglobulin more readily still. It is suggested that the slight forking of the γ-globulin line often seen in immunoelectrophoretic patterns near the cathode side may be due to the existence of antigenically related, but not identical, γ globulins whose range of electrophoretic mobilities does not quite overlap there and may vary in different subjects. The previously suggested spontaneous or enzymatic cleavage remains another possible explanation.

INTRODUCTION

Immunoelectrophoretic studies have shown that γ globulins cannot be distinguished from other proteins solely on the basis of electrophoretic mobility (Grabar, 1959). Since antisera produced by injecting rabbits, horses, goats and other animals with human sera or purified γ globulins form a very long, simple, unbranched line, which extends from the lowest electrophoretic mobility to the zone of α globulins immunologists have considered that the globulins with which this line is formed are a family of γ globulins, that are antigenically identical although their electrophoretic mobilities are spread over a wide range.

In this paper human γ globulins which give a single γ globulin line with the usual antisera will be shown to consist of at least three immunologically distinguishable, although related families of proteins.

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Oudin (1956 a, b), Dubiski, Dudziak and Skalba (1959), Dray and Young (1959) and others have shown slight but constant antigenic differences between the γ globulins of individual rabbits. This suggests that individuals of the same species may have γ globulins of a slightly different determinant structure, but does not tell us whether the γ-globulin molecules of a single individual are all antigenically alike or must be thought of as several species of molecules that are antigenically only partially related or unlike.

There have been suggestions that the γ globulins of a single individual are antigenically not completely alike. Immunoelectrophoretic patterns with lines parallel to the usual γ-globulin line have been reported, e.g. by Goodman (1960) who found in certain human sera a second line by means of fowl antisera for human sera; and we discovered three lines in place of the usual single γ-globulin line by means of a rabbit antiserum for a Waldenström macroglobulin (Augustin and Hayward, 1960a). Since then Edelman, Heremans, Heremans and Kunkel (1960), in an immuno-chemical study similar to ours, found one additional γ-globulin line.

The present work has already been reported (Augustin and Hayward, 1960b) but was not published in detail. It is concerned with the demonstration of three immuno-chemically distinguishable species of normal human γ-globulin molecules, the enzymatic cleavage of two of these and attempts to establish a minimum number of determinants for human γ globulins.

In a previous publication (Augustin and Hayward, 1960a) our finding two lines parallel to the usual γ-globulin line with a special antiserum (R89) was interpreted to mean that possibly even the purest γ-globulin preparations may be contaminated with proteins other than γ globulin which happen to have mobility ranges similar to those of the γ globulins and do not usually induce antibody formation. The present communication will show that the three lines we found are formed by the usual 7S γ globulins which give no more than one line with the usual antisera. Relationships between normal and abnormal serum γ globulins were uncovered incidentally and it is suggested that the lability of highly purified reagins (the skin-sensitizing non-precipitating antibodies occurring in subjects suffering from asthma and hay fever) is due to their being γ globulins and therefore likely to suffer easily enzymatic — and perhaps spontaneous — cleavage like other γ globulins (Augustin and Hayward, 1960c).

MATERIALS AND METHODS

HUMAN γ GLOBULINS. These were prepared by ion exchange chromatography on DEAE-cellulose, as described for reaginic sera (Augustin and Hayward, 1960c). Fractions eluted at pH 7.4 with sodium phosphate (50 ml. of 0.01 M NaH_2PO_4 to about 36 ml. of 0.01 M NaOH) consisted of pure γ globulins; they formed in immunoelectrophoresis a single line of precipitate in the γ-globulin position when antisera against whole human serum, such as R72, were used for development. Rabbits immunized with the first pH 7.4 eluate yielded antisera (R92) giving a single line with whole human serum. It was possible to select γ globulins with special ranges of mobility, as previously shown for reaginic sera (Augustin and Hayward, 1960c); one such preparation of medium fast γ globulins is largely used in the present experiments; ultracentrifugation showed them to be 7S globulins.

MACROGLOBULINS. The serum of a subject (Redwood) suffering from Waldenström's disease was ultracentrifuged for 5 hours at 40,000 rev./min. The pellet was then

redissolved in buffer and this process was repeated four times, according to the method described for the attempted isolation of a 19S reagin (Augustin and Hayward, 1960c); this macroglobulin was a cryoglobulin. We are indebted to Dr. Kawerau of St. Mary's Hospital for its preparation.

MACROGLOBULINAEMIC SERA. We thank Dr. Kawerau for supplying the sera — Redwood, Walkoff, Blindert and Smith.

ANTISERA. Rabbits were immunized with whole human serum, pure γ globulin and the macroglobulin fraction described above, by means of a multiple injection technique (Hayward and Augustin, 1957), yielding immune sera R72, R92 and R89 respectively. The particular R89-bleeding used in the present investigation did not have antibodies specific for β_2M, although later bleedings from the same rabbit did have such antibodies.

ENZYMIC DIGESTION. This was carried out as described by Porter (1959) using 1 mg. of a commercial preparation of crystalline papain (Armour Ltd.) per 100 mg. of protein.

GEL DIFFUSION TESTS. Micro-Ouchterlony patterns were formed on suitable glass sheets, in flat Petri dishes or on microscope slides by punching holes into agar-gel layers of 1–1.5 mm. thickness. One per cent solutions of agar (New Zealand, pure granulated, Davis Ltd., N.Z.) in 0.9 per cent saline was employed as in previous investigations (Hayward and Augustin, 1957). The diffusion patterns were allowed to develop in moist chambers at room temperature.

IMMUNOELECTROPHORESIS. The modification of Scheidegger's micromethod described previously (Augustin and Hayward, 1960c) was used. The electrode vessels were charged with a mixture of 0.05 M Veronal buffer (2 parts — 0.5 M was quoted in error in the previous publication, Augustin and Hayward, 1960c) and 0.1 M sodium borate buffer (1 part), both of pH 8.6. The Veronal buffer was made up from 20.62 g. sodium barbiturate and 3.68 g. barbituric acid in 1 litre of distilled water and the boric-acid buffer by adding 24 ml. of 0.1 M NaOH to each 100 ml. of 0.1 M boric acid. Both buffers were saturated with chloroform to prevent bacterial contamination. Microscope slides were covered with a thin film of 0.2 per cent aqueous agar, followed after drying by 2 ml. of a mixture of liquified 2 per cent aqueous agar (containing 0.02 per cent sodium-azide) and 0.05 M Veronal buffer (equal volumes). The apparatus described previously was used for the electrophoresis, which was allowed to proceed for 1 hour (rather than 45 minutes quoted previously) at 6 volts/cm. Under these conditions we usually registered a current of about 20 milliamps.

Antigen-antibody precipitation lines usually began to appear a few hours after the antisera had been added. They were drawn, photographed and stained (amido black according to Scheidegger, 1955) after development for 24–36 hours.

RESULTS

IMMUNOELECTROPHORETIC ANALYSES WITH ANTISERA R72 AND R92

Changes on Keeping

Fig. 1 gives the immunoelectrophoretic patterns of two normal human sera, the lower showing a slight forking of the γ-globulin line on the cathode side. Such slight forking must have been seen by many people during the routine examination of sera, but has probably always been dismissed as an 'artefact'. However, when examining more carefully sera that had been stored (4°), we found an increasing tendency to such forking in

the sera that had been stored the longest. The phenomenon proved to be reproducible; sera showing forkingonce always did so on future occasions, whereas normal (unhaemo-

FIG. 1. Immunoelectrophoretic patterns of two normal sera, the lower showing slight forking of the γ-globulin line on the cathode side.

lysed) sera, prepared from freshly collected blood and examined within 48 hours, never did; in fact, out of thirty-four sera stored up to 4 months at 4° only one showed slight forking; incubating fresh sera at 37° for 72 hours did not produce any forking, nor

TABLE I

VARIATIONS IN IMMUNO-ELECTROPHORETIC PATTERNS OF 'NORMAL' SERA

	Numbers examined	*Storage years*	ρ	β_2M	*Post γ or 'forking'*
	40	0–1	2	33	3 (slight)
	20	5–6	18	16	12 (pronounced)
Total	60	0–8	20	49	15

did intermittent violent shaking at room temperature for 48 hours. Out of a total of fifty-four randomly selected, fresh and stored routine samples of sera, thirteen showed a forking of the γ-globulin line. This is comparable to fifteen out of sixty of another series,

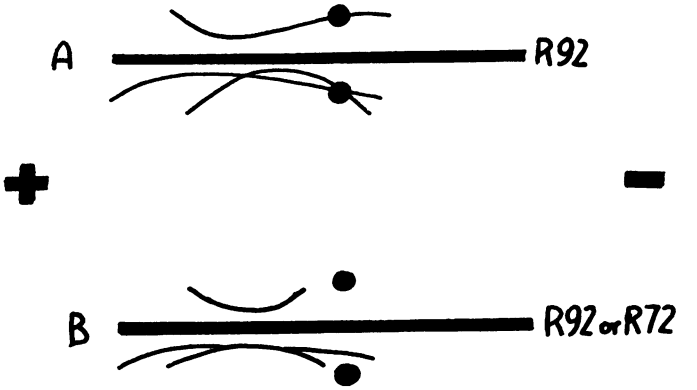


FIG. 2A. Immunoelectrophoretic patterns of a fresh normal serum (above) and one that had been stored for many years at 4° (below).
FIG. 2B. Immunoelectrophoretic pattern of a freshly isolated γ-globulin preparation and a similar one after storage for 8 weeks at 4°.
Patterns developed with antiserum for whole human serum (R72) and to immunoelectrophoretically pure γ globulin (R92). Note that R92 contains antibodies for γ globulins only.

of which the times of storage were known and are tabulated together with the appearance of cleavage in the γ globulin (Table I). In one of the oldest sera the γ globulins appeared to be completely cleaved into two parts (Fig. 2A). The table shows also that with anti-

serum R72 the β_2M line could be discovered in only forty-nine out of the sixty sera. Variations were noted, but not examined in detail, amongst the α-globulins of these sera. Table 1 records also the frequency with which a pre-albumin line was found; it always appeared as a very strong, easily discernible line and is referred to as ρ line since it is most probably identical with the ρ line reported by Grabar (1959).

Two γ-globulin preparations obtained by fractionating serum on DEAE-cellulose (Augustin and Hayward, 1960c) originally gave only one line when tested with antiserum R72 or with a rabbit antiserum specific for globulins only (R92). However, after only a few weeks at 4° the immunoelectrophoretic patterns showed three partially related γ-globulin lines (Fig. 2B). Similar patterns were later obtained after enzymic digestion (Fig. 4).

IMMUNOELECTROPHORETIC AND OUCHTERLONY ANALYSES WITH ANTISERUM R89

The Antibodies in Antiserum R89

When we examined normal and macroglobulinaemic sera immunoelectrophoretically by means of R89, we found that three lines parallel to each other were always obtained in the γ-globulin position (Fig. 3A), instead of the usual single line (as obtained with R92 and R72 and numerous other anti-human sera and anti-human-γ-globulin sera tested by us), the middle line always being much thicker and less sharp than the other two. These three parallel lines indicate the presence of at least three different types of protein molecules in the γ-globulin range of normal, as well as abnormal, sera. It is not possible to say from Fig. 3A (and 3B) whether any or all of the three lines are identical with the single line formed by the γ globulins with R72 and R92.

R89 can further be seen to possess antibodies for one α globulin, presumably (from its method of preparation) α_2M globulin. Cryoglobulinaemic sera usually caused the deposition of a thick precipitate around the point of application of the serum. Realizing that this was due to the low ionic strength used in the gel we increased the ionic strength four-fold to 0.1 and obtained the pattern given in Fig. 3B. In this particular gel pattern the weakest of the three lines could not be seen, as happened on other occasions without increase of ionic strength. Increased electroendosmosis led to a slight displacement of the lines, but otherwise the patterns of Fig. 3A and 3B are similar (note, however, the slight displacement in both of the mobility ranges of 'Redw' compared with the normal serum RA); the macroglobulin had remained in solution (Fig. 3B) and can be seen to have formed a well-defined strong line on the cathode side in Fig. 3B (Redw). The normal serum (RA) gave a slight haze of a line in a similar position, which does not appear to be in the β_2M position. Since later bleedings of rabbit R89 gave heavy lines in the usual β_2M position, one may conclude that serum R89 used here did not have antibodies specific for β_2M.

R89 thus contained antibodies for serum proteins in the γ-globulin position, for α_2M globulin and for macroglobulins roughly in the β position (Fig. 3B) and only the faintest traces of antibodies against other serum proteins. It was at this stage not possible to say whether R89 had antibodies for normal γ globulins, i.e. for those giving the unbranched single line with the usual anti-human sera.

Fig. 3C gives an Ouchterlony comparison of normal (Augustin and Demmy) and macroglobulinaemic (Smith, Blindert, Walkoff and Redwood) sera in terms of R89. Lines of variable density can be seen to have formed nearest the antigen cups and some of these lines are only strong with the macroglobulinaemic sera; these lines must therefore be due to the special macroglobulins in approximately the β_2M position in the immunoelectro-

phoretic analyses (Fig. 3B). Since the normal sera (Augustin and Demmy) contain faint traces of lines related to the thick lines of macroglobulinaemic sera it is possible that normal sera contain traces of macroglobulins of which the macroglobulinaemic sera contain large amounts. Other workers have pointed out that abnormal macroglobulins are partially related to the β_2M globulins of normal sera, but this has not been investigated in the present study.

All the sera in Fig. 3C share a sharp line near the antigen cups, seen either inside or

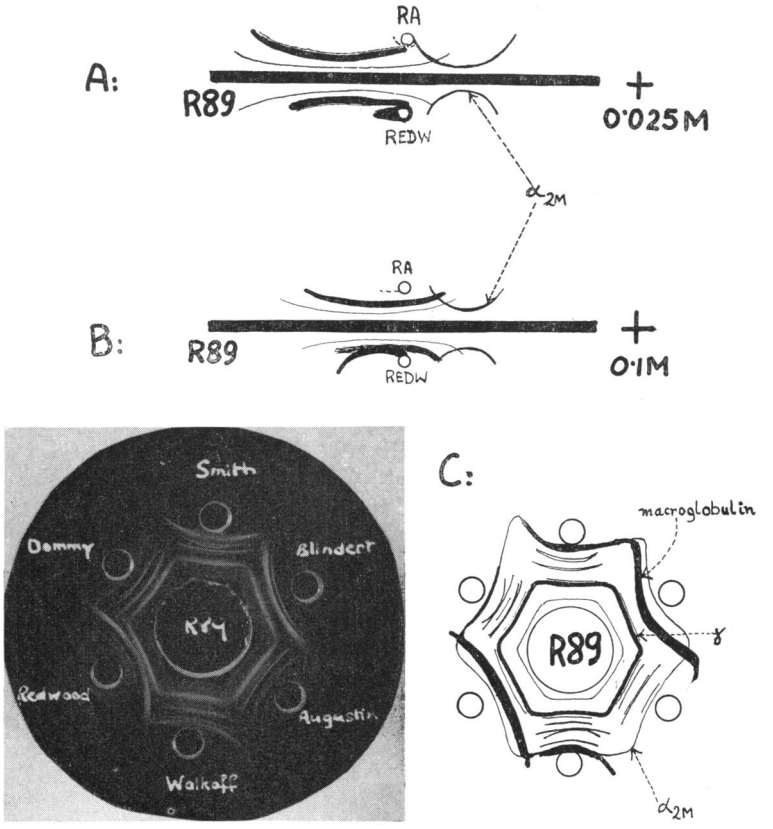


FIG. 3, A and B. Immunoelectrophoretic patterns of a normal human serum (RA) and the serum of a Waldenström patient ('Redw') developed with R89.
A: agar gel made up in 0.025 M Veronal buffer
B: agar gel made up in 0.1 M buffer
C: Ouchterlony comparisons of four macroglobulinaemic sera (Smith, Blindert, Walkoff and Redwood) with two normal human sera (Augustin and Demmy) in terms of R89.

outside the line formed by the other macroglobulins, close to them and of equal density with all the sera. This line is, therefore, likely to be due to α_2M globulin. There is another strong inner line of even density, common to all the six sera, which is flanked by two thin lines, the inner thin line nearest the antibody cap being much better defined than the outer. The thick line is therefore most probably identical with the thick γ-globulin line (present in normal and abnormal sera) of the immunoelectrophoretic patterns (Fig. 3 A and B). Some of the sera gave additional lines between the γ-globulin and the macroglobulin lines, which have not been investigated.

THE PRODUCTS OF HYDROLYSIS WITH PAPAIN

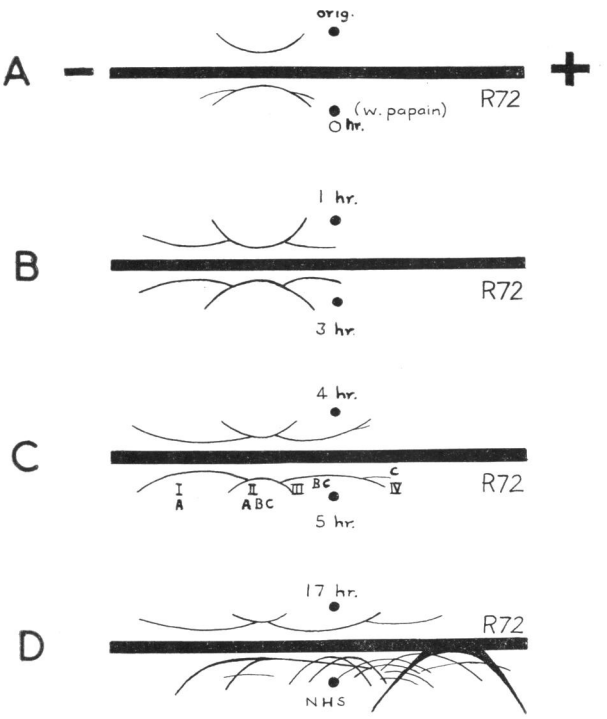
Evidence of Three Determinants in normal γ globulin

About 30 mg. of electrophoretically medium fast γ globulins in 1.5 ml. saline were mixed with 0.35 ml. of 0.63 M sodium phosphate buffer of pH 7.0 containing 0.06 M cysteine and 12.5 mM ethylene-diamine tetra-acetate (EDTA). To this 0.15 ml. of the papain suspension (2 mg. per ml.) was added. For the control, 10 mg. of the same globulin preparation in 0.5 ml. saline were mixed with 0.12 ml. of the buffer, the enzyme being replaced by an equal volume of saline. Both mixtures were incubated at 37°. Samples were withdrawn immediately after the components were mixed, before the incubation began (time 0), after 1, 2, 3, 4, 5, 17 hours and 1 week of digestion with sterile precautions; immunoelectrophoretic analyses were performed on these samples immediately after their withdrawal. No interference due to the presence of enzyme, cysteine or EDTA could be observed; control samples, after 5 and 17 hours gave exactly the same immunoelectrophoretic patterns before and after the removal of cysteine and EDTA by dialysis against distilled water in the cold and further inactivation of the enzyme by oxidation by aeration. However, slight forking at both ends of the γ globulin line was already observed in the sample that had been taken immediately after adding the papain (but not in the controls containing cysteine and EDTA alone); although digestion may in fact have started immediately after addition of the enzyme, the possibility of some little residual digestion during the electrophoresis cannot be excluded.

The immunoelectrophoretic patterns of the digests given in Fig. 4 have been developed with R72, R92 and R89. We had the impression that the post-γ-globulin line (component I) formed rather earlier than the pre-γ-globulin line (component III), and it can be seen that the post- and pre-γ-globulin increased during the digestion at the expense of the middle component (II). The latter continued to occupy a position compatible with its being identical with the original γ globulin. After 4 hours at 37° a further component (IV) began to appear on the anode side. It continued to get stronger during further digestion, but even after 17 hours it was not as strongly developed as the post- and pre-γ-lines. After 48 hours of digestion (Fig. 4H) the middle piece (II) was still left, while the component IV had completely disappeared. It was necessary to continue the digestion for a whole week for the middle piece to disappear completely (Fig. 4H, top).

Rabbit immune serum for γ globulin alone (R92) gave patterns identical with those obtained with R72, except that IV was always missing, i.e. R92 must have lacked antibodies specific for IV which were present in R72. Further, since R92 as well as R72 gave lines I, II and III, and since III and IV can be seen to give a reaction of partial identity (Fig. 4 D, F, G), it follows that the additional antibodies in R72 must be directed to one — or several — determinants common to III and IV. Further, III must have at least one other determinant which it shares with II (reaction of partial identity in Fig. 4D) and to which R72 and R92 both have antibodies. This gives, therefore, a minimum of two determinants for III, at least one of which is shared with IV.

The γ globulins appear to be the most easily attacked serum proteins. No changes other than in the γ globulins were observed after digestion for as long as 4 hours. After 17 hours digestion of whole serum pictures like those of Fig. 4K were obtained, i.e. some α globulins and probably albumin appear to be split, whereas the γ globulins can be seen to have been dissociated completely into two parts (compare with Figs. 4J and 2A). This complete splitting only occurred after a week's digestion of the isolated γ globulin (Fig. 4H); since we always used the same amount of papain per milligramme of protein



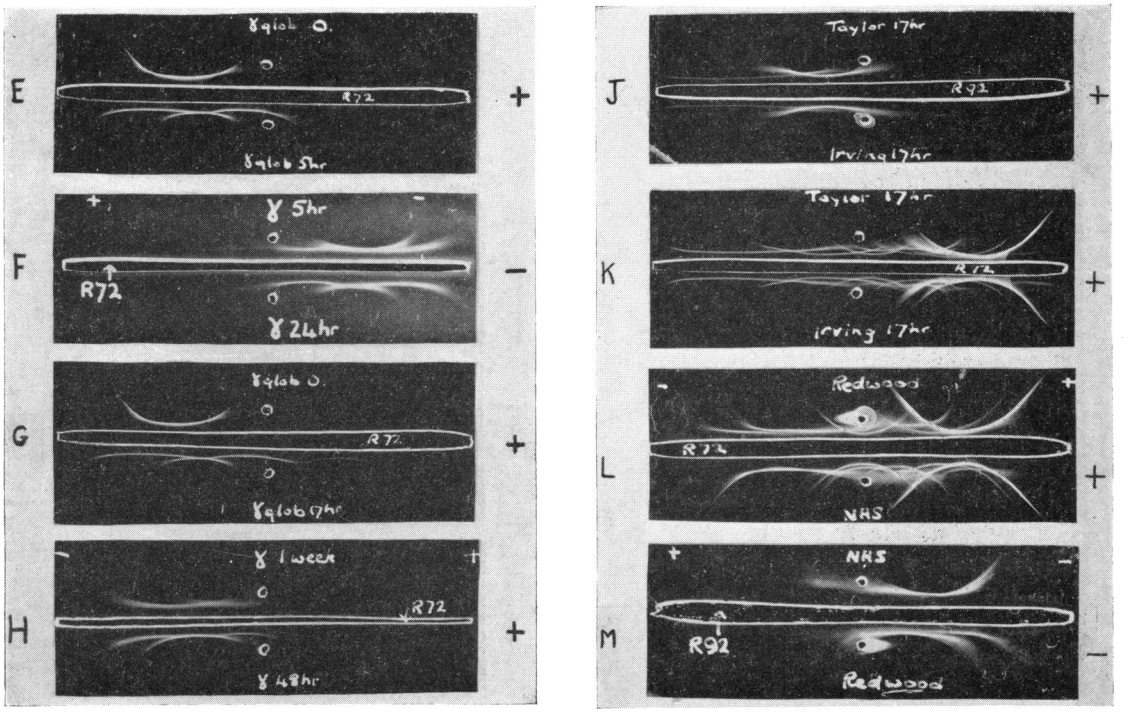


FIG. 4, A–H: Immunoelectrophoretic patterns given by a highly purified fraction of γ globulins (note the short range of electrophoretic mobilities in 4A and 4E compared with that of unfractionated γ globulins in Fig. 2) with R72 before any additions (orig.), after the addition of papain (0 hour), and after 1, 3, 4, 5, 17, 24, 48 hours and 1 week's digestion. NHS = whole human serum (for comparison purposes), I, II, III and IV are digestion fragments with determinants A (on I), A B C (on II), B C (on III) and C (on IV).

A–D: diagrammatic drawings.

E–H: photographs; fragment IV is not very clearly shown on the photograph after 5 hours' digestion and has practically disappeared after 24 hours. (Fig. 4F.)

J–M: photographs of immunoelectrophoretic patterns of whole normal sera digested for 17 hours (J and K) and undigested macroglobulinaemic serum compared with normal serum (L and M). The patterns have been developed with anti-γ-globulin serum (R92, J and M) and antiserum to whole human serum (R72, K and L).

and only some of the serum proteins were attacked (and those, other than the γ globulins, very slowly), more papain must have been available for the digestion of the γ globulins of whole serum than for the digestion of the isolated γ globulins.

Gel-covered microscope slides were charged in triplicate with the 17-hour digests. After the usual 1-hour electrophoresis the gel of the lower halves was sectioned blind as indicated in Fig. 5 and only the upper halves were developed with immune serum as

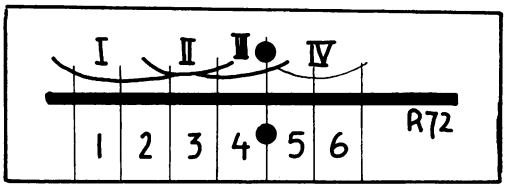


FIG. 5. Immunoelectrophoresis of the γ-globulin preparation. Before adding R72, the lower gel half was fractionated blind, as indicated and the gel sections from the lower half were used to form the Ouchterlony patterns of Fig. 6. The immune pattern (upper half) was developed after removing the gel sections 1, 2, 3, 4, 5, 6 from the lower half.

usual. The gel sections were immediately (i.e. before developing the immunoelectrophoretic patterns) filled into the peripheral cups of micro-Ouchterlony plates, as indicated in Fig. 6. From the patterns developed, as usual, by placing immune serum (R72) in the central compartment it can be deduced (cf. Augustin and Hayward, 1960a) that I has at least one determinant (say A), II three (say A, B and C), III two (say B plus C) and

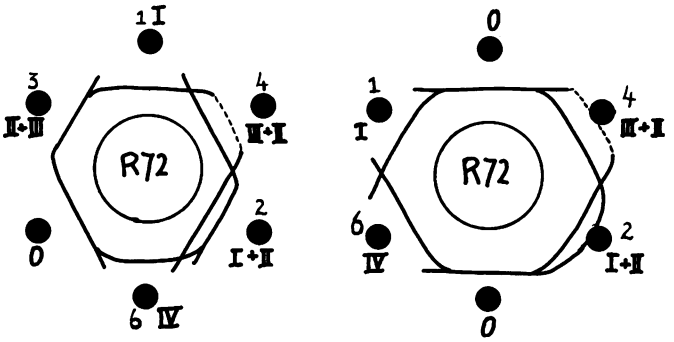


FIG. 6. Ouchterlony patterns obtained with the γ-globulin digest fractions of Fig. 5. The roman figures identify the digest fractions, the arabic figures the gel sections.

IV one (say C). R72 can therefore be credited with at least three antibodies for human γ globulins, a, b and c and R92 with at least two, a and b. According to the lattice theory IV must have several determinants C per molecule and I several determinants A in order to be able to form a precipitate with the corresponding antisera. However, three different determinants on the undigested γ globulin are sufficient to explain all the results obtained so far with the help of R72 and R92 alone.

ABSORPTION EXPERIMENTS

(1) *Antiserum Excess*

Absence from R89 of antibodies against some normal γ-globulin determinants. Three aliquots of the 17-hour digest of our γ-globulin preparation ('γ Dig') were treated with the three

antisera (R72, R92 and R89) until no further precipitation occurred and the antibodies were considered to be in slight excess. The supernatants were placed in the central cups of Ouchterlony plates (Fig. 7) and four peripheral cups were filled respectively with the three antisera and with untreated γ-globulin digest (adjusted by means of added saline to the concentration of the solution put in the central cup). No supernatant gave a line with the antiserum that had been used to prepare it, thus demonstrating the absence of unprecipitated antigen. All the supernatants in the central cups formed lines with the

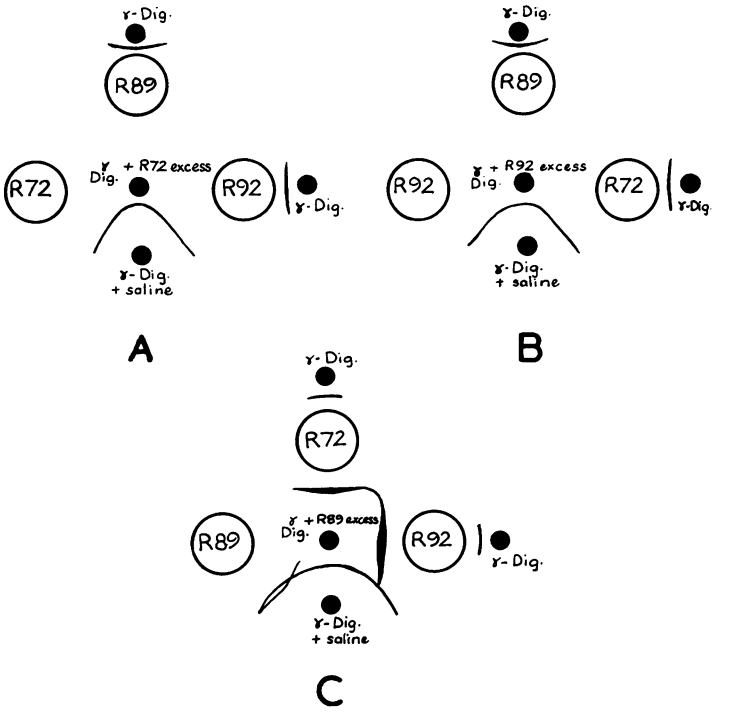


FIG. 7. Ouchterlony patterns of absorption experiments using excess antiserum. The centre cups contain 17-hour γ-globulin digest with excess of R72 (A), excess of R92 (B) and excess of R89 (C). These and the contents of the other cups are indicated in the figure. γ dig. = γ-globulin digest. The several digest lines seen in Figs. 5 and 6 were mostly superimposed under the conditions of the experiments depicted in this figure.

γ digests, i.e. the central cups contained excess of antibodies. When R72 or R92 had been used in the preparation of the fluid in the central cup, the antigens for all the three antisera can be seen to have been removed, including those for R89. However, R89 was only capable of removing part of the antigens of the γ digest, for heavy precipitation lines can be seen to have formed with R72 and R92 (Fig. 7C) in spite of antiserum excess in the central cup.

R89 thus lacks antibodies for the determinants of one or more of the pieces of normal γ globulins for which R72 and R92 contain antibodies.

(2) *Antigen excess*

Determinants lacking in the hydrolysed γ-globulin sample. In Fig. 8 the γ-globulin components of a normal human serum 'Irv', of Waldenström serum 'Redw' and of our γ digest are

compared in terms of R89 and R92. In Fig. 8A the 'Redw' serum can be seen to have formed a spur against the γ digest. This could represent a reaction of partial identity between 'Redw' γ globulins and the normal γ-globulin digest, or it could mean that 'Redw' possesses an additional globulin, that formed a line superimposed on that due to normal γ globulins, although unrelated to normal γ globulin.

Excess of 'Irv' (Fig. 8B) can be seen to have removed all the R89 antibodies for the γ digest, but not those for one of the components of 'Redw'; i.e. 'Redw' can be seen to possess one protein that is not present in the normal γ globulins of 'Irv'. However, this residual line was very thin, certainly very much thinner than the line — and spur — formed between R89 and 'Redw' in Fig. 8A. From this it appears that most of the 'Redw' γ globulins, including the macroglobulins, are at least partially related to normal γ globulins.

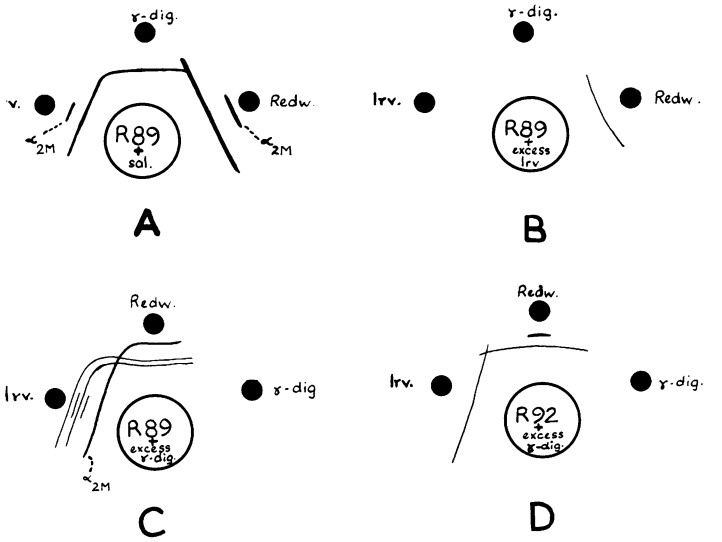


FIG. 8. Ouchterlony comparisons of absorption experiments using excess antigen.
Irv = a normal human serum
Redw = a macroglobulinaemic serum
α_2M = α_2M globulin
γ-dig. = 17-hour γ-globulin digest
sal. = physiological saline

Treatment of R89 with γ digest in place of 'Irv' (Fig. 8C) must leave antibodies for the α_2M globulins and can be seen to have also left antibodies for two other proteins which have a much higher concentration in 'Redw' than in 'Irv', since these lines are much nearer to the cup containing Irv serum than to the cup containing Redw serum. It is therefore likely that these two fine lines are due to macroglobulins of 'Redw' (traces of which are also present in 'Irv', according to Fig. 3C), which have determinants additional to those present in the γ-globulin digest. The special 'Redw' component (Fig. 8B) must be superimposed on one of the two residual lines in Fig. 8C.

We may conclude from Fig. 8A–C that 'Irv' possesses more γ-globulin determinants than are present in the γ-globulin digest and that 'Redw' possesses more than 'Irv'.

Treatment of R92 (the serum containing only antibodies specific for γ globulins) with excess γ digest (Fig. 8D), left traces of antibodies for constituents of 'Irv' and 'Redw'

which appear to be unrelated (reaction of non-identity). This confirms that both 'Irv' and 'Redw' possess some γ globulins with determinants other than those present in the γ digest and that 'Redw' has a small amount of a component not found in 'Irv'.

We conclude that the bulk of the Waldenström γ globulins, including the macroglobulin, are related to normal γ globulins, but that there are additional determinants in the macroglobulins for which R89 (and not R92 and R72) has antibodies and that 'Redw' has in addition a small amount of a component unrelated to normal γ globulins (Fig. 8B).

Fig. 8 deals only with the main γ-globulin line formed by R89. The two subsidiary γ-globulin lines could not be seen with the dilution of R89 used in these experiments.

IMMUNOELECTROPHORETIC COMPARISONS

Absence from R89 of Antibodies against Two Determinants of Normal γ Globulin

Fig. 9 gives the immunoelectrophoretic patterns of our isolated γ-globulin preparation, whole serum 'Irv' and their 17-hour digests in terms of R72, R92 and R89. R92 was used in place of R72 when whole serum 'Irv' was electrophoresed (Fig. 9A) to avoid interference by serum components other than γ globulins.

Neither R72 nor R92 ever gave more than one γ-globulin line with undigested γ globulin or with normal sera. R89, on the other hand, always gave three γ-globulin lines (Fig. 9 B, D, E and G) as described before (Fig. 3).

The question arises whether these three R89 lines are formed with the same components of the γ globulins that are precipitated by R72 and R92. We will assume for the moment that the same γ-globulin molecules are involved in the formation of the three lines given by R89 and the one line given by R72 and R92.

On all of the slide patterns of Fig. 9 A to E the patterns of the undigested γ globulins are compared with the patterns of the digests. The three proteins giving the three lines in the γ-globulin position (Fig. 9B) have not been separated and we can therefore not be sure to which of the original protein lines the digest lines belong. However, it seems likely that the digestion products of the protein giving the very fine line furthest from the antibody trough (Fig. 9 B, D, E and G) — and often too faint and too close to the thick middle line to show up at all in immunoelectrophoretic diagrams — would certainly be in too low a concentration to show up in the diagrams of the digestion products. We have further considered it likely that the thickest of the three γ-globulin lines (Fig. 9B, top) would give the thick digestion lines and that the protein giving the other fine line (nearest the antiserum trough) would have its own fine line digestion products.

The 17-hour digests can be seen to give two lines with R89 on the cathode side (Figs. 9 B, D and E), a thin forked line and a thick line indented and roughly in the position of fragments I and II formed with R72 (Figs. 9 C and F). This indentation in what appears to be a common thick line, in place of I and II formed with R72, was not always clearly marked (e.g. Fig. 9E) and the part of the line in the position equivalent to II was always fainter than the line in the position equivalent to I.

The forked thin digest line (Figs. 9 B, D and E) — the fork on the cathode side not always clearly visible — crossing the thick digest line arises most probably from the thin γ-globulin line nearest the antibody trough (Figs. 9 B and D, top; also Figs. 3 and 9G). Once more there is no digest line corresponding to that formed by fragment III with R72 (Fig. 9C). These results confirm our previous conclusion that R89 lacks antibodies for some determinant or determinants of normal γ globulin and show that these are located on piece III.

According to our previous terminology, the cleavage product III possesses determinants B and C, and R89 therefore lacks antibodies b and c. This would also explain the reaction of identity given by I and II in terms of R89; for, according to our terminology, B and C are the determinants that distinguish I and II from each other.

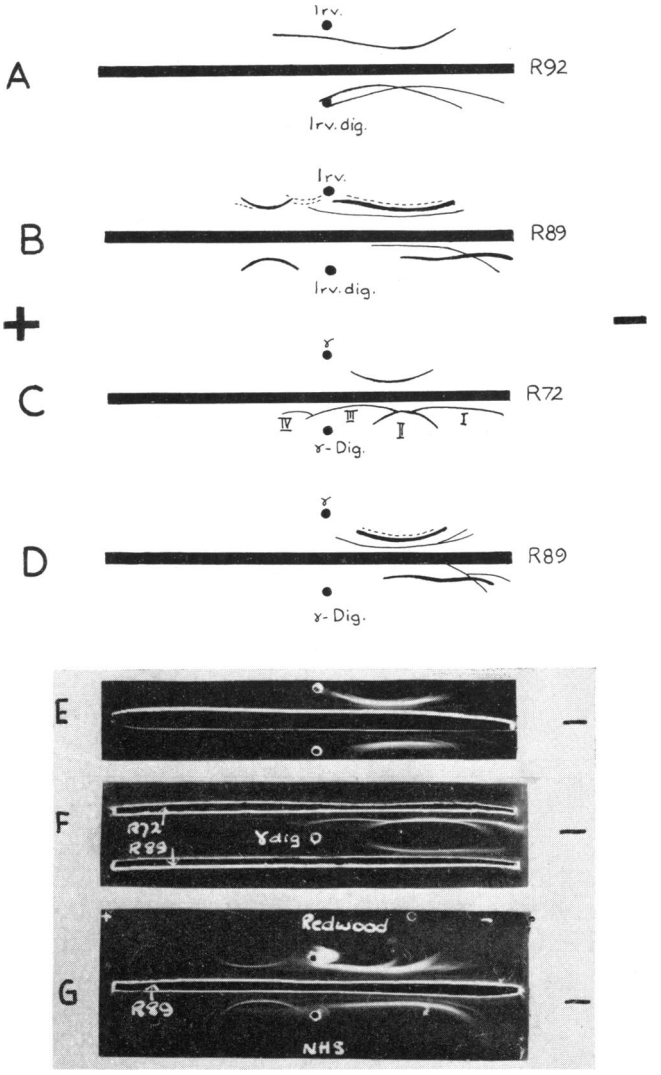


FIG. 9, A–F. Immunoelectrophoretic comparisons of normal serum 'Irv', our γ-globulin preparation and their 17-hour papain digests in terms of R92, R89 and R72. E: captions identical with D. G: Immunoelectrophoretic comparison of normal serum (NHS) with the Waldenström serum 'Redwood' in terms of R89.

The results given so far are compatible with R89 possessing antibody a; we have further to assume that II has much less determinant A than I, and we know already that R89 has at least one antibody, say m (for the macroglobulin) in addition to those present in R72 and R92.

OUCHTERLONY COMPARISONS

Lack of Determinant A in 'Thin Line' γ Globulin and Presence of a Determinant Y that is lacking in the Bulk of the γ Globulin

In Fig. 10 the three antisera are compared in terms of our γ-globulin preparation, normal human serum 'Irv', the macroglobulinaemic serum 'Redw' and the 17-hour γ digest of our γ-globulin preparation (medium fast γ globulins).

Fig. 10A shows that both the thin and the thick line formed by R89 with normal γ globulins fuse with the γ-globulin lines formed with R92 and R72 (reaction of identity) — which confirms the absorption experiments. Knowing already that R89 lacks antibodies b and c, we can assume that the thick line R89 of Fig. 10A has been formed with

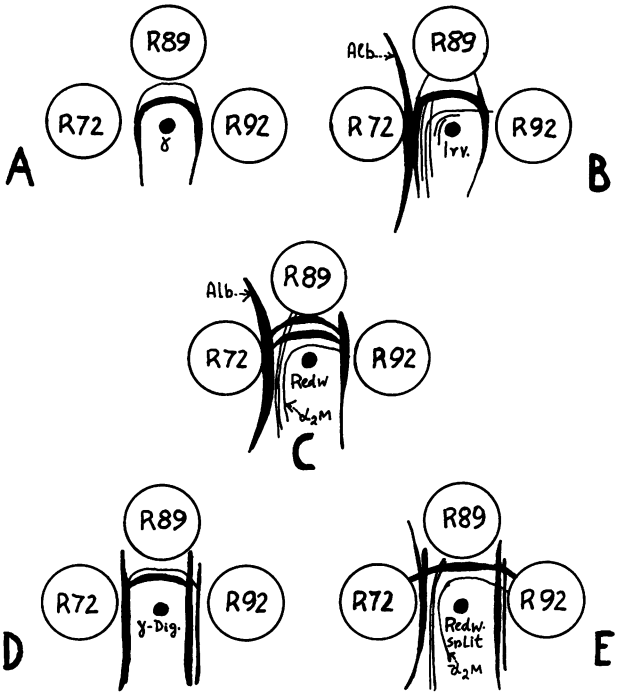


FIG. 10. Ouchterlony comparisons of the pure γ-globulin preparation, normal human serum 'Irv', the macroglobulin preparation 'Redw' and their digestion or split products in terms of R72, R89 and R92.

γ globulins possessing determinant A, i.e. with the bulk of the γ globulins, ABC. The thin R89 line must therefore have been formed by γ globulins lacking in A (since they could otherwise not have passed the ABC-a barrier), yet sharing at least one other determinant with normal γ globulins as well as possessing at least one further determinant which the bulk of the γ globulins lack and for which R89 has antibodies. Let us call the special determinant Y, thus giving R89 a minimum of antibodies a and y. The shared determinant must be B since R92, as well as R72, gives a reaction of identity with the thin line although R92 lacks c. The precipitation lines formed by ABC and BY with R72 and R92 are superimposed on each other, as would be expected since the concentration of ABC exceeds the concentration of BY. ABC is thus the first γ-globulin species to meet antibodies and form a precipitation line. Regardless of which of the three antibodies of R72 starts the

precipitation (i.e. is in excess of the others), the ABC line must form a barrier for b-antibodies and BY is thus forced to precipitate in the same antibody 'sink' (Spiers and Augustin, 1958). The corresponding argument holds true for R92 with antibodies a and b, but not for R89 lacking in b and c. ABC-a (formed by R89) gives a reaction of identity with ABC-abc (formed by R72) and ABC-ab (formed by R92), but is no barrier for BY which precipitates behind ABC-a with y of R89. BY-y gives a reaction of identity with BY-b (formed by R72 and R92) superimposed on ABC-abc and ABC-ab respectively. We considered at one time the possibility that the thin line formed with R89 was due to γ globulins with determinants AY and a very much higher diffusion coefficient than ABC; however, in this case one would expect to get two lines not only with R89, but also with R92 and R72.

Fig. 10B produced by 'Irv' in place of the γ-globulin preparation closely resembles Fig. 10A except that there is some interference by serum components other than γ globulins.

A further Determinant X in Normal γ Globulin

Fig. 10D assesses the γ-globulin digest in terms of the three antisera. We know already that the digest contains at least A+ABC+BC+C, the latter on piece IV (Fig. 9C) in too small a quantity to be considered in the present context, as well as the split products of BY (which gives the thin line with R89). The latter will be remembered (Fig. 9D) to have produced a two-pronged pattern during immunoelectrophoresis, which, in the simplest case, would be met by, say, a piece containing Y-determinants only being split off BY and thus giving a reaction of partial identity with the parent molecule and another with B. However, both the lines formed by R89 in Fig. 10D (one of them, presumably the thicker nearest the antigen cup, necessarily formed with the a antibodies of R89, i.e. with ABC and A of the γ digest) can be seen to give a reaction of partial identity with the line formed by R72. Yet a line Y-y would give a reaction of non-identity with the precipitates formed with antibodies a, b and c of R72. The two split products of the γ globulins giving the thin line with R89, must therefore *both* have a determinant for which R72 has antibodies. If one of the split products is BY, the other split products must have at least one more determinant (to give a two-pronged pattern) for which R89 has antibodies. Let this third determinant be X, thus attributing to R89 antibodies a, x and y. At its simplest the split products can then be BYX and BY, which is compatible with Fig. 9D as well as 10D.

In Fig. 10D all the γ-split-products can be seen to have produced only one single line with R72, which is likely to happen if the concentrations of a, b and c are not too different. From Fig. 9 split products with A-determinants are probably in excess of the others, i.e. A-a will precipitate first and cause ABC to accumulate as ABC-a in the 'sink' thus formed, which will then form also a barrier for a and b antibodies, which, in their turn, will draw BC, BXY and BY into this identical 'sink'.

A further Determinant D in Normal γ Globulin

Two lines can be seen to have been given by R92 in Fig. 10D, the line nearest the antigen cup being the thicker line and therefore most likely formed with ABC+A; R92 must therefore contain a significantly larger amount of a than of b antibodies. A-a and ABC-a can thus form well before the arrival of the b antibodies, allowing BC, BXY and BY to pass and form a second line nearer the R92 cup. It is compatible with these assump-

tions that the inner R89 line (ABC-a superimposed on A-a) should show antigenic affinity with the inner R92-line (again ABC-a superimposed on A-a) while the outer, upper R89 line (BXY-y superimposed on BY-y — by arguments as before) shows a similar relationship to the thinner R92 line (BXY-b superimposed on BY-b and BC-b). The spurring of the thin R92 line beyond the R89 lines would be expected since the A-a + BXY-xy + BY-y lines of R89 do not form a barrier for BC. However, the corresponding R72 spur would seem to be too pronounced for this and if the thicker of the R92 lines represents ABC-a + A-a, the extension of this line beyond the R89 lines cannot be caused by BC and indicates the existence of a further split product of γ globulins, capable of passing the barriers formed by ABC-a + A-a + BXY-xy, for which R92 and R72 (but not R89) have antibodies. To be superimposed on the A-a + ABC-a line of R92 it must further have a determinant in common with A and/or ABC. If we let this further split product be D, we must give R72 and R92 a further antibody d. In addition we must agree that split products I and II of γ globulins are in fact AD and ABCD and not A and ABC as assumed hitherto.

This gives us so far a total of six determinants for γ globulins, AD + ABCD + BC (+C) as the split products of the bulk of the γ globulins and BXY + BY as the split products of one of the minor species of human γ globulins; with antibodies a + x + y for R89, a + b + c + d for R72 and a + b + d for R92.

A Seventh Determinant E in Normal γ Globulin

Fig. 10C shows two thick lines for the Waldenström serum 'Redw' with R89, both of them giving a reaction of identity (or partial identity) with the γ lines given by R72 and R92, as would be expected from the absorption experiments (Fig. 8). The pattern is a little confused by the non-γ-globulin lines formed with R72 and R89, but it looks as if 'Redw' did not contain AXY globulins (unless their line is obscured by the one of the thick R89 lines), but had instead a further species of γ globulins capable of passing both the R89 lines (the upper one of which must have been formed by the Waldenström macroglobulin) and for which only R92, and not R72, has antibodies. This would give us at least one further determinant say E for the γ globulins, and antibodies e for R92. However, the picture is a little complicated.

A Specific Determinant in a Waldenström Macroglobulin

It was most interesting to find such a clear indication that the Waldenström macroglobulin should be related to the ordinary 7S γ globulins (Fig. 10C). When the experiment was repeated some weeks later (the serum had been stored at 0–4°) the picture given in 10E emerged, whereas fresh samples of 'Redw' gave once more Fig. 10C (the two R89 lines were occasionally fused), only to revert once more to 10E after a period of storage. In 10E the R89 line crosses the R92 and R72 γ lines in a reaction of non-identity. It seems obvious that the 'Redw' Waldenström macroglobulin is easily split to give one split product that has no immunological similarity to 7S γ globulins, at least not in terms of R92 and R72. R89 must have a large amount of an antibody w specific for the Waldenström macroglobulin — which is not surprising since R89 was prepared by immunizing a rabbit with the macroglobulin largely freed from 7S globulin.

It is possible that the unrelated thin lines formed in the absorption experiments and seen in Fig. 10C are due to a beginning of this spontaneous splitting process of the rather labile Waldenström macroglobulin.

An Eighth Determinant F in Normal γ Globulin

In Fig. 11 further attempts have been made to sort out the immunological relationships between normal γ globulins (as represented by unfractionated 'Irv'), our isolated γ-globulin preparation (covering a limited range only of the electrophoretic mobilities of γ globulins) and split (i.e. stored) and unsplit (fresh) Waldenström macroglobulin ('Redw').

Fig. 11A shows once more that the strong W component of split 'Redw' is related and/or superimposed on a γ-globulin component, which, in terms of R89, is immunologically identical with 'Irv' γ globulins.

In Fig. 11 A and B the three R89 γ-globulin lines have been obtained as in Fig. 3, and are seen to give a reaction of partial identity with fresh 'Redw'. The small spur on the

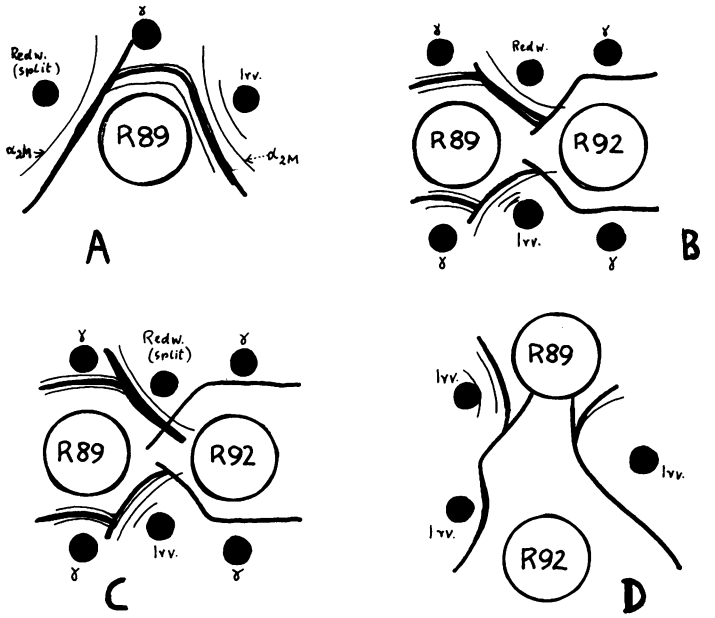


FIG. 11. Ouchterlony comparisons of normal ('Irv') and pathological ('Redw') sera and the pure γ-globulin preparation in terms of R89 and R92.

R89 side against γ globulin is similar to that given by 'Irv' and corroborates the absorption experiments; both 'Redw' and 'Irv' γ globulins have determinants over and above those present in the γ-globulin preparation, which we may refer to as F (with additional antibodies f in R89). Unsplit 'Redw' and 'Irv' give both a reaction of identity with the γ-globulin preparation in terms of R92 (antibodies a, b, c, d); the spur formed by R92 with both 'Redw' and 'Irv' against their γ-globulin lines formed with R89 could be due to BXY and BXYW respectively. This is also in agreement with Fig. 11D; R92 gives with 'Irv' ABCD-abd superimposed on BXY-b. 'Irv' forms an ABCD-a-line with R89 giving a reaction of identity with ABCD-abd and allowing BXY molecules to pass through and combine with b from R92, as well as with x and y from R89. Unsplit fresh 'Redw' in place of 'Irv' gives a picture identical with 11D (Fig. 12), but split 'Redw' in terms of R89 once more gives a strong reaction of non-identity (Fig. 11C and Fig. 12) with

γ globulins in terms of R92 and a reaction of partial identity with all three γ globulins in terms of R89.

In conclusion, this analysis establishes the existence of three to five species of normal γ globulins (three forming the three lines with R89 plus one to two further γ globulins carrying determinants E and F), two of which have been examined in further detail. These two species were found to possess at least four (A+B+C+D) and three (B+X+Y) different determinants respectively, the bulk of the γ globulins being made up of the ABCD species. The allocation of the further two determinants, E and F, has not been established. The γ-globulin species isolated by us lacked F (and possibly E) — which would account for Fig. 8D, if we assume that 'Redw' has E only and 'Irv' F only. Most likely, there are more determinants still.

The Waldenström serum 'Redw' has been found to contain in addition to the usual γ globulins ABCD (plus BXY) a macroglobulin BXYW which easily splits to BXY+W.

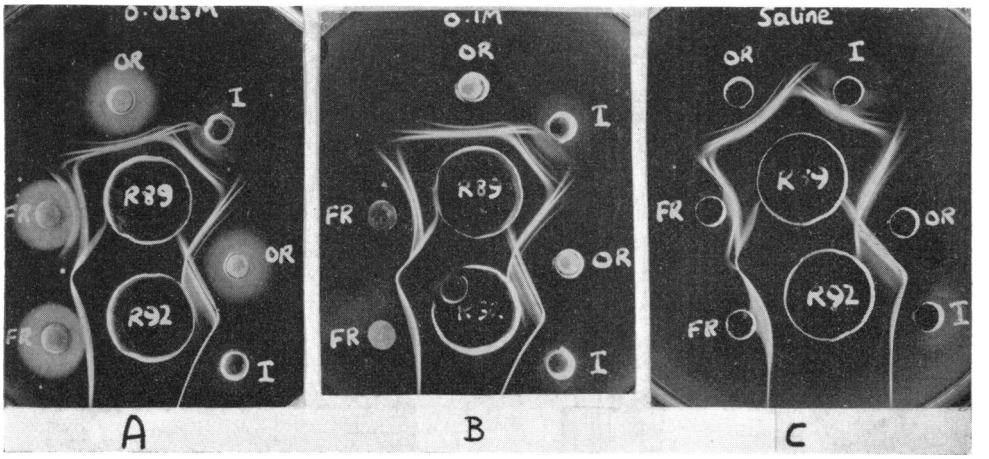


FIG. 12. Ouchterlony comparisons of normal ('I') and pathological (FR = fresh 'Redw', O.R. = Old 'Redw', i.e. serum containing split macroglobulin) sera. Agar in A contains 0.025 M, in B 0.1 M Veronal buffer and in C physiological saline. Opaque halos around the cups containing the macroglobulinaemic sera in A are due to the Waldenström macroglobulin.

BXY may be the carrier of a further determinant E (for which R92 has antibodies and which must thus also occur in some normal γ globulins, although not necessarily in 'Irv'). The 'Redw' macroglobulin is thus composed of units which occur — in small quantities — also as a subspecies of normal γ globulins, but in combination with other units W which do not occur in normal γ globulins. W determinants do not appear to be immunologically related to any of the other serum proteins since R89 does not give lines with any serum components other than γ globulins (including the Waldenström macroglobulin) and α_2M for which it has also antibodies: α_2M is not related to any of the other macroglobulins against which R89 contains antibodies (Figs. 3, 8, etc.).

The ultracentrifugally prepared macroglobulins of 'Redw' consist of BXYW, α_2M and, presumably, β_2M; perhaps a very small amount of normal 7S ABCD molecules could have given rise to antibodies b, x, y and w, of which in fact the latter three were found in R89. R89 also contained a antibodies, A being probably the chief determinant of normal 7S γ globulins. R89 may have contained antibodies for other determinants of

7S γ globulins (not b, c or d) and the relationship to normal β_2M has not been investigated. R89 appears in fact not to possess antibodies for normal β_2M since no β_2M lines were formed with normal sera (Fig. 3 A and B). It is, however, possible that the third γ-globulin line given with R89 (which has not been investigated) and also given by our pure γ-globulin preparation, is related to β_2M (Franklin and Kunkel, 1957) and it has already been pointed out that later sera from rabbit 89 gave strong β_2M-lines.

DISCUSSION

The work described here is a further contribution to the already large literature devoted to the immunological relationship of human 7S γ globulins and the pathological macroglobulins, which has been excellently reviewed by Hässig, Gugler and Scheidegger (1960) and by Putnam (1960). In addition we have attempted to find out how many determinants human γ globulins have and have further shown that normal human γ globulins consist of at least three to five immunologically related species.

Most workers agree that Waldenström macroglobulins are related to normal 19S (= β_2M) globulins (e.g. Franklin and Kunkel, 1957; Morton and Deutsch, 1958) and cross react with the normal 7S γ globulins (Korngold and van Leeuwen, 1957 a, b). There is, however, still considerable controversy as to whether the Waldenström macroglobulins contain determinants that are absent in normal 19S proteins.

With the antisera at their disposal, Burtin, Hartman, Heremans, Scheidegger, Westendorp-Boerma, Wieme, Wunderly, Fauvert and Grabar (1957) found Waldenström macroglobulins to be qualitatively identical with the normal β macroglobulins although increased in quantity; like Deutsch, Morton and Kratochvil (1956) before them they concluded that the abnormal macroglobulins are identical with macroglobulins found in small quantities in normal sera. Burtin *et al.* became finally convinced that the abnormal macroglobulins were immunologically identical with the normal β_2M globulins and suspected that the individual specificities attributed by other workers (Weber and Hässig, 1956 and others) to Waldenström sera were of a quantitative nature only. This was strongly disputed by Korngold and Leeuwen (1957; cf. also Korngold and Lipari, 1956 a, b) who showed by means of Ouchterlony tests that although there were some determinants shared with 19S and 7S normal serum proteins, these abnormal macroglobulins lacked some determinants present in normal sera and had others absent from normal sera. These workers have recently succeeded (1959) in splitting normal and abnormal macroglobulins, according to the method of Deutsch and Morton into 7S units, some determinants of which cross reacted with normal γ globulins while others had no counterpart in normal sera.

The work of Korngold *et al.* is entirely supported by the results given here. In fact our macro-cryoglobulin split spontaneously (after twice freezing and thawing) into one unit antigenically related to normal γ globulins and another not found in normal sera. This is in good agreement with chemical evidence obtained by Putnam (1960). The relationship with β_2M globulins has not been investigated by us, but we found that the cleavage product that was antigenically related to normal γ globulins shared at least three determinants with one of the subsidiary species of normal γ globulins and only one also with the bulk of the normal γ globulins. Our work thus reconciles the different viewpoints and also supports in part the view of Deutsch, Morton and Kratochvil (1956), that abnormal macroglobulins are antigenically identical with heterogeneous portions of

normal sera, possessing a smaller contingent of normal determinants than the normal γ globulins. At least in this one instance a cleavage product of a Waldenström macroglobulin* is seen to be identical with a subspecies of normal γ globulins.

One may speculate on the ease with which the different molecules antigenically related to γ globulins split into subunits. It seems that the body is capable of producing a large variety of subunits and a variable capacity to join them together, e.g. by S-S-bonds, possibly in many different ways; it may be this property that makes γ globules capable of becoming antibodies. Metabolic faults may result in over-polymerization and/or the production of excessive amounts of a particular type of building stone (usually only found in traces in the normal organism or even not at all) during the production of pathological macroglobulins and/or Bence-Jones proteins (Putnam, 1960). The most highly polymerized molecules are not unexpectedly split most readily by reducing agents and even spontaneously as reported here. Nisonoff, Wissler, Lipman and Woernley (1960) have recently shown that the remarkable cleavage of rabbit γ globulin by papain (Porter, 1959) is really a two-stage process that can be imitated also by pepsin in the presence of cysteine. The proteolytic enzyme cleaves the molecule into 5S fragments which are then readily broken down to 3.5S units by reducing agents and even by ion-exchange chromatography. Grabar (1959) was the first to describe spontaneous alterations of immunoelectrophoretic patterns of isolated γ-globulin preparations and we found that the most highly purified γ globulins also have a tendency to split spontaneously (Augustin and Hayward, 1960a) into fragments similar to those formed by papain digestion. Presumably admixed serum proteins afford some protection against this, since the immunoelectrophoretic γ-globulin patterns of whole sera are not readily changed in a similar way although lines characteristic of cleaved γ globulins are occasionally found, particularly after prolonged storage. Presumably some unfolding may occur even with very gentle procedures during the purification of γ globulins, particularly in dilute solution, resulting in the exposure of the same labile S-S bonds which can be exposed by enzymic action. Other serum proteins are not as easily split, either spontaneously (Table 1) or by digestion (Fig. 4). Korngold and van Leeuwen (1959) have shown that not only depolymerization but also re-aggregation of subunits into macroglobulins occurs very readily. In this connection it is interesting to speculate on the difficulties experienced by various workers in recovering reaginic antibodies in a biologically active form (Stanworth, 1959; Augustin and Hayward, 1960c) which have usually been attributed to some special lability of these antibodies. Activity was usually lost during the last stages of purification which we found to be associated with the appearance of two immunoelectrophoretic lines in place of one. We have shown that reagins are 7S γ globulins with a special limited range of mobilities (Augustin and Hayward, 1960c); as γ globulins, reagins would be expected to share the lability peculiar to γ globulins, particularly in dilute solution, and no other special lability need be evoked to explain their behaviour. It remains to be seen what subspecies of γ globulins reagins belong to, to give them a greater tendency to adhere to tissues than other proteins, and whether they have fewer or more — or, possibly, some determinants different to those of normal γ globulins.

What is the relationship of the work reported here to the somewhat similar studies of Edelman *et al.* (1960) which has meanwhile appeared?

* The Waldenström serum investigated in detail here has two features, either of which is, according to Hässig, Gugler and Scheidegger, 1960, characteristic of only a minority of Waldenström sera; Fig. 4 L and M show a stronger than usual β2A line as well as a macroglobulin remaining at the place of origin during gel electrophoresis at the customary low ionic strength.

Edelman *et al.* obtained two closely adjacent parallel γ-globulin lines with some antisera. Papain digestion yielded mainly two non-identical split products, one fast, the other slow, often together with a small midpiece of only partially degraded γ globulins — exactly as reported by us here and in a previous publication (Augustin and Hayward, 1960a). The workers showed that each of the two original γ-globulin lines had contributed only one of the two electrophoretically different split products; but it is a little difficult to understand why both the original γ lines should under these circumstances have equal mobilities. Edelman *et al.* did not find the very fast split product discovered by us with R72 after a limited period of digestion.

It is possible that the two closely adjacent γ-globulin lines obtained by us with R89 (together with the easily distinguishable third thin line nearest the antibody reservoir) correspond to the two closely adjacent lines of Edelman *et al.* However, the explanation given by these workers for the appearance of the two split products (looking so very much like our own) cannot hold for our fragmentation; for, since R89 does not contain antibodies against the fast split product, the reaction of R89 with any one of the three γ-globulin lines cannot be due to the presence of a fast piece in any one of them.

Several attempts have been made to estimate the minimum number of determinants for human γ globulins. Scheidegger and Buzzi (1957) found a minimum of four by analysing sera with the help of Bence-Jones proteins. Korngold and Lipari (1956b) postulated seven from the cross reactions of abnormal macroglobulins belonging to three antigenic groups, and two of Bence-Jones proteins with normal γ globulins. Antigenic relationships of the four split products obtained by us by proteolytic cleavage of normal γ globulins revealed a minimum of three determinants (Augustin and Hayward, 1960a).

In the work presented here we were able to attribute a minimum of eight determinants A B C D E F X Y to normal 7S γ globulins. This was possible by comparing a highly purified γ-globulin preparation of a limited range of electrophoretic mobility and its split products with a specific pathological macroglobulin and its (spontanteous) split products in terms of three different antisera, one lacking antibodies to three of the common 7S γ globulins, but possessing others for their more unusual determinants. This third antiserum has been the keystone to the present work since it was thus able to distinguish between three species of normal 7S γ globulins whose precipitation lines normally overlap to produce the familiar single unbranched γ-globulin line of the usual immunoelectrophoretic patterns. This is possible although they share only some of their determinants because they cover practically the same range of electrophoretic mobilities and because the usual antisera have antibodies only for the determinants of the bulk of the γ globulins and, it appears, for only a limited number of these. Differences between the various species of γ globulins are thus obscured and probably many more than the three to five species suggested here exist. However, the mobility ranges of the different globulin species do not quite overlap and we think now that the slight forking that is often observed at the cathode end during routine immunoelectrophoretic examinations can be due to the fact that the range of the BXY molecules does not completely overlap the range of the ABCD molecules. At that end only can antibodies a, c and d (if present in addition to b) pass the BXY-b line to form a spur with the ABCD molecules. It is possible that the spontaneous or enzymatic cleavage previously thought responsible for this phenomenon (Augustin and Hayward, 1960a) adds to this, but it is also not unlikely that the overlap of the mobility ranges is not quite the same in different individuals.

And this brings us to the much debated question of the nomenclature of γ globulins.

Here we have three or more species of γ globulins whose existence is usually obscured by the type of antisera we use to analyse them; it has been shown that β_2M globulins (Franklin and Kunkel, 1957) as well as β_2A globulins (Heremans, 1959) and even abnormal macroglobulins also share some of their determinants with what we at present call γ globulins. Particularly, the 7S β_2A globulins are related in the same manner to the bulk of the γ globulins — and these may yet be resolved into many sub-species — as are the two special subsidiary γ-globulin species described here; the β_2A-globulin line also stretches for a long distance and also overlaps, usually for most of its length, the γ-globulin line (this will depend on the antiserum used and cannot easily be seen unless the other serum components are removed) and has only been so readily accepted as a separate protein because of a long strong spur due to its having a shorter range of mobility than other γ globulins. Heremans (1959) has proposed the name 'immune globulins' to cover slow and fast γ globulins together with β_2M and β_2A globulins; Schultze (1959) considers that some antibodies of human serum may be β_2A globulins, although this evidence cannot be regarded as conclusive, since his β_2A fraction also contained β_2M globulins. All three types of globulins are lacking in agammaglobulinaemic patients who are incapable of forming serum antibodies; i.e. it is believed that all three types of γ globulins are produced by the same cells — and the antigenically related myeloma macroglobulins by plasma cell tumours. Although it is thought that plasma cells are probably not or only rarely involved in the production of Waldenström macroglobulins.

The names γ_1 and γ_2 globulin are still given to two groups of proteins distinguished solely on the basis of electrophoretic mobility. This classification gives rise to still further confusion, as from immunoelectrophoresis studies it is known that the mobilities of proteins, antigenically similar to (if not identical with) slow γ globulin, overlap those of various β_2, β_1, α_2 and α_1 globulins, which are proteins antigenically and functionally entirely unrelated to the slow γ globulin. This heterogeneity is of quite a different order to the limited heterogeneity of the antigenically related γ globulins. At the present time it appears most logical to call all serum proteins γ globulins for which it can be shown that they share at least some determinant with the slow γ globulins of Tiselius, even if they do give distinct lines with some antisera lacking antibodies to some common determinants or because they have very different diffusion constants from the 7S γ globulins. β_2A and β_2M globulins are well established easily recognizable entities and it would be unreasonable to change the names now, but they should certainly be included in the family of γ globulins, together with the myeloma and the Waldenström globulins.

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