Preparation and Analysis of Peptide Fragments produced by Pepsin Hydrolysis of Human Plasma Albumin and their Relationship to its Structure

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Human plasma albumin was prepared and subjected to proteolysis by pepsin at pH 2.45 at 25° for 10 min. with albumin/pepsin ratio 3000:1. Five peptide fragments were detected in the proteolysate by means of zone electrophoresis and gel filtration; these were separated and purified. Molecular weights, amino acid composition and disulphide bond content of the purified fragments were determined. The results show that a high proportion of the polypeptide chain of albumin appears to have a low cystine content, and at low pH values the molecule would be expected to have a considerable degree of freedom in its structure in these regions of the chain. A tripartite model for the structure of plasma albumin is proposed.

Plasma albumins of different mammalian species show a high degree of similarity in their chemical and physical properties, and it is probable that their molecular structures have many features in common. In the different albumins so far examined, the primary structure has been found to consist of a single polypeptide chain of about 600 amino acids. Molecular weights usually lie between 65 000 and 70 000 and there is no structural lipid nor carbohydrate.

From a study of bovine plasma albumin Harrington, Johnson & Ottewill (1956) concluded that the molecule exists at acid pH values as two compact, reasonably symmetrical, sub-units flexibly joined by a peptide segment. Under neutral conditions these sub-units are held together by secondary forces so that their relative rotation is not possible, and the molecule forms a compact globule. Supporting chemical evidence for this hypothesis was provided by Adkins & Foster (1965, 1966). Digestion of bovine plasma albumin by a bacterial proteinase (subtilisin) at pH 8.9 in the presence of a high concentration of a detergent resulted in the rapid liberation of two major fragments with molecular weights roughly half that of the intact molecule. Though the amino acid compositions of these two fragments were distinctly different, their sum accounted for virtually all the amino acid content of the albumin molecule. It seems therefore that the fragments had been formed

by cleavage of the molecule at a central point in the polypeptide chain, and thus constituted the two Harrington-model sub-units.

Foster (1960) expanded the hypothesis of Harrington *et al.* (1956) through a consideration of the electrophoretic and solubility behaviour of bovine albumin at acid pH values and through various titration and interaction experiments. He proposed that the molecule consists of four compact regions connected by relatively short segments of polypeptide chain. Above pH 4 close association of the sub-units confers a globular structure on the molecule. This globule, the *N* form, consists of two *F'*-form globules bonded by electrostatic forces, and these two globules (corresponding to the two halves of the Harrington model) are each composed of two hydrophobically bonded *F*-form sub-units. At low pH values the molecule expands fully and the four sub-units are joined only by short peptide links.

Weber & Young (1964*a,b*) pointed out that, if the albumin molecule were to consist of a mixture of globular and flexible parts, 'an almost inescapable consequence . . . is that a protease acting in acid solution should preferentially act on the peptide bonds of the linking segments, thus liberating a small number of globular units'. They accordingly treated bovine albumin briefly with pepsin at acid pH and were able to separate from the dialysed hydrolysate two fractions of globular fragments, one with an average molecular weight of 12 500 and the other of about 30 000. Though two distinct components were found in the heavy fraction, they were

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found to be so similar in composition as to suggest that they were both derived from a single entity. The light fraction, however, was manifestly a mixture of molecular species with differing physical properties and amino acid compositions. Since the light fraction formed about 55% of the recovered digest, these workers concluded that the albumin molecule consisted of a single large globule from the heavy fraction connected with and surrounded by two or three smaller globules from the light fraction, thus in principle supporting the Foster hypothesis.

Luzzati, Witz & Nicolaieff (1961) proposed a different model after a thorough investigation of bovine albumin by low-angle X-ray scattering. At pH 5.3 the molecule was found to be compact, and the values of various physical parameters agreed closely with those given by other techniques. At pH 3.6 changes appeared in the X-ray-diffraction pattern that suggested that about 35% of the polypeptide chain had unwound to take up a loose configuration around a compact globule formed by the rest of the molecule. Bloomfield (1966) in a re-examination of the same data concluded that they were compatible with a variation of the Foster-Weber model if the molecule were to consist of two spherical globules of radius 19.0 Å separated by one of 26.6 Å. His model, however, accounted for only about 80% of the molecular weight of albumin, and he suggested that the remaining 20% formed the polypeptide chains connecting the sub-units.

Thus if the general form of the Foster-Weber hypothesis for the structure of plasma albumin is accepted, present speculation is conflicting on the proportion of polypeptide chain present in a non-globular form. Are the flexible joints between the sub-units as short as implied by Foster (1960) and by Weber & Young (1964a,b), or do they constitute 20–35% of the intact molecule as suggested by Luzzati *et al.* (1961) and by Bloomfield (1966)? In connexion with this it should be remembered that the albumin molecule has 16–18 intramolecular disulphide bonds, which considerably restrain the unfolding of the polypeptide chain. Since at low pH values the hydrophobic and electrostatic forces bonding the sub-units are mostly broken, an almost inescapable consequence of the Foster-Weber model is that the structure of any parts remaining as globular units at such pH values would be maintained essentially by the presence of the disulphide cross-linkages of the cystine residues. We therefore briefly treated human plasma albumin with pepsin in acid conditions by the method of Weber & Young (1964a,b), and from the undialysed hydrolysate we isolated five fragments. As reported in the present paper, analyses of these fragments showed that a high proportion of the polypeptide chain is devoid of cystine residues, and it follows that at low pH values the albumin molecule should have a consider-

able degree of freedom in its structure in these regions of the chain.

MATERIALS AND METHODS

All salts used were reagent grade chemicals. Distilled water was used in the preparation of all solutions except for those needed for the amino acid analyser, where deionized water was used.

Phosphate and acetate buffers. These were prepared as described by Boyd (1945, 1965).

Human plasma albumin. This was prepared from outdated heparinized blood by the cold-ethanol fractionation method of Cohn *et al.* (1946).

Protein-concentration determinations. These were carried out by the micro-Kjeldahl procedure of Markham (1942), the nitrogen values being multiplied by 6.25 to give the protein values, and by a biuret method modified from those of Gornall, Bardawill & David (1949) and Rosenthal & Cundiff (1956) with human plasma albumin as a standard.

Fatty acid content of albumin. This was determined by the method of Chen (1967), with specially pure palmitic acid (British Drug Houses Ltd., Poole, Dorset) as a standard.

Viscosity determinations. These were made on five different concentrations of the albumin sample over the range 0.5–2.0 g./100 ml. in 10 mm-sodium acetate buffer, pH 5.70, with an Oswald viscometer maintained at $25 \pm 0.1^\circ$.

Optical-rotatory-dispersion measurements. These were determined on a 0.01% (w/v) solution of the albumin sample in 0.15 M-sodium acetate buffer, pH 5.28, over the range 223–400 m μ , at 20° in a 1 cm. cell on a Polarmatic 62 spectropolarimeter (Bellingham and Stanley Ltd., London, N.15).

Ultracentrifugal analysis. This was performed at the Lister Institute of Preventive Medicine, London, S.W. 1, by kind permission of Professor R. A. Kekwick, by his standard technique in 0.1 M-sodium phosphate buffer, pH 7.40, containing 0.1 M-NaCl.

Pepsin. This was a twice-crystallized sample from porcine mucosa (L. Light and Co. Ltd., Colnbrook, Bucks.) with an activity of 2860 units/mg. determined by the method of Anson (1939). It was made up as a stock solution of 0.033% (w/v) in 1 M-sodium acetate buffer, pH 5.40. Optimum activity with the human albumin sample as substrate was found to occur at pH 2.45 by the method of Seijffers, Miller & Segal (1964).

Pepsin hydrolysis. This was carried out at $25 \pm 0.1^\circ$ on an aqueous solution of the albumin sample adjusted to pH 2.45 with 1 N-HCl and with an albumin/pepsin ratio 3000:1 (w/w). Initially albumin concentrations of 0.5–2.0% (w/v) were used in experiments to follow the course of the reaction. Samples were withdrawn at intervals for up to 70 min., and examined by zone electrophoresis and for the release of small peptides containing tyrosyl residues. The latter were determined by precipitating the protein with enough 0.3 M-trichloroacetic acid to give a final concentration of 0.2 M and treating the supernatant with Folin-Ciocalteu reagent by the method of Seijffers *et al.* (1964). The course of the reaction was not found to be altered at higher concentrations of albumin examined subsequently, and the following method of preparing the fragments was finally used. The albumin sample (1 g.), dissolved in 10 ml. of water and adjusted to pH 2.45 with 1 N-HCl, was treated with pepsin stock solution (1 ml.) for 10 min. at $25 \pm 0.1^\circ$. During this time the pH

remained constant to within 0.1pH unit. The digestion was stopped by adding 5ml. of 1M-K₂HPO₄. The extent of the hydrolysis was checked by zone electrophoresis, and the complete volume of hydrolysate was immediately subjected to filtration on Sephadex dextran gel G-75 (Pharmacia, Uppsala, Sweden).

Zone electrophoresis. This was carried out at room temperature in 2.5cm. × 12.5cm. cellulose polyacetate strips (Medical Division, Oxo Ltd., London, E.C. 4) either in 40mm-pyridine adjusted with acetic acid to pH 6.75, or in 10mm-5,5'-diethylbarbituric acid-60mm-sodium 5,5'-diethylbarbiturate buffer, pH 8.60. The normal load was 1μl. of solution containing 10–20μg. of material. The electrophoretograms were stained with Procion Brilliant Blue RS (Edward Gurr Ltd., London, S.W. 14) by the method of Fazekas de St Groth, Webster & Datyner (1963) followed by clearing in Whitmor 120 transparency oil (Shandon Scientific Co., London, N.W. 10). This method of staining has the particular advantage that the dye is linked to the protein through the peptide bond, and thus gives an accurate estimation of the protein present, irrespective of its class. Separations were also conducted in 5% (w/v) polyacrylamide gel by using the techniques and apparatus of Lorber (1964). Densitograms of the strips and gels were obtained with a Chromoscan densitometer (Joyce, Loeb and Co. Ltd., Gateshead, Co. Durham) by using a 1mm. scanning slit, an Ilford no. 608 dark-red filter and the B cam of the instrument.

Preparative zone electrophoresis was used to purify the crude fractions isolated by gel filtration. This was carried out in a 76cm. × 10cm. × 1.5cm. slab of Sephadex gel G-25 in either the pyridine buffer or the diethylbarbiturate buffer described above. Bacterial growth was controlled by the addition of sodium azide (0.01%, w/v) to the buffer solutions. The slab was connected to 2l. tanks of buffer by ten thicknesses of Whatman no. 3MM filter paper. The sample was dissolved in 5ml. of buffer and applied to a slot cut in the centre of the gel for the pH 6.75 buffer or 6cm. from the cathode end of the block for the pH 8.60 buffer. Electrophoresis was conducted at a potential gradient of about 4.6 v/cm. at about 10°. The progress of the separation was followed by occasionally making prints of the surface of the block on to strips of Whatman no. 1 filter paper and staining these with acidified Ponceau S (Edward Gurr Ltd.). Electrophoresis was usually terminated by 39hr. The positions of the separated components were determined by making a final print, after which they were individually eluted from the gel with buffer, desalted where necessary and freeze-dried.

Immuno-electrophoresis. This was conducted in the apparatus of LKB Instruments (Stockholm, Sweden) on the albumin sample and on the purified fragments according to the manufacturer's general instructions with the discontinuous buffer system, pH 8.60, of Laurell, Laurell & Skoog (1956) and 1% (w/v) Ionagar no. 2 (Medical Division, Oxo Ltd.) as the supporting medium. The usual load was about 10μg. of material. Horse anti-(human serum) serum (Burroughs Wellcome Laboratories, Beckenham, Kent) and rabbit anti-albumin serum were used to produce the precipitin lines, which were subsequently stained with Amido Black 10B (Edward Gurr Ltd.).

Gel filtration. This was carried out in Sephadex gels G-25, G-75 and G-200. In all cases the temperature of the gel was maintained at 10°. Bacterial growth was controlled by the

addition of sodium azide (0.01%, w/v) to the buffer solutions. Analysis of the intact albumin was conducted on a column (97cm. × 5cm. diam.) of Sephadex G-200 in 0.1M-tris-HCl buffer, pH 8.00, to which was added 0.2M-NaCl. The load (100mg.) was applied in 5ml. of solution to the top of the column. Effluent fractions (8.0ml.) were collected and their extinctions were individually determined at 280mμ with a Unicam SP.500 spectrophotometer. Preliminary separation of the fragments produced by pepsin hydrolysis was conducted on a column (46cm. × 9.7cm. diam.) of Sephadex G-75 in 50mm-sodium phosphate buffer, pH 7.70, containing 0.15M-NaCl. The total hydrolysate from 1g. of albumin was applied in 15ml. of solution. Effluent fractions of volume 7.6ml. were collected and their extinctions were individually determined at 220 and 280mμ. Fractions containing the different fragments were appropriately pooled from a consideration of the elution curve, and desalted and freeze-dried before further purification by zone electrophoresis.

Desalting. This was effected by passing the solution through a column (80cm. × 1.9cm. diam.) of Sephadex G-25 gel in 10mm-pyridine-acetic acid buffer, pH 4.93. The part of the effluent containing the sample was freeze-dried.

Apparent molecular weights. The apparent molecular weights of the purified fragments were determined from their elution volumes on a column (60cm. × 2.9cm. diam.) of Sephadex G-75 at 10° in 0.1M-tris-HCl buffer, pH 8.0, containing 0.2M-NaCl (Andrews, 1964). This column was calibrated before and after the determinations by measuring its void volume with Blue Dextran 2000 (Pharmacia) and the elution volumes of the following proteins: lysozyme, trypsin, ovalbumin, bovine plasma albumin and human plasma albumin. These proteins were taken as having the following molecular weights respectively: 14800 (Warner, 1954), 23500 (Neurath, 1957), 45000 (Warner, 1954), 66000 (Spahr & Edsall, 1964) and 66000 (Spahr & Edsall, 1964). The samples were provided by respectively Armour Pharmaceutical Co. (Eastbourne, Sussex), Seravac Laboratories (Maidenhead, Berks.), British Drug Houses, Stayne Laboratories (High Wycombe, Bucks.) and this Laboratory. The sample (5mg.) was applied in 1ml. of buffer to the top of the column. Effluent fractions (2.9ml.) were collected and their extinctions were individually determined at 220 or 280mμ.

p-Chloromercuribenzoate. p-Chloromercuribenzoate from Hopkin and Williams Ltd. (Chadwell Heath, Essex) was purified by the method of Boyer (1954). In amperometric titrations, a concentration of 5mm-p-chloromercuribenzoate was used for the determinations of thiol groups and disulphide bonds on albumin and one of 2mm for those on the purified fragments. These concentrations were checked by titration with GSH (Benesch & Benesch, 1962).

Polarographic determinations of thiol groups and disulphide bonds. These were made by the dropping-mercury-electrode method (Cecil & Loening, 1960) with an A1630 manual polarograph (Southern Instruments Co., Camberley, Surrey). The base electrolyte for thiol determinations was 0.10M-sodium phosphate buffer, pH 7.40, containing 0.15M-KCl and 0.005% (w/v) methylcellulose. For determination of disulphide bonds the base electrolyte contained in addition 50mm-Na₂SO₃ and 8M-urea. The sample was dissolved in 3ml. of solution at concentrations of 0.25–0.50% (w/v) for the albumin sample and 0.05–0.08% (w/v) for the purified fragments. Voltage/current curves with and without added

p-chloromercuribenzoate were plotted to determine the voltage at which titrations should be made. This was found to be 0.50 v for the albumin sample and 0.30–0.35 v for the fragments. All determinations were carried out under O₂-free N₂. The maximum difference between triplicate determinations was 2.5%.

Amino acid analysis. Oxidation of 1–2 mg. of purified fragment was effected with cold performic acid (Bidmead & Ley, 1958). The oxidized material was dissolved in 10 ml. of 6 N-HCl, in a Pyrex-glass Carius tube. The solution was degassed by freezing, evacuating the tube and then slowly thawing. After a further freezing and evacuation, the tubes were sealed and placed in an oven at 105–110° for 22 hr. The residue from the dried hydrolysate was dissolved in 1 or 2 ml. of 0.10 N-HCl containing 0.10 mM-norleucine. Any insoluble material was removed by centrifugation. Quantitative amino acid analysis of the hydrolysate was performed on a Technicon autoanalyser with a resin column (125 cm. $\times$ 0.6 cm. diam.) of Chromobeads type A, according to a slight modification of the manufacturer's instructions for the 21 hr. method of analysis; this modification consisted of increasing the pump rate so that the run was completed in 18 hr., and including 6.7% (v/v) methanol in the first four chambers of the gradient mixer. The Technicon standard amino acid mixture with the addition of cysteic acid, methionine sulphone and norleucine was used to identify and determine the peaks of each amino acid in the hydrolysates. Since the amount of purified material was limited, it was not possible to determine the amount of amino acid destruction by carrying out hydrolyses for various times, and accordingly correction factors for the loss of serine and threonine were taken from Tristram (1964). The correction found by Hirs (1956) was used in the calculation of the cysteic acid results. Tyrosine and tryptophan were also determined in fragments P₁, P₃ and P₅ by the method of Goodwin & Morton (1946).

N-Terminal amino acid analysis. This was made on 0.2 μ mole of purified fragment by reaction with 1-fluoro-2,4-dinitrobenzene by the quantitative procedure of Fraenkel-Conrat, Harris & Levy (1955). In determining the identity of the DNP-amino acids derived from each fragment, suitable two-dimensional paper-chromatographic systems taken from the above authors were used. As a final check the DNP-amino acid was mixed with an authentic sample of the DNP-amino acid that it was considered to be and the mixture was subjected to chromatographic analysis. The quantitative values were used to check the molecular weights obtained by gel filtration. The correction factors given by Porter (1950) were used in the calculations.

RESULTS

Albumin

Zone electrophoresis in cellulose polyacetate strips showed that the prepared sample of human plasma albumin was contaminated with not more than 1–2% of other proteins. Immunoelectrophoresis showed that this contaminant was mainly transferrin. The intrinsic viscosity of the sample was 0.044 dl./g. [cf. the value of 0.042 dl./g. found by Oncley, Scatchard & Brown (1947)]. Assuming a mean residue weight of 118, optical-rotatory-

dispersion measurements gave an α -helical content of 46% from b_0 calculated from Moffit–Yang plots of the protein dispersion, assuming that a value for b_0 of –630 corresponds to 100% α -helix [cf. the value of 40–50% given by Callaghan & Martin (1962)]; the minimum of the Cotton effect was found at 233 $m\mu$ [cf. the values of 235 $m\mu$ given by Callaghan & Martin (1962) and 232 $m\mu$ by Jirgensons (1962)]. About 93% of the sample was present as monomer albumin, the remainder being primarily dimer. This was calculated from the relative proportions of the appropriate peaks on the 280 $m\mu$ elution curve given by gel filtration of the sample on Sephadex G-200. This was confirmed by zone electrophoresis in polyacrylamide gel and by ultracentrifugal analysis, which showed the presence of a small amount of 6s material, probably dimer albumin. The result is similar to those given by other samples of freshly prepared, freeze-dried, human plasma albumin examined in this Laboratory, and is within the range reported by Adkins & Foster (1966) for their sample of commercial bovine albumin.

In structural studies the protein under examination should be free from significant amounts of adsorbed materials, in particular from peptides. Rubin, Lubash, Aronson & Davidson (1963) reported that 10% (w/w) of human albumin prepared by electrophoresis in alkaline buffers can be bound polypeptides. Since such polypeptides are released in acid conditions, 0.5 g. of the sample was dialysed against 1 N-acetic acid, pH 2.36, at 4° for 24 and 48 hr. As a control a second sample was dialysed against 0.9% (w/v) sodium chloride adjusted to pH 7.2 with pyridine. Electrophoresis in cellulose polyacetate strips showed no difference in mobility of the two samples after this treatment. The concentrated diffusates showed no significant extinction at 280 $m\mu$, and, when dried on to chromatography paper or cellulose polyacetate sheet, did not stain with either Procion Brilliant Blue RS (which is sensitive for large polypeptides as well as for proteins) or with ninhydrin. Lipid contamination measured by the method of Chen (1967) was low, being not greater than 0.06 mole of free fatty acid/mole of protein; accordingly the bulk of the sample was not treated by the technique of Williams & Foster (1960) to remove such material.

Fragmentation of albumin by pepsin

The course of pepsin proteolysis of the albumin sample was followed in two ways: first, by measuring the release of small peptides containing tyrosine; secondly, by observing the appearance of extra components on the electrophoretogram at pH 8.60.

Determination of the release of small peptides showed that digestion of the albumin proceeded

Fig. 1. Time-course of pepsin proteolysis of 1% (w/v) human plasma albumin at pH 2.45 and 25° with albumin/pepsin ratio 3000:1. Material containing tyrosine was estimated with the Folin-Ciocalteu reagent after precipitation of undigested protein with 0.2 M-trichloroacetic acid.

uniformly for about 40 min., after which the rate decreased considerably (Fig. 1). At this point about 20% of the total tyrosine appeared to have been separated from the precipitable protein. This observation of regular proteolysis is based on the Folin-Ciocalteu reaction and is useful only in so far as peptides containing tyrosine are representative of all the peptides released. Clearly, had some other method been used, the pattern of proteolysis might have appeared different. However, in very similar experiments on bovine albumin Peters & Hawn (1967) found that both the colour yield with ninhydrin and the hydrogen ion uptake of their reaction mixture increased linearly for at least 30 min., suggesting that over this period the release of peptides, irrespective of their size, was regular with respect to time.

The rate of production of large fragments as demonstrated by electrophoresis was not found to

Fig. 2. Densitograms of electrophoretic analyses of human plasma albumin subjected to pepsin proteolysis for different lengths of time. Electrophoresis was carried out in 70 mM-diethylbarbiturate buffer, pH 8.60, in cellulose polyacetate strips, and the strips were subsequently stained with Procion Brilliant Blue RS. (a) Control human serum protein; (b) unhydrolysed albumin; this run was deliberately overloaded to show the small proportion of β -globulins; (c) 5 min. proteolysis mixture; (d) 10 min. proteolysis mixture (A, fraction A; P_1 - P_5 , fragments produced by pepsin proteolysis); (e) 15 min. proteolysis mixture; (f) 20 min. proteolysis mixture; (g) 30 min. proteolysis mixture; (h) 40 min. proteolysis mixture.

be uniform, but showed three distinct phases (Fig. 2). The initial rate of breakdown was rapid, and a spreading of the albumin band was usually detected after only 1 min. of proteolysis. During the first 5 min. three fragments were released, apparently together, from the native albumin. Two of these fragments, fragments P_1 and P_2 , had mobilities close to that of the intact albumin, whereas the third, fragment P_3 , was distinctly slower. Two other fragments, fragments P_4 and P_5 , were then detected, so that at the end of 10 min. five extra components were found in the mixture. Fragments P_1 and P_2 formed the major part of these, and, as far as could be judged from the densitograms, appeared to be present in roughly equal proportions. However, these two components lay in that part of the electrophoretogram occupied by the intact albumin, so this estimate should be regarded with caution.

The second phase followed immediately, being a period of about 10 min. during which no significant amounts of new major components were detected. At the end of this time, i.e. about 20 min. from the beginning of the experiment, the third phase began, with additional components appearing on the electrophoretograms, so that by 40 min. eight or more were regularly present. Beyond this period the electrophoretic pattern degenerated rapidly and it became impossible to distinguish the various components unequivocally. Peters & Hawn (1967),

working under very similar conditions, found that six to nine peptide bonds were ruptured/mol. of bovine albumin in 33 min.

The initial action of pepsin on albumin thus appears to consist of two parts: (1) a steady sloughing of small peptides from the molecule, together with (2) a rapid fragmentation of the albumin into five main components. This is followed by a quiescent period in which little major breakdown occurs but during which small peptides continue to be released. A 10 min. period of hydrolysis was chosen for the preparation of the albumin fragments, since this time allows the completion of the initial fragmentation of the molecule into major components, but keeps the production of minor peptides to a minimum. Peters & Hawn (1967) followed the course of major fragmentation of their bovine albumin sample by measuring its fluorescence after interaction with 1-anilinonaphthalene-8-sulphonic acid. Their results showed that the pattern of breakdown was biphasic and that, after about 10 min. under conditions similar to those used in the present work, the rate of major fragmentation of bovine albumin decreased quite suddenly to about one-fifth of its initial value.

Albumin fragments

Preparation. Filtration on Sephadex G-75 of the mixture obtained by a 10 min. digestion of the albumin gave an elution curve with five peaks when the effluent was monitored at $280\text{m}\mu$ but six when it was monitored at $220\text{m}\mu$ (Fig. 3). This extra peak was subsequently found to be composed of fragment P_5 , which was deficient in tyrosine and tryptophan and low in phenylalanine. Traces of material were detected after the emergence of fragment P_5 , but their positions varied from run to

Fig. 3. Elution from a column (46 cm. $\times$ 9.7 cm. diam.) of Sephadex G-75 of the mixture of fragments formed by digesting 1 g. of human plasma albumin with 0.33 mg. of pepsin at pH 2.45 and 25° for 10 min. Elution was effected with 50 mM-phosphate buffer, pH 7.7, containing 0.15 M-NaCl. Fractions (7.6 ml.) were collected and pooled as shown. A, Fraction A; P_1 - P_5 , fragments formed by pepsin proteolysis.

Fig. 4. Electrophoretic distribution on cellulose acetate of fractions produced by proteolysis of human plasma albumin with pepsin at pH 2.45 and 25° for 10 min. with albumin/pepsin ratio 3000:1: (a) in diethylbarbiturate buffer, pH 8.60; (b) in pyridine-acetic acid buffer, pH 6.75. A, Fraction A; P_1 - P_5 , fragments produced by pepsin proteolysis.

run and the quantity was too small for further analysis. The first peak to emerge from the column, fraction A, behaved as native albumin in which no major fragmentation had occurred; this fraction was not investigated further. It was found that the order of elution of the fragments was the same as their order of electrophoretic mobilities at pH 8.60, thus conveniently simplifying their numbering.

The fragments were crudely isolated by pooling the fractions found by gel filtration, as shown in Fig. 3. The pattern of electrophoretic separation of the fragments at pH 6.75 was usefully different from that at pH 8.60, particularly with respect to the separation of fraction A from fragments P₁ and P₂ (Fig. 4). Purification was therefore effected by subjecting the isolated fragments to another passage through the Sephadex G-75 column. Fragments P₁ and P₂ were subsequently submitted to preparative electrophoresis at both the pH values mentioned above. Sufficient purification of fragments P₃–P₅ was found to occur by electrophoresis

at pH 6.75 alone. Losses had to be considerable to obtain sufficiently pure samples of the fragments for further analysis, and it was not possible to estimate the relative proportions of the fragments in the original mixture from the weights of the purified fragments. A rough idea of this, however, was obtained from the proportions of the peaks on the densitograms and on the gel-filtration elution curves. As a check on the efficiency of the purification the fragments were analysed by zone electrophoresis in polyacrylamide gel. The purity of the separated fragments was found by this means to be at least 95%, and, in particular, the separation of fragments P₁ and P₂ from each other and from the unfragmented albumin was found to be maintained at this value. This was important, since it was found subsequently that the compositions of these two fragments were similar.

Amino acid composition. The amino acid compositions of the purified fragments are given in Tables 1 and 2, together with that of human

Table 1. *Amino acid compositions, molecular weights and end-group analyses of fragments P₁ and P₂*

Tryptophan and tyrosine were determined by the method of Goodwin & Morton (1946). The remaining amino acids were determined on hydrolysates of oxidized fragments on a Technicon autoanalyser. The amino acid compositions of human mercaptalbumin (HPA) and bovine mercaptalbumin (BPA) determined by Spahr & Edsall (1964) and of the bovine fragments IIa and IIb analysed by Weber & Young (1964b) are included for comparison. The values of the latter workers have been recalculated from their original results.

	Composition (residues/mol. of fragment or protein)					
	P ₁	P ₂	HPA	IIa	IIb	BPA
Gly	5.6	6.2	11.8	8.4	7.3	15.9
Ala	28.5	27.3	61.7	20.4	16.8	46.1
Val	17.4	13.4	41.5	9.7	7.6	36.5
Leu	27.7	21.8	60.0	27.8	22.0	61.7
Ile	4.3	4.0	8.0	2.8	3.4	14.0
Pro	10.9	11.0	25.8	12.5	11.6	29.9
Phe	10.6	9.0	30.1	12.6	11.5	26.7
Tyr	9.7	9.8	17.5	13.9	11.0	20.2
Trp	0.8	0.0	1.0	—	—	2.0
Ser	10.5	8.3	19.1	10.2	9.2	26.0
Thr	9.9	8.6	24.6	9.1	8.6	34.2
Cys	19.7	12.4	36.9	16.6	13.7	36.1
Met	1.1	0.9	5.7	1.4	1.3	3.8
Arg	9.2	7.4	23.8	7.0	6.1	22.4
His	4.2	5.4	16.0	8.4	6.4	16.8
Lys	21.8	21.5	61.7	26.5	21.6	61.7
Asp	24.0	29.9	51.2	30.6	26.3	54.1
Glu	33.1	35.5	78.6	37.6	33.7	77.6
No. of integral residues	244–254	228–237	608–613	250–260	212–224	617–621
Mol. wt. by gel filtration	34 000	29 000				
			66 000*	34 000†	29 000†	66 000*
Mol. wt. by end-group analysis	33 000	30 000				
N-Terminal residues	Thr 1.03	Arg 0.97				
(moles/mole of fragment)		Asp 0.05				

* Values from Spahr & Edsall (1964).

† Values from Weber & Young (1964b).

Table 2. *Amino acid compositions, molecular weights and end-group analyses of fragments P₃–P₅*

Tryptophan and tyrosine were determined by the method of Goodwin & Morton (1946). The remaining amino acids were determined on hydrolysates of oxidized fragments on a Technicon autoanalyser. The amino acid composition and molecular weight of bovine albumin 'inhibitor' determined by Porter (1957) are included for comparison. The values for this fragment have been recalculated from the original results.

	Composition (residues/mol. of fragment)			
	P ₃	P ₄	P ₅	'Inhibitor'
Gly	4.5	3.9	2.4	3.0
Ala	13.5	7.8	2.8	7.5
Val	10.0	8.1	1.7	9.5
Leu	13.0	8.2	2.4	10.6
Ile	2.3	1.1	0.5	2.6
Pro	5.6	4.0	1.2	7.6
Phe	2.9	3.1	0.9	4.4
Tyr	4.8	0.0	0.0	0.8
Trp	0.0	0.0	0.0	0.0
Ser	7.5	3.4	1.8	2.3
Thr	6.7	4.5	1.1	7.3
Cys	2.3	0.6	0.6	2.7
Met	3.2	0.4	1.3	0.6
Arg	4.4	1.5	0.9	3.7
His	2.6	3.0	0.5	1.6
Lys	20.2	9.3	2.1	10.6
Asp	13.8	9.6	2.3	8.1
Glu	19.8	14.2	3.2	13.3
No. of integral residues	132–141	78–85	22–30	89–102
Mol.wt. by gel filtration	18200	10800	3200	12000
Mol.wt. by end-group analysis	20900	—	2600	
N-Terminal residues (moles/mole of fragment)	Arg 0.90	Thr 0.69	Ser 1.20 Phe 0.33	

mercaptalbumin (Spahr & Edsall, 1964). Fragments IIa and IIb found by Weber & Young (1964b) in their peptic hydrolysate of bovine albumin appear to be similar in many features to fragments P₁ and P₂. The original values given by Weber & Young (1964b) for the amino acid analyses of the fragments IIa and IIb have been recalculated as residues/mol. of fragment, and are included in Table 1 with those given by Spahr & Edsall (1964) for bovine mercaptalbumin. Since there is a possibility of fragment P₄ being similar to the 'inhibitor' fragment found in a chymotryptic digest of bovine albumin by Porter (1957), the values given by him for the composition of his peptide were recalculated and have been included in Table 2 for comparison.

The N-terminal residues found in each fragment are given in Tables 1 and 2. Essentially single residues in approximately molar proportions were found for fragments P₁–P₃, indicating a high degree of purity. Fragment P₄ consistently gave a low yield for its N-terminal residue, threonine, but there was no evidence of the presence of any other. Since there are no masked end groups in human

albumin (Hunter & McDuffie, 1959), it would seem that, despite this low yield, this fragment was essentially pure. Although no evidence of heterogeneity was observed in electrophoresis, end-group analysis indicated that fragment P₅ consisted of at least two components. This was confirmed by the bimodal shape of its peak on the Sephadex gel-filtration elution curve and by the presence of a fraction of a disulphide bond in its composition.

None of the fragments was found to contain significant amounts of aspartic acid as the N-terminal residue. It would appear therefore that the original N-terminal residue of the intact molecule was lost in the process of breakdown, probably in the form of a small peptide released by the generalized proteolysis mentioned above.

Molecular weights. The molecular weights of the fragments determined from their elution volumes on a column of Sephadex gel G-75 and from their end-group analyses are given in Tables 1 and 2. Fig. 5 shows an Andrews (1964) plot of the elution volumes of the standardizing proteins and of the purified fragments. Fragments P₄ and P₅ lay

respectively just outside and distinctly outside the range covered by the standardizing proteins, and consequently the estimates of their molecular weights are less reliable than those for the remainder of the fragments. However, Andrews (1964) showed that the linear relationship between the elution volume of a molecule and the logarithm of its molecular weight was maintained down to a molecular weight of 2000 in Sephadex gel G-75. In general the agreement between the results of the two methods was good. Since fragment P₄ gave a poor yield for its end-group analysis, its molecular weight was determined by gel filtration only.

Gel filtration also showed that fragments P₁ and P₄ partially aggregated during 3 months' storage at -20° (Fig. 6). This was associated with a loss of thiol titre, and, with fragment P₁ only, with the appearance of a new, slow-moving, component in zone electrophoresis at pH 6.75. It seems that dimerization occurred through the formation of

disulphide bonds. No aggregation was found to occur with the remaining fragments.

Thiol titres and disulphide bond analyses. The polarographic analyses of the fragments for thiol titre and disulphide bond content are given in Table 3. As a check on their reliability Table 3 also includes the corresponding values of half-cystine measured as cysteic acid from the amino acid analyses of the oxidized fragments. There is good agreement between the two sets of results. Normal preparations of albumin usually give yields of 0.6-0.7 mole of thiol/mole; this group probably

Fig. 5. Andrews (1964) plot of standardizing proteins and isolated fragments produced by pepsin proteolysis of human plasma albumin at pH 2.45 and 25° for 10 min. with albumin/pepsin ratio 3000:1. Further experimental details are given in the text. P₁-P₅, Fragments produced by pepsin proteolysis of albumin; A, bovine and human plasma albumins; O, ovalbumin; T, trypsin; L, lysozyme.

Fig. 6. Elution of fragment P₁ from a column (60 cm. × 2.9 cm. diam.) of Sephadex G-75 with 0.1M-tris-HCl buffer, pH 8.00, containing 0.2M-NaCl. The load was 1ml. of a solution containing 2% (w/v) of the fragment in the eluting buffer. Each fraction contained 2.6 ml. (a) Kempt for 1 week after preparation; (b) kept for 3 months at -20° after preparation.

Table 3. Disulphide bond and thiol group contents of the fragments and of human plasma albumin determined by polarography

	Composition (moles/mole of fragment or protein)					
	P ₁	P ₂	P ₃	P ₄	P ₅	HPA
Disulphide group	10.1	6.0	1.3	0.0	0.3	18.0
Thiol group	0.3-0.5	0.0	0.0	0.2-0.5	0.0	0.6
CyS content	19.7	12.4	2.3	0.6	0.6	36.9

partially reacts with cysteine and GSH in the body (King, 1961; Andersson, 1966). Only fragments P₁ and P₄ showed the presence of this reactive group. The values obtained in these two fragments did not exceed that of the parent albumin, and a loss of up to 50% of the original titre occurred during 3 months' storage at -20°. As mentioned above, this was associated with the appearance of aggregated material in the fragments.

Immunological properties. On immunoelectrophoresis only fragments P₁-P₃ gave detectable precipitin lines with the anti-albumin sera. This was demonstrated both with the isolated fragments and with the unseparated hydrolysate. Immunological reaction was thus confined to the three largest fragments, which were also the only ones containing disulphide bonds. The precipitin lines were distinct though slightly more diffuse than those formed by native albumin. Fragment P₁ continued to give a visible precipitin line after aggregation on storage had occurred; the line was rather sharper than that given by the fresh material and included a spur line in the position of the newly formed slow-moving component. Fragments P₁ and P₂ gave a reaction of partial identity by agar-diffusion precipitin analysis.

DISCUSSION

Fragments P₁-P₃ are released together as the first sign of major fragmentation of the albumin molecule. The densitogram of the electrophoretic analysis and the gel-filtration elution curve show that fragments P₁ and P₂ are present in the hydrolysate in roughly equal amounts. This proportion is close to that of the two 'hemimers' isolated by Adkins & Foster (1966) from a subtilisin hydrolysate of bovine albumin; the molecular weights are also similar, fragments P₁ and P₂ both being slightly smaller than the hemimers, as though they had undergone mild degradation during the proteolysis. There is strong evidence that the hemimers represent the Harrington-model sub-units of the bovine albumin molecule, and it might be thought therefore that the two P fragments are the corresponding sub-units of human albumin. Examination of the amino acid analyses show, however, that these fragments cannot be these sub-units nor even degraded versions made through peptic proteolysis attacking the sub-units during their release. The amino acid compositions of the two hemimers are distinctly different and sum consistently to that of the intact molecule (Adkins & Foster, 1966). In contrast, those of the two P fragments are similar, suggesting that they have part of the polypeptide chain in common, rather than that they are derived from opposite halves of the molecule. More important, as shown in Table 1, the sums of both the

aspartyl and the tyrosyl residues in these fragments are significantly greater than those in the intact albumin, and hence fragments P₁ and P₂ cannot exist as separate entities in the same molecule.

Comparison of the analyses of fragments P₁ and P₂ with the fragments IIa and IIb found by Weber & Young (1964b) in a peptic hydrolysate of bovine albumin suggests that the former are the human versions of fragments IIa and IIb. Their molecular weights are the same within experimental error. The amino acid compositions are similar, when allowance is made for species differences as indicated by the analyses of the parent albumins. Though it is not immediately obvious in the analyses as originally presented by Weber & Young (1964b), recalculation of the results given by them shows that in fragments IIa and IIb, as in fragments P₁ and P₂, the sums of the aspartyl and tyrosyl residues exceed those of the intact molecule (Table 1). These fragments, both human and bovine, have high cystine contents and thus a high degree of tertiary structure. They would therefore be expected to resist the perturbation produced by lowering the pH and maintain whatever 'globular' structure they may have.

Since the albumin molecule cannot contain both fragments P₁ and P₂ as discrete portions of the polypeptide chain, we considered how they could have arisen. They are very similar in composition, but fragment P₂, though slightly smaller in molecular weight, has a higher content of aspartic acid. In view of this distribution, fragment P₂ cannot have been derived from fragment P₁ through breakdown of the latter; this is unlikely, in any case, since the two fragments appear simultaneously during proteolysis. As shown below, and also by the work of Peters & Hawn (1967) on bovine albumin, these fragments lie within the body of the molecule and two points of rupture in the polypeptide chain would be needed to release them. They could be formed from a population of identical molecules if albumin had two separate points of initial peptic attack. Identical molecules could thus give rise to a mixture of the two fragments, their ratio determined by the relative accessibility of the initial rupture points. Enzymic splitting following different routes to produce different end products has been shown to occur in subtilisin digestion of melanophore-stimulating hormone (Harris & Roos, 1959), and it may occur in chymotryptic digestion of human albumin (Press & Porter, 1962).

An alternative hypothesis is that the albumin molecules have slight variations in structure with corresponding variations in their points of attack by pepsin. Fragments P₁ and P₂ would then be related to differences of structure in the parent molecules. Human albumin can be converted into a mixture of slightly aggregated and unaggregated

molecules with a ratio of about 1:1 by heating at 58° at pH 6.5 (Mackay & Martin, 1957). Work in this Laboratory has shown that this is due to the presence of two classes of normal monomer albumin differing slightly in their mode of denaturation by heat (G. Franglen & S. A. Taylor, unpublished work; cf. Štokrová & Šponar, 1963). Since these two classes differ physicochemically, they could also differ in some aspects of their chemical behaviour. The fragments P₁ and P₂ could thus each arise from one of these two classes of albumin, rather than being due to alternative routes of peptic breakdown of identical molecules. Whatever the explanation, fragments P₁ and P₂ can only come from separate molecules and not from the same one.

The densitogram of the electrophoresis strip stained with Procion Brilliant Blue (Fig. 2) suggests that the concentration of fragment P₃ in the 10 min. hydrolysate was approx. 75% of that of either fragment P₁ or fragment P₂; it may have been more, since fragments P₁ and P₂ were poorly separated from each other and included fraction A. This estimate is crudely confirmed by the gel-filtration curve monitored at 220 mμ (Fig. 3). Fragment P₃ cannot be derived by breakdown from the other two fragments, since it contains three methionyl residues/mol. (half the total in the intact molecule), whereas fragments P₁ and P₂ have only one each. Since its molecular weight is about half those of fragments P₁ and P₂, these rough estimates suggest that more fragment P₃ is released in molar proportions than either fragment P₁ or fragment P₂ alone. Fragment P₃ is thus probably associated with both fragments P₁ and P₂ in the intact molecules, i.e. the parent molecules contain either fragments P₁ and P₃ or fragments P₂ and P₃. The possibility that more than one fragment could be associated with only one of the other fragments is unlikely in view of the evidence from tryptic hydrolysates of oxidized human albumin that there are no large repeating sequences of amino acid residues in the polypeptide chain (Gitlin, Schmid & Earle, 1960).

Fragment P₃ contains only one disulphide bond, though it has about 140 residues in its composition and makes up about 27% of the intact molecule. At neutral pH its internal structure would depend largely on the formation of α-helices, modified by the presence of the five or six proline residues in the fragment. At present the positions of its two half-cystine residues are not known but, irrespective of these, the fragment cannot have a particularly rigid structure. By using the method of Hatch (1965), the ratio of polar to apolar residues was found to be 1.96:1.00, higher than that of the intact molecule (1.51:1.00). This fragment is thus more likely to lie on the outside of the molecule than elsewhere. Its early appearance during hydrolysis shows that,

at least in the acid-expanded form of albumin, it lies in a position where it can be readily detached from the rest of the molecule.

Peters & Hawn (1967) isolated from a pepsin hydrolysate of bovine albumin two small peptides that most probably represent the C- and the N-terminal sections of the polypeptide chain. The general conditions of proteolysis used by these workers were very similar to those used in the present study and, though the activity of their pepsin was not given, the general pattern of breakdown that they reported appears sufficiently close to that described above to suggest that the two samples had essentially the same activity. Their larger peptide, the C-terminal 'Phe' fragment with a molecular weight of 8553, might be thought, from its size, to be a slightly degraded version of fragment P₄, but the amino acid analyses show considerable differences, particularly in the contents of threonine and cystine. The period of hydrolysis used by Peters & Hawn (1967) was over three times as long as that used here, and a comparison of the amino acid analyses suggests that this extended period resulted in a marked breakdown of the bovine fragment P₃ to give the 'Phe' fragment. These workers showed that general fragmentation was occurring at the particular stage of proteolysis used for the preparation of their peptides, since they found a component associated with their 'Phe' fragment that appeared to be a degraded version of it. If the 'Phe' fragment was indeed derived from the bovine fragment P₃, this would indicate that fragment P₃ lies in the C-terminal section of the polypeptide chain. At present it is not possible to relate the other peptide found by Peters & Hawn (1967), the N-terminal 'Asp' fragment, with any of our P fragments. It may have part of the polypeptide chain in common with fragment P₄, since it is similar in being a peptide without cystine residues and without immunological activity, but it could equally well be among the peptides contained in the mixture of fragment P₅. However, fragments P₁ and P₂ are unlikely to form part of the 'Asp' fragment, since their compositions appear to exclude any long straight-chain sequences. In the circumstances fragments P₁ and P₂ are therefore more likely to be central rather than terminal sections of the polypeptide chain.

Fragment P₄ makes up about 16% of the albumin molecule and contains about 84 amino acid residues. It has no disulphide bonds and at low pH would exist as a loosely structured chain similar to the 'Asp' fragment described by Peters & Hawn (1967) but without the N-terminal residue of the intact molecule. It contains the single thiol group of human plasma albumin as determined directly by polarographic measurement and by estimation of cysteic acid after oxidation of the fragment.

Fragment P₁ also contains this group and it seems that part of the polypeptide chain is common to both fragments. Since fragment P₄ was not found in significant amounts until some time after the appearance of fragment P₁, it is possible that fragment P₄ is contained within fragment P₁ and cannot be released until fragment P₁ has been freed from the rest of the molecule. Such an explanation would mean, however, that the ten disulphide bonds of fragment P₁ would be contained within 160–170 residues, implying a rather localized large amount of tertiary structure. Moreover, extended peptic hydrolysis of isolated fragment P₁ did not produce any fragments resembling fragment P₄, nor has it shown that the disulphide bonds are confined within a particular section of fragment P₁.

An alternative hypothesis is that fragment P₄ represents the section of polypeptide chain containing the reactive thiol group associated with fragment P₂ and not with fragment P₁. The two classes of albumin molecules would then consist of (a) fragments P₃, P₂ and P₄, containing the thiol group, and (b) fragments P₃ and P₁, containing the thiol group, together with some other fragment similar to fragment P₄ but without the thiol group. So far a fragment of this size and characteristics has not been found in the hydrolysate. However, a peptide of this type could be readily hydrolysed to give smaller components. Fragment P₅ may represent this fragmented version, being a mixture of at least two different peptides with an average molecular weight of 3000.

Porter (1957) described the preparation and analysis of a fragment ('inhibitor'), similar in size to fragment P₄, that was released from bovine albumin at neutral pH values by chromatrypsin. This fragment retained part of the antigenic properties of the intact molecule in that it could inhibit the precipitation of albumin by an anti-albumin serum. Fragment P₄ appears to contain no immunological activity, though the test used, that of observing the direct precipitation of antibody-albumin complex, is much less sensitive than that used by Porter (1957). His 'inhibitor' also appeared to contain the reactive thiol group, since his preliminary amino acid analyses gave 1.4 moles of cystine/mole of fragment, suggesting that it contained one disulphide bond and one free thiol group. Like fragment P₄, the 'inhibitor' formed dimers; this was associated with a loss of its immunological activity. Both the activity and the original molecular weight were largely regained in an acid reducing medium.

It is therefore possible that, in spite of the presence of a disulphide bond in the 'inhibitor' and not in fragment P₄, these fragments come from the same part of the molecule. Comparison of the amino acid analyses, however, shows significant

differences, particularly in the contents of proline and threonine, even after allowing for the differences in compositions of bovine and human albumins. It would seem then that any overlap between these two fragments is small. When Porter's (1957) amino acid analyses are recalculated as moles of residue/mole of 'inhibitor', ten of the 18 determinations appear as values involving approximate half residues (Table 2). The half residue found for cystine may, then, be more the result of the uncertainties of a preliminary amino acid analysis than definite evidence for the presence of the thiol group in the 'inhibitor'. The molecular weight and composition are such that polymerization could be due to the processes outlined by Fisher (1964) rather than to disulphide bond formation. Such polymerization through hydrogen-bonding and the shielding of hydrophobic groups can be broken in acid media to give a reappearance of activity and a decrease in the molecular weight of the polymer. In the absence of a direct determination of the thiol group, the evidence for its presence in the 'inhibitor' would seem to be equivocal, and this peptide could come from a different part of the molecule from that occupied by fragment P₄.

Fragment P₄ is similar to fragment P₃ in its high content of straight-chain polypeptide. Since it appears later in the process of fragmentation of the molecule, its smaller size might suggest that it is formed from that part of the molecule that contains fragment P₃. Under the conditions used, however, the disulphide bond in fragment P₃ should not be ruptured during hydrolysis to generate the thiol group that appears in fragment P₄. Any overlap between these two fragments can thus only be partial, since neither the reactive thiol group in fragment P₄ nor the disulphide bond in fragment P₃ are common to both fragments. Slayter (1965) observed that in acid conditions the albumin molecule appears under the electron microscope as an extended thread. Remembering the limitations of this technique, this implies that fragments P₃ and P₄ are unlikely to overlap or even be simple continuations of one another, since such a structure would tend to appear as a chain to the ball formed by the globular fragment of fragment P₁ or fragment P₂.

Thus consideration of the present results together with those of other workers suggests that the general structure of albumin is as shown schematically in Fig. 7. A highly structured core, fragment P₁ or fragment P₂, making up about half the weight of the molecule is flanked, probably on the C-terminal side, by fragment P₃, a loosely organized section with low cystine content. At the other end of the central core is another even more loosely organized section without cystine residues, probably fragment P₅ with fragment P₁ and fragment

Fig. 7. Proposed relationships of fragments P_1 – P_5 in the intact albumin molecule in neutral (N) and acid (A) environments. 'sub', Point of proteolytic attack by subtilisin under conditions used by Adkins & Foster (1965, 1966); 'pep', points of early proteolytic attack by pepsin under acid conditions.

P_4 with fragment P_2 , with the reactive thiol group sited in the part connecting this section with the core. In contrast with the method used by Weber & Young (1964b), the hydrolysate in this study was not dialysed with a resulting possible loss of small peptides before separation of the fragments. In spite of this, it is clear from the amino acid analyses and from the loss of the original end groups of the intact molecule that not all the fragments released by the action of pepsin were recovered; this is reflected in the sum of the molecular weights of the fragments making up the albumin containing fragment P_2 as its core being 7–12% below the weight of the intact molecule. It is probable that the loss was in the form of small peptides, since, as pointed out by Laskowski (1961), the specificity of pepsin in splitting peptide linkages appears to be less restricted with natural substrates than with synthetic peptides. Nevertheless, the loss is small enough to make it reasonable to assume that the general structure deduced would not be very different had these small peptides been recovered.

This model for albumin is similar to that of Weber & Young (1964a,b) in that it consists of a sub-unit of molecular weight about 30 000 surrounded by smaller pieces. This sub-unit has a large amount of tertiary structure and in this sense is more highly organized than the other sections. Since this sub-unit is centrally placed, this model is also compatible with that of a highly structured core surrounded by loosely organized polypeptide chain deduced by Luzzati *et al.* (1961) from X-ray-diffraction data. Fragments P_3 and P_4 , both with small amounts of tertiary structure, form about 40% of the intact molecule, a value close to the 35%

given by Luzzati *et al.* (1961) for the loosely organized portion of albumin. The proposed model is similar in general structure and proportions to that of Bloomfield (1966), in which the molecule is assumed to be composed of a spherical sub-unit making up about 60% of the total molecular volume flanked by two smaller units.

A tripartite model such as proposed here and by Bloomfield (1966) does not immediately appear to be compatible with the four-part Foster model nor the evidence on which the latter is based. Moreover, it has been pointed out by Markus, McClintock & Castellani (1967) that albumin appears to have several proteolytically vulnerable regions that can give rise to the production of different fragments by different proteases. Thus the ability of subtilisin to split albumin into the Harrington-model sub-units suggests that the central core of the proposed model can be cleaved at neutral pH in spite of its organized structure. The disparity between the two different types of model can be resolved if it is assumed that at neutral pH the core of the tripartite model folds into roughly equal halves to cover hydrophobic side chains. The molecule would then have the apparent four-part structure suggested by Foster (1960) and would respond to changes of pH as shown by him. At neutral pH subtilisin could split the polypeptide chain where it is exposed at the point of folding of the central core of the molecule (point 'sub' in Fig. 7). At low pH values the core would expand and partially unfold in a manner similar to the rest of the molecule as electrovalent and hydrophobic bonds are loosened, but it would retain an essentially globular structure determined by its high content of disulphide bond. Such unfolding could shield the central section of the core from enzymic attack, so that pepsin would not immediately split the molecule into two halves, but would preferentially slough off the two flanking sections, leaving the core essentially untouched (points 'pep' in Fig. 7).

Plasma albumin is remarkable in its ability to combine reversibly with a great many compounds of biological importance and thus act as a means of transporting within the animal material that would otherwise be largely insoluble in the plasma. Though proteins can combine non-specifically with various substances, the capacity of albumin is so wide as to suggest that this is a peculiarity of this protein rather than just an extension of a property of proteins in general. Some aspects of the interaction behaviour of albumin with other compounds may be related to its 'configurational adaptability', a process involving changes in the relative positions of the side chains, leading to subtle structural changes in the organization of the molecule (Karush, 1952, 1954; Markus & Karush, 1958). Such changes would permit the protein to combine with a wide range of different substances by suitable changes of

configuration leading to appropriate alterations of the surface pattern of reactive groups. It seems that 'configurational adaptability' of this nature would occur most easily where there is a small amount of tertiary organization, i.e. in the flanking sections rather than in the structured core of the proposed model. It may be therefore that these loosely organized sections of the polypeptide chains are vital to the important physiological function of albumin in acting as a transport protein.

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