Human Serum Protein Fractionation by Gel Filtration

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The development of a quantitative immunological technique using polyvalent antiserum permits a more logical approach to the fractionation of complex protein mixtures. In this study whole serum was separated by conventional gel filtration and the fractions obtained were analysed. This demonstrates over 60 immunologically distinct serum proteins. Because the current terminology is inadequate to describe this number of proteins, a temporary numerical nomenclature has been used.

There are now a number of systems for fractionating the complex protein mixture found in biological systems. However, for the majority of proteins there has been no analytical system for measuring the relative amount of each protein in the various fractions obtained. A quantitative immunological technique using anti-(whole human serum) permits a more logical approach, and has been applied to one biological system (whole human serum) by using one fractionation system (gel filtration). The present study shows the elution characteristics of some 60 immunologically distinct serum proteins; these include 12 proteins that have been identified with certainty and whose characteristics are consistent with those in the literature. The remaining 51 proteins have not been identified. Some of their characteristics (electrophoretic mobility, solubility in ammonium sulphate) are given.

MATERIALS AND METHODS

Serum and storage of samples. Normal acid-citrate-dextrose plasma was collected from 42 healthy British blood donors with an equal sex distribution and ages ranging from 20 to 65 years. The pooled plasma was clotted by the addition of Ca^{2+} . The serum was stored at -25°C until gel filtration was performed. Fractions obtained were pressure-dialysed and stored at -25°C .

Gel filtration. Serum (150 ml; protein concentration 5.9 g/100 ml) was applied to a Sephadex G-150 column (180 cm $\times$ 8 cm), previously equilibrated with 0.5 M-NaCl–0.2 M-(Na_2HPO_4 – NaH_2PO_4) buffer (pH 7.2)– NaN_3 (0.2 g/l). A constant flow rate of 1.2 ml/min was maintained by a peristaltic pump: after passage through a u.v. recorder (254 and 280 nm) both the samples were collected by using a drop counter (M.B.I.) and fraction collector.

Pressure dialysis. Volumes were decreased to a predetermined value throughout by ultrafiltration through Visking tubing (6.3 mm diam.), by tying a glass container of appropriate volume into the bottom of the dialysis tubing. This procedure allows one to leave the system

running overnight without fear of concentrating the sample to dryness, and avoids the very high protein concentrations that can be observed at the bottom of the usual tied-off sac. Detailed analysis of whole serum before and after dilution with 100 vol. of 0.9% NaCl + 0.1 M- Na_2HPO_4 – NaH_2PO_4 buffer, pH 7.2, followed by pressure dialysis to the original volume showed significant changes only in α - and β -lipoproteins. Experiments with iodine trace-labelled proteins indicated that protein loss does occur during this procedure, but the mass lost is roughly proportional to the area of membrane, and seems to be non-selective.

Immunoelectrophoretic analysis. The method used was the same as that described by Clarke & Freeman (1966, 1968) and is a modification of the two-dimensional crossed-antigen–antibody immunoelectrophoresis described by Laurell (1965). In brief, it consists of an initial separation of proteins in agarose [by using 0.03 M-barbitone buffer, pH 8.6, containing calcium lactate (0.38 g/ml)]. After this separation is complete (10 V/cm for 1 h), a second gel containing anti-(whole human serum) is poured alongside the separated protein, and a second electrophoresis (2.5 V/cm) is performed at right angles to the first. This is allowed to continue until all protein precipitation is complete (22 h). The plate is then washed, dried and stained, and the areas under each protein peak are measured. These areas are directly proportional to the concentration of antigen applied, and inversely proportional to the concentration of antibody in the antiserum used (Clarke & Freeman, 1966).

To obtain a better separation some of the first-dimension electrophoretic runs in the present study have been longer, with plates measuring 25.5 cm $\times$ 10.5 cm. A single pool of antisera has been used throughout; this was raised in goats by using Freund's complete adjuvant, and preceding each bleed with a course of intravenous aluminium precipitated protein. The goat serum obtained was precipitated with 1.8 M- and then 1.6 M-(NH_4)₂ SO_4 to remove most of the non-immunoglobulins. In all human fractions an internal control of acetylated human albumin was used; this has a greater electrophoretic mobility but retains its antigenic properties. Thus it can be measured independently from albumin by this technique. A fixed weight of acetylated human albumin was added to each

fraction before concentration; subsequent values were then expressed in relation to this protein. By this means pipetting errors and errors due to unselective loss of protein on the dialysis membrane were eliminated.

Demonstration of identity. It is obvious that any single

protein will appear in more than one of the arbitrary fractions. Thus it was necessary to demonstrate whether or not a protein appearing in one plate was the same as that in another. Several techniques were used. (1) A number of different antisera, raised against glycoproteins

Table 1. *Analysis of the 15 protein fractions*

For experimental details see the Materials and Methods section. *, **,

Protein no.	Name	Mobility (% with respect to AcA†)	Total area (arbitrary units)	B 1	C 2	D 3	F 4
1	Thyroxine-binding prealbumin	70.9	—	—	—	—	—
3	α_1 -Lipoprotein	65.5	5374	—	—	—	14.8
5	Albumin	50.1	—	—	—	—	—
7	Orosomucoid	49.5	—	—	—	—	—
9		47.8	330	—	—	—	—
11	Caeruloplasmin (denatured)	46.9	501	—	—	—	—
13		46.3	140	—	—	—	—
15		44.7	1429	—	—	—	—
17	α_1 -Easily precipitable glycoprotein	44.5	526	—	—	—	—
19	α_1 -Antitrypsin	43.6	—	—	—	—	—
21	β -Lipoprotein	42.7	197	33.0	40.0	27.0	—
23		40.7	1162	—	—	—	—
25		39.7	507	—	—	—	—
27		38.8	23	—	—	—	—
29		38.5	1499	—	—	—	—
31	Caeruloplasmin	38.4	1242	—	—	—	—
33		38.4	250	—	—	—	—
35		38.0	1098	—	—	18.9	36.7
37		37.9	1481	—	—	33.3	66.7
39		37.2	248	—	—	—	—
41		35.4	1904	—	—	—	—
43		35.0	248	—	—	—	—
45		34.7	3803	—	—	—	—
47	α -Group specific component	34.4	1146	—	—	—	—
49		33.3	115	—	—	—	—
51		33.1	974	—	—	—	—
53		32.6	1096	—	—	—	11.0
55	α_2 -Macroglobulin	32.1	319	19.4	42.1	28.5	—
57		31.7	412	—	—	—	—
59	Haptoglobin	31.5	2169	2.9	21.2	30.2	21.2
61		31.3	87	—	—	—	100.0
63		30.8	4853	—	—	—	—
65		30.6	304	—	—	—	55.6
67		30.4	168	—	—	—	—
69	α_2 HS	30.2	—	—	—	—	—
71		29.8	809	—	—	—	—
73		29.4	—	—	—	—	*
75		26.8	72	—	—	—	—
77		26.2	105	—	—	—	—
79		26.1	4388	—	—	—	—
81		25.4	1268	—	—	6.0	33.1
83		24.5	389	—	—	—	—
85		22.2	479	—	—	—	—
87		22.0	1717	—	—	—	—
89	Haemopexin	21.3	5454	—	—	—	—
91	β_1 A-C(C'3)	19.1	486	—	—	—	—
93	Transferrin	18.2	4251	—	—	—	—
95		17.8	672	—	—	—	6.6
97		17.4	295	—	—	—	—
99		16.5	869	—	—	—	—
101		15.7	261	—	—	—	—
103		15.1	1020	—	—	—	—
105	Gamma A	12.5	1472	—	—	11.3	34.8
107		12.3	320	—	—	—	—
109		11.7	339	—	—	18.2	33.9
111		11.3	24	—	—	—	—
113		10.7	1163	—	—	—	—
115		9.9	140	—	—	—	—
117		9.7	312	—	—	—	—
119		8.6	163	—	—	—	—
121		5.4	278	—	—	—	—

† AcA, Acetylated human albumin.

Fractions from Sephadex G-150 column											Precipitation in (NH ₄) ₂ SO ₄ , pH 5.0 (% saturation)
G 5	H 6	L 7	M 8	N 9	P 10	R 11	S 12	T 13	V 14	Z 15	
—	—	—	—	—	—	*	**	***	***	**	—
21.1	18.6	15.1	16.5	6.4	7.5	—	—	—	—	—	—
—	—	—	—	—	*	**	***	***	**	*	50-60
—	—	—	—	—	—	*	***	***	***	*	40-50
—	—	—	—	5.5	32.7	43.2	14.3	4.2	—	—	40-50
—	—	12.1	60.7	27.3	—	—	—	—	—	—	30-40
—	—	—	—	—	—	100.0	—	—	—	—	—
—	14.5	15.9	31.6	26.9	—	—	—	—	—	—	—
—	—	—	—	—	13.2	42.0	24.9	15.7	4.2	—	40-50
—	—	—	—	—	—	*	*	**	***	*	40-50
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	57.3	42.7	—	—	—	—	—	—	—
—	—	—	—	—	—	49.8	50.2	—	—	—	40-50
—	100.0	—	—	—	—	—	—	—	—	—	30-40
—	—	—	—	2.0	9.3	43.4	23.9	18.9	5.4	—	40-50
—	—	3.9	32.2	38.2	25.8	—	—	—	—	—	30-40
—	—	—	—	—	100.0	—	—	—	—	—	—
18.1	22.7	3.5	—	—	—	—	—	—	—	—	40-50
—	—	—	—	—	—	—	—	—	—	—	30-40
—	—	—	—	—	100.0	—	—	—	—	—	—
—	—	—	—	—	—	9.8	2.9	15.9	48.7	22.7	40-50
—	—	—	—	—	40.5	59.5	—	—	—	—	50-60
—	—	—	—	—	—	2.0	5.6	25.0	55.2	12.2	50-60
—	—	—	—	—	—	22.5	29.9	27.8	17.1	2.6	40-50
—	58.9	41.1	—	—	—	—	—	—	—	—	30-40
—	—	—	—	—	—	45.2	22.1	24.5	8.1	—	—
—	—	—	—	—	—	—	—	25.4	74.6	—	40-50
—	—	—	—	—	—	63.7	36.3	—	—	—	40-50
8.3	5.8	5.9	2.9	0.3	—	—	—	—	—	—	40-50
—	—	—	—	—	15.8	39.2	19.6	16.7	8.7	—	40-50
44.4	—	—	—	—	—	—	—	—	—	—	0-30
—	—	—	31.9	68.1	—	—	—	—	—	—	—
—	—	—	—	*	*	**	***	***	**	*	40-50
—	—	77.8	22.2	—	—	—	—	—	—	—	—
**	**	—	—	—	—	—	—	—	—	—	30-40
31.3	32.7	36.0	—	—	—	—	—	—	—	—	—
53.8	46.2	—	—	—	—	—	—	—	—	—	30-40
—	—	—	—	—	1.2	15.9	21.3	30.5	31.2	—	50-60
46.9	14.0	—	—	—	—	—	—	—	—	—	40-50
—	—	—	—	—	—	—	—	100.0	—	—	40-50
—	—	—	—	100.0	—	—	—	—	—	—	—
—	—	—	—	—	19.4	28.6	20.6	20.2	11.1	—	50-60
—	—	—	—	—	20.3	37.4	16.5	15.8	9.9	—	40-50
23.1	49.9	18.8	8.2	—	—	—	—	—	—	—	30-40
—	—	—	—	0.5	9.6	33.6	19.5	22.1	14.6	0.4	

proteins results in a number of horizontal lines across the plate which are continuous with their respective protein peaks. In this way proteins that have antigenic identity but different mobility can be demonstrated, e.g. caeruloplasmin and α_1 -apocaeruloplasmin (a degradation product). (5) By labelling fractions with ^{125}I , with the iodine monochloride technique (McFarlane, 1958) trace amounts of radioactive proteins can be added to other fractions or to whole serum and their presence revealed by radioautography. (6) Finally, selective staining can in some instances be used, e.g. for lipoproteins, though the results can be misleading; for example the α_1 -caeruloplasmin degradation product does not show oxidase activity.

Ammonium sulphate precipitation. Whole human serum from the pool used for the gel filtration was diluted with 2 vol. of 0.9% NaCl and adjusted to pH 5.0 by the addition of acetic acid. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to bring the concentration to 1.2M (30% saturation) and stirred until dissolved. The solution was then placed in centrifuge buckets and left overnight at 4°C: it was then centrifuged at about 10000g for 1 h. The deposit was dissolved with a known volume of water, $(\text{NH}_4)_2\text{SO}_4$ was added to restore the concentration to 1.2M (30% saturation) and it was left overnight and centrifuged again. The deposit on this occasion was taken up in a small volume of water and pressure-dialysed to one-third the original volume of serum taken. The supernatant was added to that recovered on the previous day. Further solid $(\text{NH}_4)_2\text{SO}_4$ was added to bring the concentration to 1.6M (40% saturation). The remainder of the fractionation continued in the same manner. The final supernatant at 60% saturation was also pressure-dialysed to one-third the original volume of serum.

RESULTS

The u.v. (280nm) recording of the Sephadex column is shown in Fig. 1, indicating the 15 fractions that were taken. To each of these 15 fractions was

Fig. 1. Separation of 150ml of normal pooled human serum on Sephadex G-150, indicating the fractions taken for further analysis.

added a known weight of freeze-dried acetylated human serum albumin, the subsequent pressure dialysis being to 50ml (i.e. one-third of the original volume). Photographs of the Laurell plates resulting from each acetylated fraction are shown in Plates 1-4; the photograph of the first fraction (B) has been omitted since it contained the same proteins (though in a lower concentration) as in fraction C. The areas under each protein peak were measured and the results are shown in Table 1. A photograph of a model constructed from the results in Table 1 is given in Plate 5. It must be emphasized that the total area obtained, either from a single run of whole serum or from the sum of areas obtained for the same protein in different fractions, is related directly to the concentration of that protein in the pooled serum; but also, inversely to the concentration of antibody to that protein in the goat anti-(whole human serum) used. It follows that a protein that is a poor antigen will give a larger precipitation peak than the same quantity of another protein that is a better antigen. Thus this immunological technique is only quantitative if some standard can be employed. Since too few proteins exist in sufficiently pure form to use as a standard, a pool of whole human serum has been used as reference material for studies of protein concentrations in patients.

Nomenclature. This has been a constant problem throughout the study since at no stage have we obtained pure proteins to characterize by the usual physicochemical methods. Isolated pure proteins available from other sources can be added to whole serum to identify some individual protein peaks. However, there are still many proteins that we have been unable to identify. To overcome this a simple numerical system has been used: proteins with a fast mobility were given low numbers: thyroxine-binding prealbumin is given the number 1, albumin is given the number 5, etc. Only odd numbers were used so that the even numbers could be applied to proteins demonstrated subsequently without disturbing the sequence. Unfortunately, some of the proteins precipitated are in too low a concentration to be detected by the usual staining techniques, and thus are not included in this paper; for example α_1 -thyroxine-binding globulin can be demonstrated by adding ^{125}I -labelled thyroxine before or after separation of whole serum (Freeman & Pearson,

EXPLANATION OF PLATES 1-4

Quantitative immunoelectrophoretic analysis of each of the 15 fractions obtained from Sepadex G-150 (Fig. 1). The photograph of the first fraction (B) is omitted since it contained the same proteins as in the second fraction (C). For details see Table 1.

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1969) yet cannot be seen by simple staining. Thus there may be many other trace proteins; their demonstration may disrupt this numerical system.

DISCUSSION

The results given in Table 1 and Plates 1-4 raise a number of problems. It will be seen that many of the proteins, perhaps threequarters are inhomogeneous, by electrophoresis, molecular size, or ammonium sulphate precipitation. In general, where inhomogeneity is evident in one system, it is commonly found in another system. Indeed, that part of a protein that is away from the bulk of the protein on one system is frequently the same as that showing a difference by another fractionation system. This inhomogeneity can be due to a number of causes: first, the serum studied was a pooled sample, and presumably contained a number of different polymorphic forms; thus the inhomogeneity can be due to a mixture of sera. Secondly, the inhomogeneity may be due to denaturation of the protein during the fractionation procedure. Although considerable care has been taken to avoid conditions that are known to cause denaturation, it is probable that this has occurred. This denaturation may be (1) polymerization, (2) loss of peptides or (3) change in conformation. Fragmentation of proteins might give rise to an artificially high number of protein peaks, if the peptide detached from the main protein contained an antigenic site. If this has occurred it must have occurred completely, because no reactions of partial identity were observed. It is not possible to comment more fully on these possibilities.

EXPLANATION OF PLATE 5

Photograph of a model constructed from the results given in Table 1. It should be emphasized that a high peak may be due to either a high concentration of that protein in serum or to low antigenicity.

One other disturbing feature is apparent from Table 1; six proteins appear in one single fraction from Sephadex G-150. Although great care was taken with the Sephadex column, it seems unlikely that this was so perfect that a protein could be confined to one single fraction. The results obtained, however, were quite reproducible and the proteins concerned did not cross-react with any of the other proteins demonstrated. Thus we appeared to have proteins that react with anti-(whole human serum) that are different from the other proteins we have demonstrated, and yet appear in one fraction only. No explanation is given for these findings; we consider that this must be an artifact of some sort, though we were unable to explain its nature.

Finally, although immunoglobulin A is included in Table 1 and is shown in some of the Plates, this does not represent the whole immunoglobulin A spectrum of the patient, and the other varieties of the immunoglobulins are not represented at all, since the Laurell (1965) technique used is unsuitable in its present form for the study of the immunoglobulins. In addition proteins with a slow gamma mobility other than the immunoglobulins will not be demonstrated by this technique.

In conclusion, this fractionation of serum by gel filtration is the first of a series of studies of the conventional fractionation procedures, by using a quantitative immunoelectrophoretic method. It is hoped that this type of fractionation, taken together with other fractionation procedures such as column chromatography, will provide a basis for the isolation of individual protein components.

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