of iodine. Coupling in the 3:5 positions occurs only slightly or not at all.

3. This reaction has been utilized as the basis of a method for the localization of ^{131}I in the thyroxine molecule.

4. The method is applicable to biosynthesized [^{131}I]thyroxine.

I am indebted to the Rockefeller Trust of the Medical School and to the Medical Research Council for facilities for carrying out this work.

I wish to thank Miss Diana Walmsley for technical assistance.

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The Hydrolysis of Rabbit γ-Globulin and Antibodies with Crystalline Papain

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The molecular size of rabbit γ-globulin is such that any direct attempt to relate structure to biological activity is not feasible at present. An alternative approach is to degrade antibody molecules in such a way that activity will persist in smaller fragments and so to reduce the structural problems involved. In an earlier investigation of this type (Porter, 1950*b*) it was found that, if rabbit γ-globulin containing antiovalbumin was digested with crude papain, a fragment with a molecular weight of about 40 000 could be produced which retained the ability to combine specifically with ovalbumin though it would no longer form a precipitate. It seemed probable that this fragment contained an antibody-combining site.

In that work, the amounts of crude enzyme used relative to γ-globulin were large, making subsequent investigation of the products of digestion rather difficult. As crystalline papain can now be prepared easily (Kimmel & Smith, 1954) and fractionation techniques have improved, these earlier experiments have been repeated. It has been found that γ-globulin is split by papain into three large pieces with very little release of amino acids or small peptides. If the γ-globulin contains antibody against any of the several antigens investigated, then two of these pieces retain combining though not precipitating power. The third piece, which may be readily crystallized, has no antibody activity but it has most of the antigenic sites of the original molecule. The isolation and properties of these three fractions will be described.

A preliminary account of this work has been given (Porter, 1958*a*).

EXPERIMENTAL

Materials

Antisera. Rabbit antiovalbumin, antibovine serum albumin and antihuman serum albumin were prepared by intravenous injection of the alum-precipitated protein (Porter, 1955).

Rabbit antipneumococci polysaccharide type 3 was prepared by injection of a suspension of the formalin-killed bacteria in 0·9% NaCl solution.

Goat antirabbit γ-globulin was prepared by intravenous injection of alum precipitate and subcutaneous injection of γ-globulin with adjuvant (Freund & McDermott, 1942).

Rat antiserum was prepared according to the following schedule. The rats were given two intramuscular injections of protein and adjuvant into different thighs, with 1 week's interval between injection. Five weeks later they were given three injections, with 3-day intervals between injections, of alum-precipitated protein into the tail vein. They were bled by heart puncture 6 days after the last injection.

Rabbit γ-globulin. This was prepared either by chromatography, with diethylaminoethylcellulose (Sober, Gutter, Wyckoff & Peterson, 1956), or by Na_2SO_4 precipitation according to the method of Kekwick (1940).

Crystalline papain. This was prepared from crude enzyme powder purchased from Hopkin and Williams Ltd., London. The enzyme was crystallized once as the free enzyme and twice as the inactive Hg dimer (Kimmel & Smith, 1954). It was freeze-dried and stored as the dimer.

Methods

Quantitative estimation of precipitating antibody. This was carried out according to Kabat & Mayer (1948).

Estimations of inhibitory power. These were made by quantitative antibody assay in the presence of the inhibitor

or by measure of the delay in precipitation caused by the inhibitor with the antibody and antigen mixed in optimum proportions.

Chromatography. Diethylaminoethylcellulose and carboxymethylcellulose were prepared according to Peterson & Sober (1956). Column size was 2·4 cm. (diam.) by 30–35 cm. (ht.); volume of mixing chamber was 1200 ml. Buffers used on carboxymethylcellulose columns were 0·01 M-sodium acetate, pH 5·5, with gradient to 0·9 M-sodium acetate, pH 5·5. In the refractionation of fraction I on diethylaminoethylcellulose, the following system was used: 0·01 M-sodium phosphate, pH 6·4, with gradient to 0·2 M-sodium phosphate, pH 6·2. All buffers were saturated with toluene. The pH was measured with an EIL Direct Reading pH meter (Electronic Instruments Ltd.).

Enzyme digestion. γ-Globulin (150 mg.) and Hg papain (1·5 mg.) were dissolved in 10 ml. of buffer (0·1 M-sodium phosphate, pH 7·0, 0·01 M-cysteine, 2 mM-ethylenediaminetetra-acetate). This solution was incubated at 37° for 16 hr. in the presence of toluene. It was then dialysed against water with vigorous stirring and several changes of the outer liquid over 48 hr. This procedure, which removed the cysteine and ethylenediaminetetra-acetate, and facilitated oxidation, inactivated the enzyme. *N*-Ethyl maleimide was also used to inactivate the enzyme but as there appeared to be no advantage this was not continued. The non-diffusible digestion products were either freeze-dried or fractionated directly by chromatography after dialysis against acetate buffer, pH 5·5.

Protein determinations. Protein concentrations were determined by reading the absorption at 280 and 260 mμ in a 1 cm. cell in a Unicam SP. 500 spectrophotometer.

Radioactivity measurements. Injection of hydrolysed algal [^{14}C]protein and counting of the γ-globulin fraction were carried out as described previously (Askonas, Humphrey & Porter, 1956).

Analytical methods. Amino acid analysis was by the method of Moore & Stein (1951) as modified by McDermott & Pace (1957).

N-Terminal amino acids were estimated by the fluorodinitrobenzene technique (Porter, 1957*a*). Hexose as 'glucose' was estimated by the anthrone method of Mokrasch (1954). Hexosamine as 'glucosamine' was estimated, after separation from amino acids on a cation-exchange resin (Boas, 1953), by a modification of the method of Elson & Morgan (1933).

RESULTS

When a digestion mixture, prepared as described above, was dialysed against water at 2° a precipitate formed which appeared to be crystalline. If, however, the dialysis was against 0·04 N-acetic acid there was no precipitation and the recovery of the non-diffusible digestion products could be estimated either by measuring the absorption at 280 mμ or by freeze-drying and weighing the dry powder. This has been done in a number of experiments, and by either method the recovery of γ-globulin protein taken has fallen in the range 85–95%. In view of the probable handling losses in dialysis and freeze-drying, it is considered that the higher figure is the most accurate. When such a digest was examined in the ultracentrifuge in 0·1 M-phosphate, pH 6·7, some crystals again formed on dialysis but the supernatant showed only one peak ($S_{20,w}$ 3·5). As γ-globulin has $S_{20,w}$ 6·5, it was clear that the protein had been split into large fragments of similar size with very little production of diffusible peptides. Attempts to fractionate this mixture by zone electrophoresis were not successful, but resolution could be achieved by chromatography on carboxymethylcellulose. Acetate buffer, pH 5·5, was chosen because under these conditions most of the carboxyl groups of the ion-exchanger are dissociated; if the digest was brought nearer to neutrality crystals formed, indicating a low solubility of at least one component. To help to keep all the material in solution chromatography was carried out at room temperature (20–23°). With a gradient of increasing salt concentration at this pH, three components could be resolved which have been named fractions I, II and III in order of elution from the column (Fig. 1).

If the gradient on the column was reduced, fractions II and III were more spread and III 'tailed' badly, but there was no suggestion of any further resolution. Fraction I appeared very close to the solvent front and this was re-run on a diethylaminoethylcellulose column at pH 6·4. No fractionation was obtained but again with a slow gradient there was considerable tailing. By these limited criteria the three fractions appeared to be single components and to be the only significant products of the digestion of γ-globulin by papain.

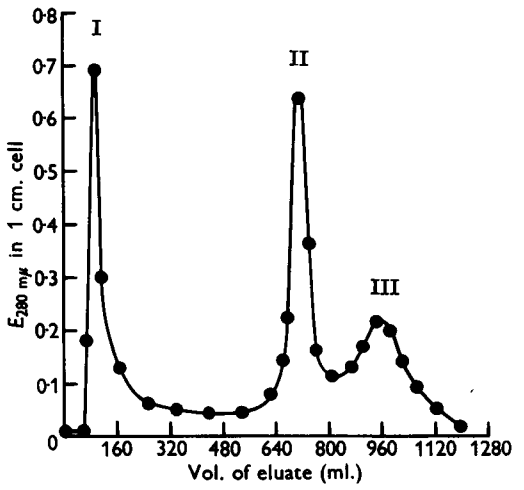

Fig. 1. Chromatography of papain-digest of rabbit γ-globulin on carboxymethylcellulose. Weight of digest 150 mg. Column 30 cm. × 2·4 cm. diam. Volume of mixing chamber 1200 ml. Gradient from 0·1 M-sodium acetate, pH 5·5, to 0·9 M-sodium acetate, pH 5·5, commencing at 200 ml. eluate volume.

Results with shorter times of hydrolysis under the same conditions suggest that the splitting is complete in very much less than 16 hr., the period which has always been used. It follows that these three fractions are exceptionally resistant to further digestion by papain.

Yields of the three fractions were measured by summing the absorption at 280 mμ in each peak. The ratios of yield varied somewhat from experiment to experiment but averaged (I:II:III) 1:0·8:0·9, and total recovery from the column was 85–90%. When re-run, fractions I and III were recovered in about 95% yield and fraction II in about 85% yield. The absorptions of peaks I, II and III at 280 mμ at a concentration of 1 mg./ml. in water or 0·02N-acetic acid were 1·4, 1·4 and 1·0 respectively. If the relative yields are corrected for the lower recovery of fraction II, and the lower specific absorption at 280 mμ of fraction III, then the corrected relative yields (I:II:III) are 1:0·9:1·25 by wt. In some experiments the fractions were concentrated by pressure-dialysis in the cold against water or 0·02N-acetic acid; insoluble material was discarded and the solution freeze-dried and weighed. The yields of I and III were similar to those above but II was somewhat lower. Considerable error can occur because of denaturation in dilute solution, and variable losses on chromatography, but it is considered that the average yields calculated from chromatography approximate to the yields of the fractions produced in the digestion.

Fraction III is readily identifiable as the material with a low solubility near neutrality. It may be crystallized and recrystallized by dialysing a solution in 0·02N-acetic acid against sodium phosphate buffer, pH 6·0–7·0, at 2°. The crystals are diamond-shaped plates, often of considerable size but thin and easily broken (Fig. 2).

Molecular weights. The three fractions were studied in the ultracentrifuge (see Addendum) and the results for normal γ-globulin are summarized in Table 1. The sum of the molecular weights of the three fragments is very close to the molecular weight of the original γ-globulin. This is in agreement with the high recovery of non-diffusible digestion products. The relative sizes of the three fragments were (I:II:III) 1:1·05:1·6, which is in approximate though not exact agreement with the calculated relative yields of the fragments.

Amino acid analysis. Amino acid analysis was carried out by Mr E. E. McDermott and Dr J. Pace at the Cereals Research Association, St Albans. The results are given in Table 2 for fractions prepared from γ-globulin from non-immune serum. The most striking feature of the analysis is the great similarity of the amino acid content of fractions I and II. With the exception of cystine,

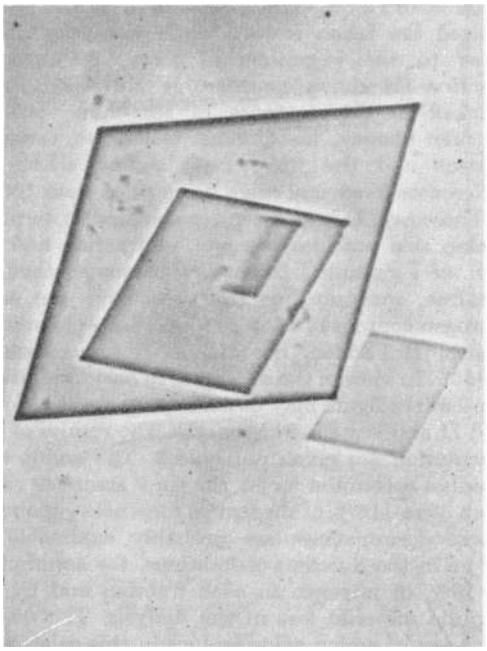

Fig. 2. Crystals of fraction III. Magnification × 500.

Table 1. *Molecular weights of fragments and γ-globulins from non-immune serum*

For details see Addendum.

γ-Globulin	188 000
I	50 000
II	53 000
III	80 000

Table 2. *Amino acid analysis of fragments from non-immune serum*

Amino acid	N (percentage of total N) I	II	III
Aspartic acid	5·66	6·01	6·18
Threonine	10·42	11·56	5·61
Serine	8·77	9·57	7·34
Glutamic acid	5·99	6·18	7·83
Proline	5·08	5·08	6·68
Glycine	7·57	6·93	3·18
Alanine	5·65	6·12	2·82
Valine	7·38	7·58	5·96
Methionine	0·64	0·75	1·48
Isoleucine	2·20	2·26	3·43
Leucine	5·17	5·39	5·19
Tyrosine	3·44	3·58	2·33
Phenylalanine	2·53	2·65	2·76
Histidine	1·59	1·87	5·23
Lysine	6·03	6·84	9·95
Arginine	5·94	6·22	13·93
Tryptophan		Not estimated	
½ Cystine	2·80	2·22	1·18

differences rarely exceed 10% and, as the results quoted are taken from a single analysis, this is close to the experimental error. In contrast, fraction III shows considerable differences, most marked in the contents of threonine, proline, glycine, alanine, methionine, isoleucine, tyrosine, cystine and the three basic amino acids, the differences in several cases being more than 100%.

These results have been recalculated in terms of amino acid residues per mol. of fraction and per mol. of γ-globulin. It has been assumed that the ash-free, moisture-free fractions have the same nitrogen content of 16% as found for γ-globulin by Smith, McFadden, Stockell & Buettner-Janesch (1955). In view of the difference in basic amino acid content the figure may be rather high for fractions I and II and low for fraction III. The results of this calculation are given in Table 3. The amino acid residues accounted for by the three fractions range from 83 to 116% of the figures for whole γ-globulin. These discrepancies are probably explicable by errors in the different estimations, the assumption of 16% of nitrogen in each fraction and by the peptide material lost in the dialysis. The overall recovery of amino acid residues in this calculation is 94%.

Carbohydrate estimations were made by Dr H. R. Perkins of this Institute, and the results with whole γ-globulin and the fractions are given in Table 4. About two-thirds of the carbohydrate of the original molecule is found with fraction III, one third with fraction I and a small amount, which may not be significant, is with fraction II. It seems likely therefore that the carbohydrate moiety of γ-globulin is in the two pieces, the larger with that part of the molecule which corresponds to fraction III and the smaller with the part corresponding to fraction I. If the total recovery of carbohydrate in the three fractions is compared with that present in the original, the yields are approximately 110 and 120% for hexose and hexosamine respectively.

N-Terminal assay has been carried out on fractions I and II, and in both alanine was found to be the main terminal amino acid (about 0·9 mol./50 000), together with smaller amounts of aspartic acid (0·2 mol./50 000) and trace amounts of serine and threonine (together about 0·1 mol./50 000). This is a very similar result to that obtained for whole rabbit γ-globulin (Porter, 1950*a*; McFadden & Smith, 1955), except that the *N*-terminal amino acid content is more than three times as great as in the whole globulin. It is also in approximate agreement with the results of the digestion of rabbit antiovalbumin with papain powder, when an immunologically active fraction containing one *N*-terminal alanine per 40 000 was reported (Porter, 1950*a*). The significance of finding *N*-terminal alanine in both I and II is not certain, as there are about 110 alanine residues per molecule of γ-globulin. An attempt to determine the *N*-terminal sequence was unsatisfactory owing to shortage of material, but it appeared that the sequence of I was alanylaspartyl and of II alanyl-leucyl. If this is correct, it suggests that II may derive from the *N*-terminal part of the molecule, as the sequence there is alanyl-leucylvalylaspartylglutamyl (Porter, 1950*a*; McFadden & Smith, 1955).

Immunological properties. The three fractions were prepared from digests of γ-globulin which had been obtained from rabbit antisera against ovalbumin, bovine serum albumin, human serum albumin and antipneumococci polysaccharide type 3. None would precipitate with the corresponding antigen but fractions I and II prepared from γ-globulin containing antiprotein antibodies inhibited the precipitation of the antigen by the homologous antiserum. This effect is specific; for

Table 3. *Recovery of amino acid residues*

Amino acid	Total no. of residues in I+II+III*	Total no. of residues in whole γ-globulin†	Recovery (%)
Aspartic acid	126	137	93
Threonine	180	207	87
Serine	175	196	89
Glutamic acid	144	150	95
Proline	121	138	87
Glycine	115	140	83
Alanine	95	111	86
½ Cystine	40	44	91
Valine	144	158	91
Methionine	22	19	116
Isoleucine	58	63	92
Leucine	110	111	100
Tyrosine	63	68	92
Phenylalanine	56	62	90
Lysine	84	83	100
Histidine	23	19	120
Arginine	66	54	120
Tryptophan		Not estimated	
Total	1582	1680	94

* Calculated from molecular weights and amino acid analysis, assuming a nitrogen content of 16% for each fraction.

† Calculated from Smith, McFadden, Stockell & Buettner-Janesch (1955), assuming a molecular weight of 188 000.

Table 4. *Carbohydrate content of γ-globulin and fractions*

	Hexosamine (%, as glucosamine)	Hexose (%)	Total carbohydrate (%)
γ-Globulin	0·6	0·5	1·1
I	0·5	0·6	1·1
II	0·1	0·15	0·25
III	1·3	0·9	2·2

example, I and II from antihuman serum albumin γ-globulin had no effect when added to bovine serum albumin and its antiserum; fraction III, on the contrary, had no effect whatever its source or whatever the test system. Testing fraction III is difficult owing to its low solubility near neutrality, where antibody–antigen precipitation is carried out, but it is soluble to about 0·3 mg./ml. at 37° at pH 7·2 and it could be shown that it had less than one-thirtieth of the activity of the corresponding fractions I and II.

Quantitative estimates of the inhibitory power of I and II are shown in Fig. 3. In order to assess this on a molar basis it has been assumed that the fractions would have the same proportion of active molecules as the proportion of antibody in the γ-globulin taken, and the weight of each fraction has been corrected accordingly when plotting the graph. On this basis it can be seen that approximately equal weights of inhibitor are required for complete inhibition of precipitation, i.e. a molar ratio of inhibitor to antibody of 3·5:1.

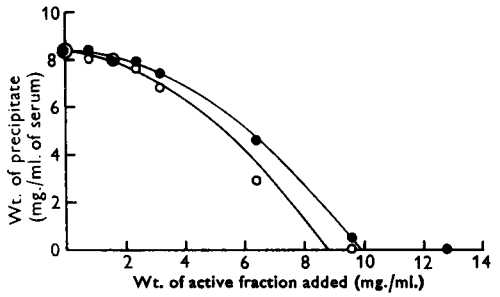

Fig. 3. Inhibition of precipitation of human serum albumin–antihuman serum albumin. Increasing weights of fractions I and II from antihuman serum albumin digest were added to antibody–antigen in equivalence. Weights of fractions were corrected for the presence of inactive material (see text). ●, Fraction I; ○, fraction II.

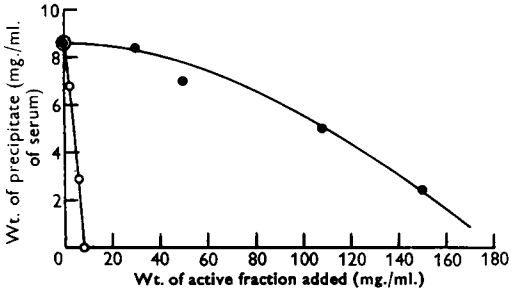

Fig. 4. Inhibition of precipitation of human serum albumin–antihuman serum albumin and pneumococci polysaccharide type 3 antipolysaccharide. ●, Fraction II from antipolysaccharide γ-globulin; ○, fraction II from antiserum albumin γ-globulin.

In contrast with these results none of the fractions from antipolysaccharide γ-globulin appeared to have any power to inhibit the combination of polysaccharide with its antiserum. However, when much higher ratios of inhibitor to antibody were used, inhibition of precipitation occurred with fraction I or II. The difference between the antiprotein and antipolysaccharide fractions appears to be quantitative rather than qualitative, as originally stated (Porter, 1958*a*). The results with fraction II from antipneumococci and antihuman serum albumin γ-globulin are shown in Fig. 4. The inhibitory power of the former is about 4 % of that of the latter, on a molar basis. Fraction III again appeared to be completely inactive in qualitative tests, but in this case the solubility limitation is greater and it could only be concluded that III has certainly less than half the activity of I or II.

Antigenic activity. The power of the fractions to precipitate with goat antirabbit γ-globulin serum was now tested and I and II showed neither precipitation nor inhibitory activity. Fraction III precipitated 70 % of the antibody precipitated by γ-globulin.

Rat antiserum was prepared against rabbit γ-globulin and fractions I, II and III. Groups of three rats were used for each antigen and, after the immunization course described above, the serum of each group was pooled, and gave antibody contents of 4·2, 7·8, 8·1 and 4·0 mg. of antibody/ml. for γ-globulin, I, II and III respectively. All the fractions were at least as effective antigens as the original molecule, and I and II were the most effective, but as only three animals were used per group the difference may not be significant.

When rat anti-γ-globulin was tested with the fractions, rather different results from those with

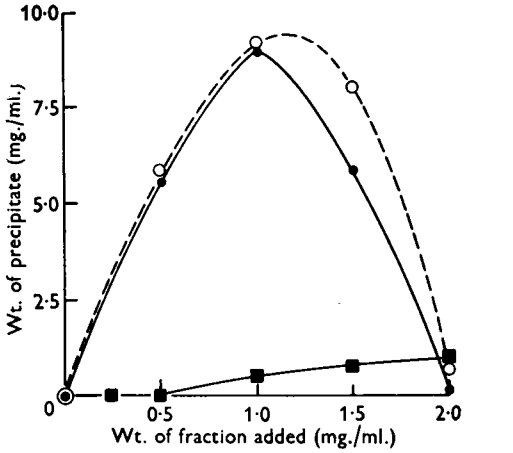

Fig. 5. Precipitation curves of I (●), II (○) and III (■) with rat anti-II serum.

goat antiserum were obtained. Fraction III precipitated 50 % of the antibody, and I and II 15 % each. The antigenic specificities of the fractions were compared by measuring the precipitates formed by I, II and III with rat anti-II serum. The precipitation curves are shown in Fig. 5. It can be seen that the curves for I and II are almost identical, but III gives almost no precipitate. This again emphasizes the great similarity of I and II and their sharp distinction from III.

Synthesis of the fraction in vivo. In view of the distinctive characters of the three fractions the possibility was considered that they might be synthesized separately and joined into the whole molecule in a separate step. If this were to happen the fractions would probably not all be synthesized from the same pool of amino acids at the same time, and hence if radioactive amino acids were injected into a rabbit the incorporation of radioactivity after a short time interval would vary from fraction to fraction. Hydrolysed algal [^{14}C]protein was used as the source of labelled amino acids; 240 μc was injected intravenously, and the rabbit was bled after 1 and 4 hr. The plasma γ-globulin is not labelled until 30 min. after injection (Askonas *et al.* 1956). The γ-globulin was prepared and hydrolysed with papain, and the fractions were isolated, precipitated with trichloroacetic acid, and counted, at infinite thickness on a 1 cm.^{2} disk. The specific activities are given in Table 5 and it can be seen that there is no significant difference between the different fractions. There is therefore no evidence to suggest that these fractions or any large parts of them are synthesized independently.

DISCUSSION

The results show that papain-digestion of rabbit γ-globulin causes limited and highly selective splitting to give three large fragments and very few small peptides. As papain shows rather a wide specificity in the digestion of the β chain of insulin (Sanger, Thompson & Kitai, 1955), it is apparent that the structure of the native molecule must be such that many potentially hydrolysable bonds are protected by steric and other factors. Ultracentrifuge studies of the splitting of γ-globulins from different species, by a variety of enzymes (Petermann & Pappenheimer, 1941; Petermann, 1942, 1946), have all shown that a small number of fragments are the main products in each case. It is possible that in γ-globulins only small parts of the peptide chain are accessible to proteolytic enzymes, so that even though different enzymes break different bonds in these vulnerable sections the principal large digestion products are similar.

Our results with the papain-digestion of rabbit γ-globulin suggest that the single polypeptide chain (Porter, 1950*a*; McFadden & Smith, 1955) has been split into three distinct sections, which together comprise some 90 % of the original molecule. However, fractions I and II are so similar that the question arises whether they could be derived very largely from the same section of the chain. They are almost indistinguishable in *N*-terminal amino acid, amino acid analysis, molecular weight, antigenic specificity and, if derived from antibody, in their antigen-binding capacity. They differ in chromatographic behaviour and carbohydrate and cystine content.

There are at least three possible ways of splitting a single chain to give results such as this, and they are illustrated in Fig. 6: A shows the obvious split into three distinct sections, B the production of two fractions from the same section of chain and C the result if γ-globulin consists of two types of molecule, such as euglobulin and pseudoglobulin, one of which gives rise to I + III and the other to II + III. If either B or C were correct then the yield of III should exceed the sum of the yield of I and II. In fact the yield of each of the three fractions is very similar, as would be expected in A. In B and C the overall recovery, in view of the molecular weights found, would be 130 000/188 000, i.e. 70 %, considerably lower than the experimental figure of about 90 %, which again is

Table 5. *Specific activities of fractions from γ-globulin after injection of algal* [^{14}C]*amino acids*

Standard error was less than 5 %.

Time after injection (hr.)	Counts/min. (on 1 cm.^{2} disk at infinite thickness) I	II	III
1	22	25	25
4	80	84	83

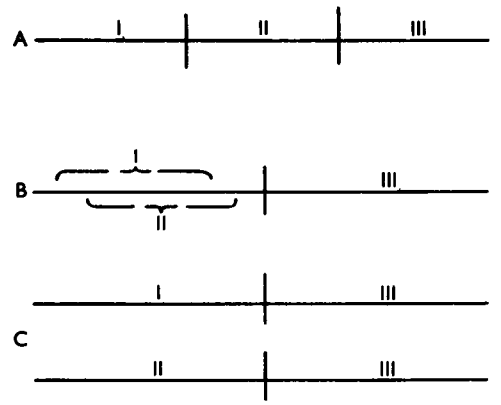

Fig. 6. Different ways of splitting rabbit γ-globulin to give three fractions of approximately equal size. For explanation see Discussion.

that which would be expected if A were correct. Similarly, with the individual amino acid residues, the recoveries would be only 70%, with the possibility of big variations in individual cases as much more material would be lost. In fact the recovery of residues is 90–95%, and the variation 83–120% in the recoveries of individual amino acids is close to the range expected from the errors in the different measurements and the assumptions made in the calculation.

Further, in B it would be expected that if I and II were digested further by papain, either interconversion would occur or both would be reduced to a slightly smaller common product. In fact both appear to be stable to a further 16 hr. digestion with papain at 37°, being unchanged in chromatographic behaviour and other properties. The simplest explanation of our results therefore seems to be that γ-globulin has been split into three distinct fractions, as shown in A. It is probable that the inhibitor described in the earlier work (Porter, 1950*b*) was a mixture of fractions I and II, and that fraction III was prevented from crystallizing by the impurities introduced with the crude enzyme preparation.

It follows that two large sections of γ-globulin (each about 30% of the whole) are extremely similar in chemical, physical and biological properties. The finding of such close agreement between the amino acid analysis of these two fragments and also an almost identical antigenic specificity suggests that there may be almost a repeat of the amino acid sequence and configuration. This is in contrast with the properties of fraction III, which differs in every respect, so that there appears to be a large repeating unit (I or II) joined to a larger section (III) of entirely different character. This unusual make-up of the γ-globulin molecule is presumably related to its antibody activity. The similarity of the pieces with mol.wt., 50 000, where the antibody-combining sites may be very much smaller (Kabat, 1956), raises the question whether large sections are required to maintain the structural integrity of a small combining site or whether antibody-combining sites may occur anywhere in these pieces.

The big quantitative difference between the inhibitory power of fractions I and II, when derived from antiprotein or antipolysaccharide antibodies, may reflect important differences between the two types of combining site, but perhaps more probably arises from differences in the speed and mechanism of precipitation between the two systems.

The significance of the part of the γ-globulin molecule represented by fraction III in antibody–antigen reactions is not known. The ease of crystallization could be taken as evidence of greater identity of structure among individual molecules in III than in the whole molecule, which appears to be complex by all available physical and biological data (see Porter, 1958*b*). Preliminary electrophoretic examination, however, suggests that it is as complex as the original molecule by this criterion. Most of the antigenic sites of γ-globulin appear to be on III, but as these sites can only be defined in relation to a given antiserum (Porter, 1957*b*), variable results might be expected, and there is in fact a difference in this respect between the goat and rat anti-γ-globulin serum. Fraction III is remarkably stable. After precipitation by 5% trichloroacetic acid at room temperature, washing with ethanol and ether and drying for 1 hr. at 110°, it will redissolve in 0·05N-acetic acid and it retains its power specifically to precipitate goat antirabbit γ-globulin.

The finding that γ-globulin appears to be built of three sections, one of exceptional stability and the other two containing the antibody-combining sites, is reminiscent of Pauling's (1940) theory of antibody formation, in which it is suggested that antibody molecules consist of a rigid centrepiece and two flexible ends capable of taking up configurations complementary to the antigen and hence forming antibody-combining sites on these flexible parts. Pauling further suggested that the flexibility might be due to a high content of proline residues. There is no evidence on the relative positions of fractions I, II and III in the whole molecule, except that II may be from the *N*-terminal end. Nor is there any evidence on the essential feature of Pauling's theory, that the amino acid sequence of all antibodies is identical and that the different antibody-combining sites are formed only by refolding of the same polypeptide chain. The proline content of I and II is less than that of III, whereas the cystine content, often an important feature in determining the stability of a protein molecule, is lower in III than in I and II.

The equal rate of incorporation of radioactive amino acids into the different fractions is in agreement with the view that the whole molecule is synthesized simultaneously from the same pool of amino acids.

SUMMARY

1. Rabbit γ-globulin, when digested by crystalline papain, gives three fragments which together form 90% or more of the original molecule.

2. If the γ-globulin contains antibodies, two fragments (I and II), of molecular weight 50 000–55 000, retain the power to combine with the antigen. The third fragment (III), molecular weight about 80 000, crystallizes easily and has much of the antigenic specificity of the original molecule.

3. I and II are extremely similar in chemical and biological properties, and III differs very widely in all respects. This has led to the suggestion that rabbit γ-globulin is formed of two pieces with very similar structure joined to a third piece of quite different character.

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ADDENDUM

Ultracentrifugal Examination of Digestion Products from Rabbit γ-Globulin

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Sedimentation-velocity experiments were carried out at 59 780 rev./min. in a Spinco model E ultracentrifuge, the technique and calculation of results being as described by Charlwood (1952, 1955). Molecular-weight determinations were by a method suggested originally by Archibald (1947), the procedure used being that of Charlwood (1957). For these determinations the ultracentrifuge was run at 8210 or 12 590 rev./min. according to the sample being studied. Protein concentrations were measured in terms of the refractive increment between the protein solution and buffer against which it had been dialysed until in equilibrium. The differential refractometer used was of the type introduced by Cecil & Ogston (1951). A cell with a Kel-F (polychlorotrifluoroethylene) plastic centrepiece was used for most experiments in acetate buffer. In all calculations the partial specific volume was taken arbitrarily as 0·735, because no experimental values were available.

When digests of the γ-globulin were dialysed against sodium phosphate buffer (pH 6·8; *I* 0·2), some material (later identified with fraction III) precipitated, but the soluble part, on examination at top speed in the ultracentrifuge, showed a single, fairly symmetrical peak of sedimentation coefficient about 3·5s. The results of some of the experiments made with the isolated fractions are shown in Table 1, together with the corresponding figures for untreated γ-globulin. In all cases, with a possible exception referred to below, preparations were monodisperse in the ultracentrifuge.

The sedimentation coefficients of all three fractions were approximately the same (~3·5s) at the concentrations used in most of the experiments (0·65–0·82 %), and measurements made at lower concentrations, but not given in Table 1, showed that the concentration-dependence was small (not more than 3–4 % for a change in protein concentration of 1 %, w/v). Molecular weights measured

Table 1. *Molecular weights of digestion products from rabbit γ-globulins*

Rabbit γ-globulin was prepared by sodium sulphate fractionation of serum (Kekwick, 1940).

Source	Sample	Buffer	Refractive increment of protein solution	$S_{20,w}$	Molecular wt. ± S.D.
Rabbit γ-globulin (batch 1)	Whole globulin	Phosphate (pH 6·8; *I* 0·2)	0·00149	6·50 s	187 600 ± 1400
Rabbit γ-globulin (batch 2)	Fraction I	Phosphate (pH 6·8; *I* 0·2)	0·00147	3·59 s	49 600 ± 1800
	Fraction II	Phosphate (pH 6·8; *I* 0·2)	0·00138	3·55 s	52 600 ± 2600
	Fraction III	Acetate (pH 4·0; *I* 0·2)	0·00149	3·40 s	80 200 ± 9100
Rabbit γ-globulin (batch 3)	Fraction I	Phosphate (pH 6·8; *I* 0·2)	0·00132	—	50 400 ± 2000
	Fraction II	Phosphate (pH 6·8; *I* 0·2)	0·00118	—	55 700 ± 2000
	Fraction III	Acetate (pH 4·5; *I* 0·2)	0·00133	—	80 200 ± 1600

at the finite concentrations indicated should therefore not differ greatly from values which would be obtained by extrapolation to infinite dilution.

The molecular weights quoted in Table 1 were each derived by taking the mean value calculated from five photographic exposures made at 8- or 16-min. intervals. The standard deviations of individual values about the corresponding mean are given in the last column as a measure of internal consistency. Comparisons between means must in addition take into account the uncertainty residing in refractometer measurements, which amounted to about ±0·6%, but which could affect a molecular weight to the extent of about ±0·9%. Agreement between different measurements on corresponding fractions is considered to be reasonably good when allowance is made for these sources of variation. The only unsatisfactory set of measurements in Table 1 refers to fraction III at pH 4·0, where very erratic results were obtained, with some suggestion of a downward trend with increasing time. Moreover, there was a detectable asymmetry of the peak obtained in the ultracentrifuge at high speed, although the sedimentation coefficient of the maximum ordinate (3·40 s) was lower than for similar preparations at pH 4·5, which gave about 3·7 s. It was concluded that complications, which may include some aggregation and some molecular-shape change, make pH 4·0 unsuitable for investigation of fraction III.

The sum of the molecular weights of the three fractions is in good agreement with the molecular weight of whole γ-globulin, the figure for which corresponds fairly well with that most commonly accepted (170 000; see Porter, 1959).

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The *N*-Acetylation and Estimation of Hexosamines

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N-Acetylglucosamine is usually determined by Morgan & Elson (1934) colour reaction, as modified by Aminoff, Morgan & Watkins (1952). A more recent modification by Reissig, Strominger & Leloir (1955) is greatly superior on grounds of sensitivity, convenience and reproducibility, and in addition is unaffected by small quantities of acid or alkali in the test sample. Glucosamine in relatively large amounts does not interfere in the Morgan–Elson reaction.

The standard procedure for the estimation of glucosamine calls for preliminary treatment with alkaline acetylacetone (Elson & Morgan, 1933; Rondle & Morgan, 1955); under the usual conditions *N*-acetylglucosamine interferes to some extent. Interference by other tissue constituents is greater than in the *N*-acetylglucosamine determination. Conversion of glucosamine into *N*-acetylglucosamine as a prelude to assay by the Morgan–Elson reaction offers certain advantages