Isolation and Characterization of Fragments of Human Serum Albumin Containing some of the Antigenic Sites of the Whole Molecule

By E. M. PRESS AND R. R. PORTER

Department of Immunology, St Mary's Hospital Medical School, London, W. 2

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In an investigation of the structural features that constitute an antigenic site in bovine serum albumin, Porter (1957) isolated a fragment of molecular weight 12 000 which retained the power to combine with the specific rabbit antiserum. Attempts to split this piece further, without loss of activity, were unsuccessful, and with material of this size it seemed probable that detailed chemical investigation to locate the combining site would give equivocal results. Lapresle and colleagues (Lapresle, 1955; Lapresle, Kaminski & Tanner, 1959; Lapresle & Webb, 1960) have shown that the antigenic activity of human serum albumin will survive digestion with rabbit-spleen extracts and other enzymes. The partial hydrolysis of this protein under conditions similar to those used for bovine serum albumin (i.e. continuous digestion with chymotrypsin in a dialysis sac) was therefore studied. In preliminary work (A. Holasek & R. R. Porter, unpublished work) it appeared that active fragments of molecular weight substantially less than 12 000 could be produced. More detailed investigation has shown that a variety of such pieces are produced from human albumin and that their molecular weights range from 7100 to 23 400. Some precipitate and some combine with but do not precipitate the rabbit antiserum. The isolation and properties of four of these active pieces will be described.

EXPERIMENTAL

Materials

Chymotrypsin was three-times-crystallized α -chymotrypsin obtained from Worthington Biochemical Corp.

Human serum albumin used for preparing the antisera in rabbits and also for the immunological tests was a sample of AP₂ 26/10 kindly supplied by Dr M. E. Mackay and prepared as described by Kekwick & Mackay (1954). The albumin used for enzymic digestion was given by Dr W. d'A. Maycock, batch AP2.E1, prepared by the same method at the Blood Products Laboratory, Lister Institute, Elstree, Herts. Antiserum (1) was prepared in rabbits by giving two injections of 1 ml. of human albumin in adjuvant (Freund & McDermott, 1942) at an interval of 1 week, followed 1 month later by three injections of 0.5 ml. at intervals of 3 days of alum-precipitated human albumin

(16 mg./ml.) (Porter, 1955). The animals were bled 1 week later. This antiserum contained 24 mg. of antibody/ml. Antiserum (2) was prepared in rabbits by six injections of 0.5 ml. of alum-precipitated human albumin (16 mg./ml.), during 2 weeks, and the animals were bled 1 week later. This antiserum contained 4 mg. of antibody/ml.

Sephadex G. 25, G. 50 and G. 75 (Porath & Flodin, 1959), and diethylaminoethyl- (DEAE-) -Sephadex A. 25 were bought from Pharmacia, Sweden. Cellulose used as supporting medium in zone electrophoresis was Cellulose-pulver (Grycksbo Pappersbruk, Sweden). Starch for electrophoresis was starch-hydrolysed (Connaught Medical Research Laboratories, Toronto, Canada). The dialysis sac was of 8/32 Visking tubing. Agar for double diffusion was a 1% solution of Japanese shredded agar (British Drug Houses Ltd.) in iso-osmotic salt solution, pH 7.2 (4.25 g. of NaCl, 2.45 g. of KH_2PO_4 and 8.092 g. of $\text{Na}_2\text{HPO}_4/\text{l.}$); it was filtered and autoclaved at 15 lb./in.² for 15 min. Agar for immunoelectrophoresis was a 1% solution of Ionagar 2 (Oxo Ltd., London) in 0.1 M-phosphate, pH 7.2.

Reagents. Phosphate buffers: 0.1 M-potassium phosphate, pH 7.2 (13.6 g. of KH_2PO_4 , 4.6 g. of KOH/l.); 0.01 M-potassium phosphate, pH 7.8 (1.36 g. of KH_2PO_4 , 0.63 g. of KOH/l.); 0.04 M-potassium phosphate, pH 8.0 (5.44 g. of KH_2PO_4 , 2.52 g. of KOH/l.). Acetate buffers: 0.05 M-ammonium acetate, pH 6.5 (3.85 g. of ammonium acetate/l.); 0.02 M-ammonium acetate, pH 7.9 (1.54 g. of ammonium acetate together with aq. 3 M-ammonia to pH 7.9). For zone electrophoresis: 0.05 M-sodium formate, pH 3.2 [1.9 ml. of formic acid (98–100%), 0.52 g. of NaOH/l.]; 25 mM-sodium tetraborate, pH 9.

For molecular-weight determinations the 0.1 M-sodium acetate, pH 4.0 (0.147 g. of sodium acetate, 4.75 ml. of acetic acid), contained 2.43 g. of cysteine and 0.372 g. of EDTA (disodium salt) in 1 l. Potassium chloride-potassium phosphate, pH 7.7, contained 1.069 g. of KH_2PO_4 , 0.49 g. of KOH and 11.2 g. of KCl in 1 l.

For starch-gel electrophoresis, at pH 8.8 'tris' contained 9.2 g. of tris and 1.05 g. of citric acid monohydrate in 1 l.; at pH 3.1, 0.05 M-formate contained 1.9 ml. of formic acid (98–100%) and 0.4 g. of NaOH in 1 l. The stain was a 1.6% solution of Amido Black in methanol-water-acetic acid (50:50:10, by vol.). The same solvent was used as wash solution to develop the gel.

Methods

Tests for inhibitory power. The two methods described by Porter (1957) were used. In the semi-quantitative method antiserum (1), 24 mg./ml., was diluted 100 times with 0.1 M-phosphate, pH 7.2, and to 0.5 ml. of this solution, in a flocculation tube, was added 0.02–0.2 ml. of test solution.

After incubation for 5 min. at 37° the optimum amount of human serum albumin (0.02 ml. of human serum albumin, 0.05% in 0.1 M-phosphate, pH 7.2) was added and the time of flocculation compared with that of a control tube to which inhibitor had not been added.

In the quantitative test, the antiserum was diluted with phosphate buffer to give a concentration of 2–2.5 mg. of antibody/ml. Increasing amounts of inhibitor were added to 0.5 ml. of the diluted antiserum incubated for 1 hr. at 37° and overnight at 2°. Any precipitate formed was centrifuged off, washed three times with salt solution and resuspended in 3 ml. of 0.1 N-NaOH and the extinction read at 280 m μ in a 1 cm. cell. The supernatants were incubated with an amount of human albumin sufficient to precipitate the remaining antibody for 1 hr. at 37° and at 2° overnight and the quantity of precipitate was estimated in the same way.

Starch-gel electrophoresis. The method of Smithies (1955) and Poulik & Smithies (1958) was used. Trays were 25 cm. $\times$ 4 cm. $\times$ 6 mm. deep and the concentration of starch in tris buffer, pH 8.8, was as recommended by suppliers of the starch, but at pH 3.1 a slightly higher concentration, e.g. 12.8% instead of 12.3%, had to be used. Concentration of buffers for bridge solutions was five times that used in the gel. Protein solutions were applied by inserting pieces of Whatman no. 3 filter paper, soaked in 1% solutions, into vertical slots cut in the gel. After electrophoresis the gel was sliced horizontally and stained with Amido Black for 5 min. and developed by washing for several hours.

Double diffusion in agar. The method was as described by Ouchterlony (1953); the antiserum was undiluted and the concentrations of human serum albumin and inhibitors were chosen to give precipitation lines midway between antigen and antibody cups. The cups were loaded with 0.15 ml. of solution in a sterile hood and the plates developed for 3 days at room temperature.

Immuno-electrophoresis. The method of Grabar & Williams (1955) was modified for use with starch gel as follows. Electrophoresis was in starch gel at pH 8.8. The gel was cut horizontally and half of it stained and strips from the unstained portion, about 6 cm. $\times$ 0.5 cm., were laid on a thin layer of agar on a photographic plate (8 cm. $\times$ 8 cm.) about 0.5 cm. from a trough (6 cm. $\times$ 1 mm.) cut in the agar and containing antiserum. The plate was developed for 24 hr. in a moist box.

Zone electrophoresis. This was carried out in cellulose columns as described by Porath (1956).

Determinations of molecular weight. Molecular weights were calculated from values of *S* and *D* obtained from double-sector synthetic boundary cell runs in the ultracentrifuge. Plots of the data according to Van Holde's (1960) modification of Fujita's method showed that there was no measurable effect of sedimentation upon boundary spreading. The molecular weight of inhibitor Ia (Scheme 1) was also calculated by Trautman & Crampton's (1959) plot of approach to equilibrium data and found to be 25 000, compared with 23 400 calculated from *S* and *D*. The value of $\bar{v}$ used (0.729) is that given for serum albumin by Jaenicke & Gehatia (1960).

Amino acid analysis. The procedure of Spackman, Stein & Moore (1958) was followed, a Spinco model MS Amino Acid Analyser (serial no. 246) being used. Weighed samples (2–4 mg.) of dried material were hydrolysed under vacuum

in sealed Pyrex test tubes at 110° for 24 and 72 hr. with constant-boiling HCl. Solutions were evaporated to dryness *in vacuo* in the presence of NaOH pellets, and were re-dissolved at suitable concentrations for analysis.

RESULTS

Preparation and fractionation of inhibitors

Digestion. Human albumin was digested inside a dialysis sac (Visking 8/32) with chymotrypsin and the diffusate was tested for inhibitory activity by the technique of delay in flocculation time. The digestion was carried out inside a dialysis sac in order to select antigenic molecules which were small enough to pass through the membrane, and also to obtain intermediate products of digestion, which might be further digested if not separated in this way from the enzyme. We found that we were able to increase the yield of inhibitory material filtering through the sac by 50%, by ultrafiltration at a positive pressure of about 65 cm. Hg. In addition we varied the temperature, pH and ratio enzyme/substrate, in order to obtain the highest yield of inhibitory activity. The conditions finally chosen were as follows: 2 g. of human albumin was dissolved in about 50 ml. of water and the solution adjusted to pH 10 with ammonia. Chymotrypsin (7.5 mg.) was added and the solution, saturated with toluene, was placed in a dialysis sac (8/32), under positive pressure of 65 cm. Hg at room temperature. The filtrate was removed twice daily and the pH of the digestion mixture was adjusted to 10 and the volume to 50 ml. each day. A worthwhile yield of inhibitory activity was obtained up to 9 days. The filtrate (about 1 l.) was concentrated to about 50 ml. on a rotary still, under reduced pressure at about 30°.

Fractionation. The crude digest was fractionated as shown in Scheme 1. It was first run through a column, 50 cm. $\times$ 20 cm.², containing Sephadex G. 50 equilibrated with 0.01 M-potassium phosphate, pH 7.8. The elution diagram (Fig. 1) has three u.v.-absorbing peaks, of which only the first had inhibitory activity. This fraction was therefore concentrated by evaporation on the rotary still to about 5 ml. and run again on another Sephadex G. 50 column of about three times the length and of only 4.3 cm.² cross-section, equilibrated with 0.04 M-potassium phosphate, pH 8.0.

In this way the inhibitory fraction was separated into two peaks, I and II, the first of which contained about 45% of the inhibitory activity and the second about 5% (Fig. 2). This loss in inhibitory power is partly explained by the finding that if the two fractions are assayed together the inhibitory power is about 140% of the sum of the two assayed separately.

Fig. 1. Elution diagram of crude human serum albumin digest on a Sephadex G. 50 column, 50 cm. long, 20 cm.² cross-section, in 0.01M-potassium phosphate, pH 7.8. ●, $E_{1\text{ cm.}}$ (280 m μ); ○, delay in time of flocculation on addition of sample (0.1 ml.) to human albumin-rabbit anti-human albumin system.

Fig. 2. Elution diagram of second run of first peak from the Sephadex G. 50 column shown in Fig. 1, on a Sephadex G. 50 column, 150 cm. long, 4.3 cm.² cross-section, in 0.04M-potassium phosphate, pH 8.0. ●, $E_{1\text{ cm.}}$ (280 m μ); ○, delay in time of flocculation on addition of sample (0.1 ml.) to human albumin-rabbit anti-human albumin system.

The yield of I is only 80 mg. from 2 g. of human albumin digest and the yield of II is about 90 mg. These two fractions were desalted by running through a column of Sephadex G. 25, 30 cm. × 4.0 cm.², equilibrated with 0.05M-ammonium acetate and the protein solution was freeze-dried three times.

Fraction I was further purified by zone electrophoresis at pH 9.0 in 25 mM-sodium borate on a jacketed column about 70 cm. long having an elution volume of 90 ml. The load was about 120 mg. of material and a current of 15 mA was passed at a voltage of 6v/cm. for 18-20 hr. The elution

diagram (Fig. 3) had two main u.v. peaks and each was associated with inhibitory activity. The two peaks, Ia and Ib, were each concentrated by freeze-drying, as the inhibitory power of these partially purified proteins was found to be partly lost if concentrated on the rotary still. They were then desalted by being passed through Sephadex G. 25 as described above, and freeze-dried. The recovery of inhibitory activity was only 30% but again if Ia and Ib were assayed together the inhibitory activity was about 180% of that when assayed separately.

Neither of these fractions was pure as judged by starch-gel electrophoresis and both could be purified by zone electrophoresis at acid pH. Electrophoresis of Ia in 0.2M-acetic acid-15 mM-sodium chloride, pH 2.7, for 10 hr. resulted in almost a threefold purification and recovery of activity of 60-70%. Alternatively Ia may be purified by electrophoresis at pH 3.2 in 0.05M-formate for 15 hr. at 6v/cm. (Fig. 4). If the eluate from 12-18 ml. was combined, a threefold purification was obtained but only 50% recovery of activity. The purity of these samples was tested by starch-gel electrophoresis at pH 8.8, and the major component in each case was shown to have inhibitory activity by eluting the protein from the gel as described by Gordon (1960) and testing. Electrophoresis at pH 3.2 in formate appears to give the purer preparation, but unfortunately it has not been found possible to separate the minor impurities from the inhibitor. It has been found, however, that the inhibitor can be fractionated into two antigenically active components by

running it through a column of Sephadex G. 75, when a component, Ic, representing about 20% of the whole, is eluted at the front followed by the major component, Ia. Ic will precipitate 80% of antibody from the antiserum used and cannot therefore properly be called an inhibitor.

The best method of purifying Ib was zone electrophoresis at pH 2.7, in 0.2M-acetic acid-15 mM-sodium chloride for 14 hr. at 6v/cm. (Fig. 5). The

Fig. 4. Elution diagram of zone electrophoresis of fraction Ia at pH 3.2 in 0.05M-formate buffer at 6v/cm. for 15 hr. in a column 74 cm. long, 1.5 cm.² cross-section. ●, $E_{1\text{cm}}$ (280 mμ); ○, delay in time of flocculation on addition of sample (0.2 ml.) to human albumin-rabbit anti-human albumin system.

Fig. 3. Elution diagram of zone electrophoresis of fraction I at pH 9.0 in 25 mM-sodium borate at 6v/cm. for 19 hr. in a column 74 cm. long, 1.5 cm.² cross-section. ●, $E_{1\text{cm}}$ (280 mμ); ○, delay in time of flocculation on addition of sample (0.2 ml.) to human albumin-rabbit anti-human albumin system.

Fig. 5. Elution diagram of zone electrophoresis of fraction Ib at pH 2.7 in 0.2M-acetic acid-15 mM-sodium chloride at 6v/cm. for 14 hr. in a column 74 cm. long, 1.5 cm.² cross-section. ●, $E_{1\text{cm}}$ (280 mμ); ○, delay in time of flocculation on addition of sample (0.2 ml.) to human albumin-rabbit anti-human albumin system.

activity was in the first peak; the eluate from 24–30 ml. was combined, desalted and freeze-dried. Starch-gel electrophoresis again showed that this purified material consisted largely of one component, which was shown to be inhibitory by elution from the gel. Of the two minor components, one could be removed by running again down a long narrow Sephadex G. 50 column, 150 cm. $\times$ 1.7 cm.² Ib was eluted first, followed by a small shoulder representing about 15% of the u.v.-absorbing material put on the column; this latter peak also had inhibitory activity and this was shown to be due to its content of a trace of another inhibitor, II. 2.

Fraction II, eluted from the Sephadex G. 50 column, had a much lower specific inhibitory activity than I, but as the material was believed to be of smaller molecular weight than fraction I, owing to its being eluted at 1.2 times the elution volume for I, it is of potentially greater interest and we have endeavoured to isolate inhibitory components from it.

Ion-exchangers Dowex 50 and IRC-50, as well as carboxymethyl- and diethylaminoethyl-cellulose, had been tried without success in fractionation of I, but as II was of smaller size their use was investigated. However, recovery of activity was so poor that we had recourse once more to zone electrophoresis at pH 9.0 in 25 mM-sodium borate, for 16 hr. at 6v/cm., which resolved the inhibitory activity into two fractions: II. 1 and II. 2 (Fig. 6). The crude fraction II was also run on DEAE-Sephadex G. 25 at pH 7.9, in 0.02M-ammonium

Fig. 6. Elution diagram of zone electrophoresis of fraction II at pH 9.0 in 25 mM-sodium borate at 6v/cm. for 16 hr. in a column 74 cm. long, 1.5 cm.² cross-section. ●, $E_{1cm.}$ (280 m μ); ○, delay in time of flocculation on addition of sample (0.2 ml.) to human albumin-rabbit anti-human albumin system.

acetate, and although the recovery of inhibitory activity was only about 10%, the material recovered was found to be pure by starch-gel electrophoresis at pH 8.8 and 3.1, and consisted of the same component as found in fraction II. 2. Therefore fraction II. 2 from zone electrophoresis has been fractionated on DEAE-Sephadex G. 25, and the fraction eluted at the front, in about 25% yield of activity, will be referred to as fraction II. 2. The inhibitor in II. 1 has not been purified further.

Properties of inhibitors

Starch-gel electrophoresis at pH 8.8 and 3.1 has been used to characterize the inhibitors and as a test of purity. Fig. 7 (a) compares the electrophoretic mobilities at pH 8.8 of human albumin and the three inhibitors. The heterogeneity of human albumin by starch-gel electrophoresis has been noted by a number of workers, and Poulik, Zuelzer & Meyer (1960) identified several albumin components separated by starch gel as antigenically identical. Inhibitors Ia and Ib contain a trace of impurity but II. 2 is pure. At pH 3.1 all three inhibitors migrate as single components (Fig. 7b).

The absorption spectrum for each of the inhibitors was determined at neutral pH and for Ia and Ib the extinction at 260 m μ was the same as at 280 m μ , which indicates a low ratio of tyrosine to phenylalanine in these inhibitors. The shape and magnitude of the absorption spectrum for II. 2 showed that there was no tryptophan in this molecule and probably no tyrosine either. For the latter, this was confirmed by amino acid analysis. The extinction at 280 m μ of Ia is 0.5, of Ib 0.3 and of II. 2 is 0.08, measured at a concentration of 1 mg./ml. in a 1 cm. cell at neutral pH.

The molecular weights of the three inhibitors have been determined at pH 4 in the presence of cysteine and EDTA, in order to prevent dimerization such as occurred with the fragment of bovine serum albumin (Porter, 1957) through disulphide-

Fig. 7. (a) Starch-gel electrophoresis of human serum albumin and inhibitors in tris-citrate, pH 8.8, at 6.5v/cm., 15 ma for 4 hr.; concentration of protein solutions 1%. HSA, Human serum albumin. (b) Starch-gel electrophoresis of inhibitors in formate, pH 3.1, at 5v/cm., 12 ma for 4 hr.; concentration of protein solutions 1%.

Table 1. *Sedimentation coefficients and molecular weights of inhibitors*

Measurements in the ultracentrifuge were made in 0.1 M-acetate buffer, pH 4, 0.02 M-cysteine, 1 mM-EDTA.

Inhibitor	Concentration (mg./ml.)	S_{25}	Mol.wt.
Ia	3	3.1	23 400
Ib	12	1.86	19 000
II.2	11	1.16	7 100

Table 2. *Amino acid composition of human serum albumin and inhibitor II.2*

	Human serum albumin*	
	(g./100 g.)	II.2 (g./100 g.)
Lysine	12.3	20.0
Histidine	3.5	3.2
Arginine	6.1	2.2
Aspartic acid	10.4	6.8
Threonine	5.0	6.4
Serine	3.7	2.2
Glutamic acid	17.4	17.8
Proline	5.1	1.8
Glycine	1.6	1.1
Alanine	—	8.3
Cystine (half)	5.6	5.1
Valine	7.7	5.8
Methionine	1.3	1.3
Isoleucine	1.7	2.5
Leucine	11.9	7.6
Tyrosine	4.7	0
Phenylalanine	7.8	6.5
Tryptophan	0.2	0†

* Brand, Kassell & Saidel (1944) and Brand (1946).
† Tryptophan could not be detected by the spectrophotometric method of Beaven & Holiday (1952).

Table 3. *Effect of chymotrypsin on inhibitors*

Inhibitors were incubated at a concentration of 1.5 mg./ml. with 0.2 mg. of chymotrypsin/ml. at room temperature.

Inhibitor	pH	Inhibitor surviving after 24 hr. (%)
Ia	7.4	50
	10.0	80-90
Ib	7.4	70-75
	10.0	90-100
II.2	8.0	60-75
	10.0	100

bond formation. The molecular weight of Ia was, however, also measured in potassium chloride-potassium phosphate buffer, pH 7.7, and the same result obtained as at pH 4. Sedimentation coefficients and molecular weights of the inhibitors are given in Table 1.

An analysis of the amino acid composition of II.2 was made and the mean values for 24 and

Fig. 8. Inhibition of precipitation of anti-human serum albumin by inhibitor Ia. Broken lines, antibody precipitated by adding Ia to 0.5 ml. of antiserum. Continuous lines, antibody precipitated from supernatants by addition of human albumin in optimum amount. ●, Antiserum (1), 24 mg./ml., diluted ten times; ○, antiserum (2), 4 mg./ml., diluted two times.

72 hr. acid hydrolyses were calculated, and these are compared with published values for human serum albumin in Table 2.

The stability of the crude inhibitory fraction from the first Sephadex G. 50 column was investigated and it was found to be stable at room temperature in the range pH 2.5-10.0, and it could be heated for 30 min. at 60° at pH 8 without loss of activity, but at 100° 85% of activity was lost in 10 min. The effect of chymotrypsin on the purified inhibitors has also been studied and, as will be seen from Table 3, they are more stable at pH 10, and in fact little if any loss of activity was detected on incubating Ib or II.2 for 24 hr. at pH 10.

The antigenic nature of the inhibitors has been compared by quantitative precipitation tests and by double diffusion in agar, as well as by immunoelectrophoresis. Pooled antisera have been used, one serum (1) of very high antibody titre (24 mg./ml.) and another serum (2) from rabbits after a first course of injections of titre only 4 mg./ml. Inhibitor Ia precipitated 13 and 18% of antibody from these two antisera (Fig. 8), and also completely inhibited precipitation of the remaining antibody with human albumin. A much lower concentration of inhibitor Ia was required to give complete inhibition with antiserum (2) than with (1). Inhibitor Ib precipitated only a trace of antibody from antiserum (1) and 10% from (2); also inhibition of precipitation of the remaining anti-

body with albumin was incomplete (28 and 40% with antisera 1 and 2; see Fig. 9). Inhibitor II.2 failed to precipitate any antibody and produced rather less inhibition than Ib (only 15 and 30%

Fig. 9. Inhibition of precipitation of anti-human serum albumin by inhibitor Ib. Broken line, antibody precipitated by addition of Ib to 0.5 ml. of antiserum. Continuous lines, antibody precipitated from supernatants by addition of human albumin in optimum amount. ●, Antiserum (1), 24 mg./ml., diluted ten times; ○, antiserum (2), 4 mg./ml., diluted two times.

Fig. 10. Inhibition of precipitation of anti-human serum albumin by inhibitor II.2. ●, Antibody precipitated from 0.5 ml. of antiserum (1) (24 mg./ml., diluted ten times) by human albumin in the presence of inhibitor II.2; ○, antibody precipitated from 0.5 ml. of antiserum (2) (4 mg./ml., diluted two times) by human albumin in the presence of inhibitor II.2.

with antisera 1 and 2; Fig. 10). It is of smaller molecular weight than Ib, and it was interesting to know whether the two inhibitors have antigenic sites in common. We therefore added 250 μg. of II.2 to a mixture of antiserum and 125 μg. of inhibitor Ib, and found that the quantity of antibody precipitated was the same as in the presence of Ib only, and also that the inhibition was only about 4% more than in the presence of Ib only; as this is within experimental error of the method, we must conclude that the antigenic site or sites of II.2 are part of the antigenic structure of Ib.

Ic, which was separated from Ia by Sephadex G. 75, has not been characterized, but it was known to precipitate antibody and in fact it was found that 80% of antibody from antiserum (1) was precipitated by an equal weight of Ic. At lower concentration of Ic, the antibody left in the supernatant was precipitable by human albumin, and therefore Ic is apparently not an inhibitor. Double diffusion in agar by the method of Ouchterlony (1953) showed that Ic contained two antigenic molecules, one of which gave a reaction of identity with Ia (Fig. 11a and b). The molecular weight of Ic has not been determined, but as it was eluted before Ia on Sephadex G. 75 it is presumably larger than Ia.

Comparison of the inhibitors Ia and Ib by double diffusion showed that they were antigenically related to the main component of human albumin (Fig. 11a and b). Ia gave a single line with antiserum (2), but a faint additional line with (1). Ia shows a reaction of partial identity with Ib, and the latter fraction apparently contains less antigenic sites than Ia. II.2 failed to give precipi-

Fig. 11. Comparison of human albumin and inhibitors by diffusion in agar gel by method of Ouchterlony (1953). (a) Centre cups contain antiserum (1) (24 mg./ml.). Concn. of human serum albumin and Ic 2 mg./ml., Ia 0.5 mg./ml. and Ib 0.25 mg./ml. (b) Centre cups contain antiserum (2) (4 mg./ml.). Concn. of human serum albumin 1 mg./ml., Ic 2 mg./ml., Ia 0.5 mg./ml., Ib 0.25 mg./ml.

tation lines with any of five different antisera tried, and therefore cannot be compared by this method. Inhibitors Ia and Ib were examined by immunoelectrophoresis in starch gel at pH 8.8 with antiserum (1). Both inhibitors gave single precipitation lines, suggesting that they were homogeneous. Human serum albumin also gave a single precipitation line in immunoelectrophoresis, with this antiserum.

The specificity of the three inhibitors has been demonstrated by the fact that they have no inhibitory effect whatsoever on the precipitation of bovine serum albumin by its antiserum. Only a very slight cross-reaction was observed between the whole human albumin and the antiserum to the bovine albumin.

DISCUSSION

The principal difficulty met in the fractionation of digests of serum albumin has been the heavy loss of inhibitory activity (often over 90 %) when chromatography on ion-exchange resin or cellulose was attempted. In many cases the recovery of material absorbing in the ultraviolet light was high, so that it appears that the loss was due in part to inactivation rather than irreversible adsorption. Repeated use was therefore made of zone electrophoresis and Sephadex columns, where recoveries of activity were higher (50–70 %), but the aggregate loss was still considerable. The additive inhibitory effect of the different fractions made exact estimates of recovery difficult. It is clear, however, that the weight yields of pure inhibitors (Scheme 1) are much below that expected on the basis of molecular weight, even with allowance for considerable loss during purification. For example, only 5 mg. of fraction II.2 was isolated from 2 g. of serum albumin digest, when on the basis of molecular weight a maximum of 200 mg. could be expected, or perhaps 30–50 mg. assuming 75–85 % loss in purification. As the final product is stable to further chymotryptic digestion under the conditions used, this low yield may have arisen because the enzymic splitting followed several different routes, producing different end products, as has been shown to occur in the subtilisin digestion of the melanophore-stimulating hormone (Harris & Roos, 1959).

The immunological evidence (precipitation curves, gel diffusion and immunoelectrophoresis) suggests that the antiserum used contained only traces of antibody against antigens other than serum albumin and also that fractions Ia, Ib and II.2 probably contain common antigenic sites derived from the same part of the albumin molecule. Thus serum albumin, Ia and Ib all show reactions of partial identity on gel-diffusion plates, and II.2 causes no additive inhibitory effect when mixed with antiserum in the presence of an excess of Ib. This again suggests that the chymotryptic digestion may follow different paths and produce different end products.

As the principal objective is the elucidation of the chemical structure of an antigenic site, II.2, with a molecular weight of 7100, is of the greatest interest. It appears to be homogeneous by the criteria applied and has an amino acid composition which differs greatly from that of serum albumin. The inhibitor contains no tryptophan or tyrosine and only one residue each of arginine, proline, glycine, methionine and isoleucine. There appears to be rather more than one residue (1.5) of histidine and serine present but it is not certain whether this anomaly is caused by a small contaminating peptide or inaccurate correction for breakdown during hydrolysis.

The failure to get complete inhibition of precipitation of the whole antigen with its antiserum with inhibitors such as II.2 is in keeping with the idea, previously suggested, that any antiserum is a mixture of antibodies directed against different antigenic sites on the serum albumin molecule (Porter, 1957). The difference in behaviour of the two pools of antiserum used presumably reflects their different ratios of antibodies directed against particular antigenic sites. Thus with II.2 the maximal inhibition is 15 % for serum (1) and 30 % for serum (2). As II.2 fails to give any precipitate with antiserum even in gel diffusion, it seems likely that it contains only one antigenic site. The molar ratio of the inhibitors to serum albumin at 50 % of the maximum inhibition can be calculated from Figs. 8–10, and they are given in Table 4. They fall in the range 0.3–1.5, in agreement with earlier work (Porter, 1958), and are in contrast with the results with haptens (Beiser, Burke & Tanenbaum, 1960), polyglucose chains with a dextran antigen (Kabat, 1960) and low-molecular-weight peptides from silk fibroin (Cebra, 1961). In these last three examples, a molar ratio of 1000–3000/1 of inhibitor/antigen is necessary to obtain 50 % maximal inhibition of precipitation. This 1000-fold difference in inhibitory power between the digest fragments of serum albumin and haptens, polyglucose chains and silk fibroin peptides suggests that the latter may form only part of an antigenic

Table 4. *Molar ratios of inhibitors to serum albumin*

Inhibitor	Molar ratio inhibitor/albumin (for 50 % of maximum inhibition)	
	Antiserum (1)	Antiserum (2)
Ia	1.5	0.75
Ib	0.5	0.3
II.2	1.5	1.5

site. It is not known if antigenic sites have a well-defined surface area but this evidence suggests that in protein antigens they may be of appreciable size, though less than the surface area of a peptide with molecular weight 7000. The equivalent antibody site is presumably of similar size and therefore considerably greater than has been suggested elsewhere (Haurowitz, 1960; Talmage, 1959).

The relationship of the inhibitory fragments described here to the enzymic digests of human serum albumin reported by Lapresle and collaborators cannot be determined at present, although the inhibitor described (Lapresle & Webb, 1960), which only precipitates a very small amount of antibody and which gave 15–40% inhibition, may be similar antigenically to our inhibitor Ib.

SUMMARY

1. Human serum albumin has been digested with chymotrypsin in a dialysis sac and from the ultrafiltrate four components having some of the antigenic sites of the original molecule have been isolated.

2. The largest of these antigenic components will precipitate antibody from rabbit anti-human serum, and is apparently a mixture of two antigenic components.

3. Two further antigenic components of molecular weight 23400 and 19000 will precipitate 10–20% of antibody and the former gives total inhibition and the latter partial inhibition of precipitation of antibody with human albumin.

4. The smallest antigenic component, molecular weight 7100, does not precipitate antibody either in solution or in agar gel but gives partial inhibition of precipitation. The antigenic site or sites of this molecule are also part of the antigenic structure of the two larger fragments.

5. The ratio of inhibitor to albumin for half the maximum inhibition varies from 0.3 to 1.5 for the three inhibitors, and the bearing this has on estimates made of the size of an antigenic site is discussed.

6. The amino acid composition of the inhibitor of molecular weight 7100 has been determined and found to contrast very sharply with the composition of the whole molecule.

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