The Fractionation of Bovine γ -Globulin by Partition Chromatography

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In earlier papers (Porter, 1955*a*; Humphrey & Porter, 1956; Askonas, Humphrey & Porter, 1956) it has been shown that rabbit γ -globulin could be partially resolved into a series of overlapping components by the use of partition chromatography. It was found that antibodies were localized in certain fractions, depending on the stage of immunization of the rabbit. Evidence that different tissues synthesized different fractions at varying rates was also obtained and this led to the suggestion that distinct cell types might be responsible for the synthesis of the individual globulin fractions.

We wondered if there were any chemical differences between the γ -globulin fractions, and chose the *N*-terminal acid as a distinctive characteristic to follow.

Rabbit γ -globulin has only one *N*-terminal amino acid, alanine (Porter, 1950), but the rabbit is unique, among the small number of species investigated, in having γ -globulin homogeneous in this respect. Bovine γ -globulin, for example, has five different *N*-terminal amino acids, none of which are present in integral molar proportions. We have tried in this work to decide whether these fractions of bovine γ -globulin with different *N*-terminal amino acids might correspond to fractions synthesized by different cells, or whether there is another explanation of their chemical differences. Bovine γ -globulin has been fractionated by partition chromatography and the *N*-terminal amino acids of different fractions have been estimated. By analogy with the results obtained with rabbit γ -globulin it would be expected that a separation into fractions related to site of synthesis would occur and hence, if *N*-terminal amino acids are characteristic of the cells synthesizing different fractions, fractionation of the terminal amino acids should occur.

In fact, fractionation of some but not all the components having different *N*-terminal amino acids was achieved and this, taken in conjunction with results on the β -globulin fraction and other work, has led to the conclusion that it may be necessary to postulate two kinds of bovine γ -globulin.

It is suggested that electrophoresis of rabbit serum at pH 8.6 separates a fraction, γ -globulin, which is biologically distinct from other fractions in that it has a different site of synthesis and is antibody, or closely related to antibody. Electrophoresis

of cattle serum under the same conditions does not give the same resolution. The antibody-type globulin appears to be principally in the γ -fraction, though this may not always be true; but there is also, in the γ -fraction, globulin which in site of origin and chemical composition is very similar to β -globulin. This seems to be the most probable explanation of the difference in *N*-terminal acid composition between rabbit and bovine γ -globulin.

EXPERIMENTAL

Materials

Celite 545 (Johns-Manville and Co. Ltd., London, S.W. 1) was washed and graded (Humphrey & Porter, 1956) and the dried Celite treated with dimethyldichlorosilane as described previously (Porter, 1955*b*).

Diethyl Carbitol (diethylene glycol diethyl ether) was purchased from Union Carbon and Carbide Co. Ltd., New York. It was distilled, after addition of SnCl_4 , at reduced pressure in an atmosphere of nitrogen. Only material with an optical density less than 0.1 in a 1 cm. cell at 2800 Å was used. Bovine γ -globulin was purchased from Armour Laboratories, Ltd., Chicago. No contamination by other serum proteins could be detected by paper electrophoresis. Other samples were also prepared from bovine serum by zone electrophoresis at pH 8.6, treated cellulose being used as support (Porath, 1956).

Chromatography

No success was achieved when the conditions described for the chromatography of rabbit γ -globulin (Porter, 1955*a*) were used with bovine γ -globulin. A variety of alternative systems were therefore investigated and satisfactory conditions were found with diethylene glycol diethyl ether as the organic solvent together with potassium phosphate (pH 9) and a 10% (w/v) solution of urea in water. The final concentration of urea was about 5% and at this strength it increased the solubility of the γ -globulin without causing any detectable denaturation. This is similar to the effect of urea on pepsin reported by Steinhardt (1938).

The composition of the phase mixture was 2.5 M-potassium phosphate at pH 9 (2.12 kg.), 10% (w/v) aqueous urea (2.672 kg.) and diethylene glycol diethyl ether (548 g.). The two liquid phases produced are temperature labile and chromatography was therefore carried out in a room at $2 \pm 1^\circ$. The column and phase mixture were jacketed as previously described (Porter, 1955*a*) and were held at $6 \pm 0.1^\circ$. Under these conditions the bovine γ -globulin partitioned in favour of the diethylene glycol diethyl ether-rich phase and this phase was therefore made the stationary phase, silane-treated Celite 545 being used as support.

Silane-treated Celite (60 g.) was used to prepare a column (2.4 cm. $\times$ 33 cm.) and 40 mg. of bovine γ -globulin, dissolved in 20 ml. of the aqueous phase, was fractionated on this column. Fractions (16 ml.) were collected by an automatic fraction collector and measured quantities were diluted to estimate the protein content of each fraction.

The protein content was estimated by measuring the absorption at 2800 Å in a spectrophotometer. The fractions were combined in five groups and concentrated to about 3 ml. by dialysis under a pressure of 1 atmosphere at 2°. The concentrated solution of globulin was precipitated with ethanol (2 vol.) and the precipitates were washed with ethanol and ether.

Assay of N-terminal amino acids

The N-terminal amino acids were estimated by the fluorodinitrobenzene (FDNB) method of Sanger (1945). The technique used has been described (Porter, 1957). Dinitrophenyl (DNP) globulin (100–150 mg.) was hydrolysed by boiling in 5.7N-HCl for 24 hr. The hydrolysate was extracted with ether and the ether extract, which contained the acidic DNP amino acids, was fractionated by paper chromatography on Whatman no. 4 paper with the solvent system *tert.*-amyl alcohol (2-methylbutan-2-ol)–0.05M-potassium phthalate at pH 6 (Blackburn & Lowther, 1951).

Fig. 1. Chromatography of bovine γ -globulin: (a) 40 mg. of globulin run on a column (diam. 2.4 cm.) containing 60 g. of silane-treated Celite 545; (b) fraction x rerun on the same column; (c) fraction y rerun on the same column; (d) fraction z rerun on the same column.

RESULTS

In Fig. 1 is shown the chromatogram obtained when bovine γ -globulin is fractionated according to the method described. The curve is similar to that obtained with rabbit γ -globulin (Porter, 1955a) in that a broadly spread peak is produced, but the bovine globulin gives a more symmetrical peak with the maximum in the earlier running fractions. It can be seen that when individual fractions were run a second time on the same column they appeared with a distinct characteristic *R* value, and it is therefore concluded that a genuine fractionation had occurred although no components were completely separated from each other. The recovery of

Table 1. N-Terminal amino acids of bovine γ -globulin

Calculated on a molecular weight for γ -globulin of 160 000.

Amino acid	Recovery* (%)	Amount of N-terminal acid (mole/mole of protein)	
		Commercial preparation	Electrophoretic preparation
Aspartic acid	50	0.14	0.23
Glutamic acid	60	0.05	0.06
Serine	70	0.11	0.14
Alanine	60	0.10	0.09
Valine	75	0.38	0.35
Total		0.78	0.87

* These values are the over-all recovery of DNP-amino acids after hydrolysis in the presence of DNP-bovine- γ -globulin and paper chromatography (Porter, 1957).

Fig. 2. Chromatography of bovine γ -globulin, showing cuts made to give the five fractions used for N-terminal acid assay. (Conditions were as for Fig. 1.)

Table 2. N-Terminal amino acids of bovine γ -globulin fractions

Calculated on a molecular weight for γ -globulin of 160 000.

Amino acid	Amount of N-terminal acid in fractions (mole/mole of protein)				
	A	B	C	D	E
Aspartic acid	0.17	0.14	0.13	0.08	0.07
Glutamic acid	0.04	0.04	0.04	0.04	0.04
Serine	0.13	0.09	0.08	0.06	0.07
Alanine	0.14	0.10	0.06	0.00	0.00
Valine	0.33	0.32	0.39	0.38	0.36
Total	0.81	0.69	0.70	0.56	0.54

γ -globulin from the column was 60–70%, rather lower than that obtained with rabbit globulin.

The results of assay of the *N*-terminal amino acids of bovine γ -globulin are given in Table 1. They are in qualitative agreement with those reported by McFadden & Smith (1953) but show some quantitative differences, particularly in the quantities of valine, for which we found a higher value, and of glutamic acid, for which we obtained a lower value. These differences may be due to the samples used, though the agreement between the results obtained with the commercial product prepared in America by ethanol fractionation and our own preparation made by zone electrophoresis is comparatively good.

Fig. 2 shows the cuts made to give the five fractions used for terminal amino acid assay, and the results obtained are given in Table 2. As no clear-cut separation into individual fractions was obtained by chromatography, no sharp localization of the terminal acids could be expected. The early fractions contained all of the terminal alanine and most of the terminal serine and aspartic acid. The terminal valine is present almost equally in all fractions. Terminal glutamic acid is also present in all fractions, but the small amount present makes the quantitative significance of these figures doubtful.

DISCUSSION

A feature of the chromatography was the very marked difference in the behaviour of γ -globulins from different species in the phase systems investigated. Human, bovine and rabbit γ -globulins showed quite different solubilities, partition coefficients and stabilities in these systems and it was not possible to find a common chromatographic system. These differences were unexpected, as γ -globulins from these species are very similar in molecular weight, amino acid composition and solubility in aqueous organic solvents and aqueous salt solutions.

The total number of *N*-terminal amino acids in the original material is less than 1 mole/mole of protein. Neither of the labile DNP amino acids (DNP-proline and DNP-glycine) could be detected under appropriate conditions. Denaturation of the protein, by ethanol or 2½% trichloroacetic acid for 1 hr., before reaction with FDNB did not influence the values obtained. No significant content of protein with a molecular weight higher than 160 000 was found in the preparations when examined in the ultracentrifuge. It is therefore concluded that the α -amino groups of some molecules fail to react with FDNB because they are blocked, perhaps by carbohydrate; alternatively, these molecules are cyclic and contain no α -amino groups.

The *N*-terminal amino acids aspartic acid, serine

and alanine are concentrated in the faster-running fractions whereas valine is not. In view of the finding that rabbit β -globulin runs faster in this type of partition chromatography than γ -globulin, the *N*-terminal acids of β -globulin were estimated. The β -globulin was prepared by zone electrophoresis at the same time as the γ -globulin whose *N*-terminal acids are given in Table 1. The results are given in Table 3. Threonine was found to be present, also aspartic acid, glutamic acid, serine and alanine were in much higher concentration than in γ -globulin, and valine was absent.

This suggests that the complexity of the *N*-terminal acids in the γ -globulin may have resulted from overlap with β -globulin. However, electrophoresis experiments at pH 8.6 showed that our γ -globulin preparations could not have contained significant β -globulin contaminants.

It is probable that in bovine serum, in contrast to rabbit serum, electrophoresis at pH 8.6 produces a fraction (γ -globulin) which is a mixture of two types of protein. One is antibody-type globulin, which alone forms the γ -globulin in rabbit serum, and the other is similar in chemical composition and probably in origin to the β -globulins. Partition chromatography has produced a partial resolution of these two types, which are not separated by electrophoresis. If this were correct, then antibody-type bovine globulin would appear simple by *N*-terminal assay as does rabbit γ -globulin. The end group would be valine but it would be present in less than 1 mole/mole of protein, owing to the presence of a component without reacting α -amino groups. Support for this suggestion is given by *N*-terminal acid assay, by McFadden & Smith (1953), on γ -globulin from a cow which had been strongly immunized with mixed antigens. They reported a much higher content of *N*-terminal valine and only traces of other *N*-terminal amino acids, as would be expected if, under these circumstances,

Table 3. *N*-Terminal amino acids of bovine β -globulin

Calculated on a molecular weight for β -globulin of 160 000.

Amino acid	Recovery* (%)	Amount of <i>N</i> -terminal acid (mole/mole of protein)
Aspartic acid	50	0.68
Glutamic acid	60	0.14
Serine	70	0.40
Alanine	60	0.15
Threonine	95	0.17
Total	—	1.54

* These values are the over-all recovery of DNP-amino acids after hydrolysis in the presence of DNP-bovine- γ -globulin and paper chromatography (Porter, 1957).

the γ -globulin consisted very largely of the antibody-type globulin. If bovine antiserum against a pure antigen were available, end-group assay of the specific precipitate would be a satisfactory test of this idea, but so far such material has been unobtainable.

SUMMARY

1. Bovine γ -globulin has been fractionated into a series of overlapping fractions by partition chromatography.

2. *N*-Terminal amino acid assay of the fractions shows that a partial fractionation of components having the *N*-terminal acids, aspartic acid, serine and alanine, has been achieved, but the γ -globulin with *N*-terminal valine has not been fractionated.

3. Comparison of the *N*-terminal assay of β - and γ -globulins, and other evidence, has led to the suggestion that in the cow a part of the γ -globulin may be closely related to β -globulin.

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An Improved Method for the Microdetermination of Arginine by Use of 8-Hydroxyquinoline

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The colorimetric reaction of arginine with α -naphthol (Sakaguchi, 1925*a, b*) is simple, specific and sensitive but not reliable as a quantitative method. The colour yield is not reproducible nor is there linearity between colour yield and amount of arginine, mainly because of the instability of the coloured product under the usual conditions of the reaction.

There is some improvement when 8-hydroxyquinoline is substituted for α -naphthol (Sakaguchi, 1948), for the blank then remains almost uncoloured and the reagent is more stable. The reaction is similar for both reagents, which in the presence of alkaline hypobromite or hypochlorite form an intensely coloured compound with arginine. This compound is subsequently rapidly destroyed by hypobromite and the destruction is delayed, but not blocked, by addition of urea in excess. The rate of disappearance of the colour is quite different with different batches of urea, and this is the major drawback of the method with 8-hydroxyquinoline. There are other drawbacks to the original method (Sakaguchi, 1948) which hinder its quantitative application, namely the variability of the amount of hypobromite used and the formation of nitrogen bubbles on the walls of the cuvettes.

In the present work the colour formed by the reaction of arginine with 8-hydroxyquinoline has been stabilized by extraction into butanol, the addition of urea being avoided. The different variables which may interfere in the reaction have been studied and the most suitable conditions for quantitative application have been determined.

EXPERIMENTAL

Materials

8-Hydroxyquinoline (Carlo Erba). A solution (0.4%) of 8-hydroxyquinoline is made in 95% (v/v) ethanol. To 5 ml. of this solution 60 ml. of *N*-NaOH is added immediately before use.

Sodium hypobromite. The hypobromite is prepared freshly by dissolving 0.7 ml. of Br_2 in 50 ml. of 10% (w/v) NaOH. The solution is then diluted tenfold with water (final concentration of hypobromite, 0.15%).

Arginine hydrochloride (Merck). The product was stored in a desiccator for several days and its purity checked by paper chromatography and by N determination. Standard solutions at concentrations from 1 to 8 $\mu\text{g./ml.}$ were prepared each time.

Butanol (Carlo Erba). No purification was found necessary.