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Large Fragments of Human Serum Albumin

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Large fragments of human serum albumin were produced by treatment of the native protein with pepsin at pH 3.5. Published sequences of human albumin [Behrens, Spiekerman & Brown (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 591; Meloun, Moravek & Kostka (1975) *FEBS Lett.* **58**, 134–137] were used to locate the fragments in the primary structure. The fragments support both the sequence and proposed disulphide-linkage pattern (Behrens *et al.*, 1975). As the pH of a solution of albumin is lowered from pH 4 to pH 3.5, the protein undergoes a reversible conformational change known as the N–F transition. The distribution of large fragments of human albumin digested with pepsin in the above pH region was critically dependent on pH. It appeared that this distribution was dependent on the conformation of the protein at low pH, rather than the activity of pepsin. The yields of the large fragments produced by peptic digestion at different values of pH suggested that the *C*-terminal region of albumin unfolds or separates from the rest of the molecule during the N–F transition. The similarity of peptic fragments of human and bovine albumin produced under identical conditions supports the proposed similar tertiary structure of these molecules.

The publication of the complete amino acid sequences of human and bovine serum albumin (Brown, 1975; Behrens *et al.*, 1975; Meloun *et al.*, 1975) has encouraged further studies of the structures of these well-known proteins. Foster (1960) proposed that the 580 residues of albumin formed separately organized regions of tertiary structure (domains). The sequences provide some good evidence for this proposition, since they contain three repeating patterns of disulphide links (Brown, 1975) and areas of internal amino acid homology (Meloun *et al.*, 1975). It seems likely that the present protein results from a gene expansion.

Before the availability of sequence information, several workers found that fragments of bovine albumin produced by limited enzymic hydrolysis of the native protein would bind ligands normally bound by the parent molecule. For example, King (1973) split bovine albumin into two fragments, which bound L-tryptophan and octanoic acid respectively. This behaviour might be expected of a protein with a domain substructure, where ligands were bound to separate domains. The idea of using enzymes as probes of the tertiary structure of albumin has been followed up by Braam *et al.* (1974), Hilak *et al.* (1974, 1975), Feldhoff & Peters (1975), Peters & Feldhoff (1975) and Reed *et al.* (1975). All these authors produced large fragments of bovine albumin by treatment of the undenatured protein with pepsin or trypsin. Peters & Feldhoff (1975) and Feldhoff & Peters (1975) were able to locate their fragments by using the sequence data of Brown (1975), Behrens *et al.* (1975) and Meloun *et al.* (1975). Reed *et al.* (1975) described the physical and binding properties of some of these fragments. The interaction of ligands with fragments of bovine albumin has permitted a tentative allocation of the positions of ligand-binding sites to regions of the primary structure.

The present paper describes the preparation of large fragments of human serum albumin and describes their location within the sequence of human albumin. Although there are numerous differences in the amino acid sequences of human and bovine albumin, some of the fragments of human albumin are similar to those obtained from bovine albumin by Feldhoff & Peters (1975) and partially confirm sequence and proposed disulphide-linkage data. The symbolism of Peters & Feldhoff (1975) has been adopted for the fragments described. For example 'P44' denotes a peptic peptide of mol.wt. 44000.

Experimental

Human serum albumin (fraction V; lot no. 62C-1301-8) was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. The free thiol group of human albumin was blocked with L-cystine as described by Andersson (1966). After blocking, 1–2g of human albumin was freed of residual γ-globulins and human albumin dimer by passage through a column (140cm × 3.5cm) of Sephadex G-150 equilibrated with 0.05M-Tris/HCl, pH 7.0, containing 0.3M-NaCl, at a flow rate of 55ml/h. Purified half-cystinyl albumin was pooled, concentrated to 30mg/ml and dialysed against two changes of 4 litres of water for 2h at 4°C. After dialysis the protein solution (pH 5) was either used directly or freeze-dried and stored at −20°C.

Pepsin was obtained from Sigma (twice crystallized and freeze-dried, from pig mucosa, lot no. 22C-2650). Solutions were made up in 1mM-HCl just before use and adjusted to give an absorbance of 1.0 in a 1cm cell at 279nm. Albumin concentration was measured by using $A_{1cm}^{1\%} = 5.34$ at 279nm.

N-Terminal amino acids were determined by the dansyl chloride method described by Gray (1972*a*). *N*-Terminal sequences were determined as described by Gray (1972*b*) by using the dansyl–Edman technique.

C-Terminal residues were determined by treatment of peptides with carboxypeptidase A (Sigma; di-isopropyl phosphorofluoridate-treated; 50μmol/min per mg; lot no. 54C-8021) as outlined by Ambler (1972). Peptide (0.2μmol) was treated with carboxypeptidase A at a ratio of 1:30 (w/w) enzyme/peptide. L-Norleucine was added as an internal standard. After incubation at pH8.5 in 0.2M-*N*-ethylmorpholine buffer, portions (0.02–0.05μmol) of peptide were removed at 0.5, 1, 2 and 4h. After acidification, precipitated peptide was sedimented and the dried supernatant hydrolysed for 24h with 6M-HCl at 110°C. Amino acids were determined on a BIOCAL BC 100L analyser (LKB Instruments, South Croydon, Surrey, U.K.) by using a programme supplied by the makers. All other amino acid analyses were carried out on a two-column system on a Beckman 120C amino acid analyser.

Peptide samples were dried and weighed (1–2mg) before being hydrolysed for 24, 36 and 72h at 110°C in sealed evacuated ampoules containing 1.0ml of constant-boiling HCl. A single crystal of phenol was added to each ampoule to improve recovery of tyrosine.

Polyacrylamide-gel electrophoreses were performed in 10% (w/v) polyacrylamide gels containing 2.6% (w/v) bisacrylamide and 0.2% (w/v) sodium dodecyl sulphate in 0.025M-sodium phosphate buffer, pH8.0. Protein or peptide samples were mixed with 5% (w/v) sodium dodecyl sulphate and 5% (w/v) sucrose in an appropriate dilution of sodium phosphate buffer, pH8.0, and heated in a boiling-water bath for 2min. When it was necessary to reduce disulphide bonds the solution also contained 1% (w/v) 2-mercaptoethanol. The above procedure avoided the aggregation of peptides reported by Braam *et al.* (1974). Gels were stained and destained as described by Weber & Osborn (1969).

Iodo[1-^{14}C]acetamide (57μCi/μmol, from The Radiochemical Centre, Amersham, Bucks., U.K.) was dissolved in water and added to 10mg of human serum albumin in 0.4ml of 0.5M-Tris/HCl, pH8.5, to give a ratio of 1mol of label/mol of protein thiol group (determined by the method of Ellman, 1959). After 30min a 10-fold molar excess of unlabelled iodoacetamide was added and the solution left for 30min. The protein was extensively dialysed against water, before being digested with pepsin under the standard conditions described in the Results and Discussion section. The peptides were separated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Replicate gels were sliced into 2mm-thick discs and heated in scintillation vials at 60°C with two successive 0.25ml batches of 30% (w/w) AnalaR H_2O_2 until the slices had been digested. The vials were filled with the 2:1 (v/v) toluene/Triton scintillant described by Patterson & Green (1965). ^{14}C radioactivity was measured in a Beckman LS 200 B scintillation counter.

Results and Discussion

Choice of cleavage conditions for human serum albumin

Workers attempting to prepare large fragments of bovine albumin have digested the protein for periods up to 1h by using pepsin/bovine albumin ratios of 1:1000 (w/w) or less. There seems to be agreement on the choice of pH3.7 as an optimum pH of producing high yields of large fragments. Below pH4.5, the extent of digestion of the protein is correlated with the conformation of bovine albumin, rather than the activity of pepsin (Braam *et al.*, 1971). The well-known N–F transition of albumin (a change from a Normal to a Fast electrophoretically migrating form of albumin), which is thought to involve separation of domains (Foster, 1960), is complete at pH3.7–3.6. Below pH3.6, further expansion of bovine albumin seems to occur and peptic digestion becomes more extensive (Braam *et al.*, 1971). The control of pH can therefore be used to limit the digestion and possibly to regulate the species of peptides formed. At pH3.7, Braam *et al.* (1971) obtained a highly reproducible pattern of ten peptic fragments of bovine albumin. Although the high-molecular-weight fragments were eventually digested by pepsin, no new fragments of intermediate molecular weight were formed, suggesting that there were a limited number of well-defined sites of peptic hydrolysis in the bovine albumin molecule. Fig. 1 shows the results of digesting human albumin and bovine albumin under conditions described by Braam *et al.* (1971) for bovine albumin [0.02M-KSCN, pH3.6; 1:3000 (w/w) pepsin/albumin; 30min]. At pH3.7, up to 14 components can be identified in the human albumin digest. Some of these correspond very closely in molecular weight to components in the bovine albumin digest. A considerable amount of effort failed to separate the components of high mol.wt. (39000–66000) from the human albumin digest. It was found, however, that by lowering the pH to 3.5 most of the high-mol.wt. species (45000–66000) were removed without markedly affecting the yield of peptides of intermediate molecular weight (Fig. 2).

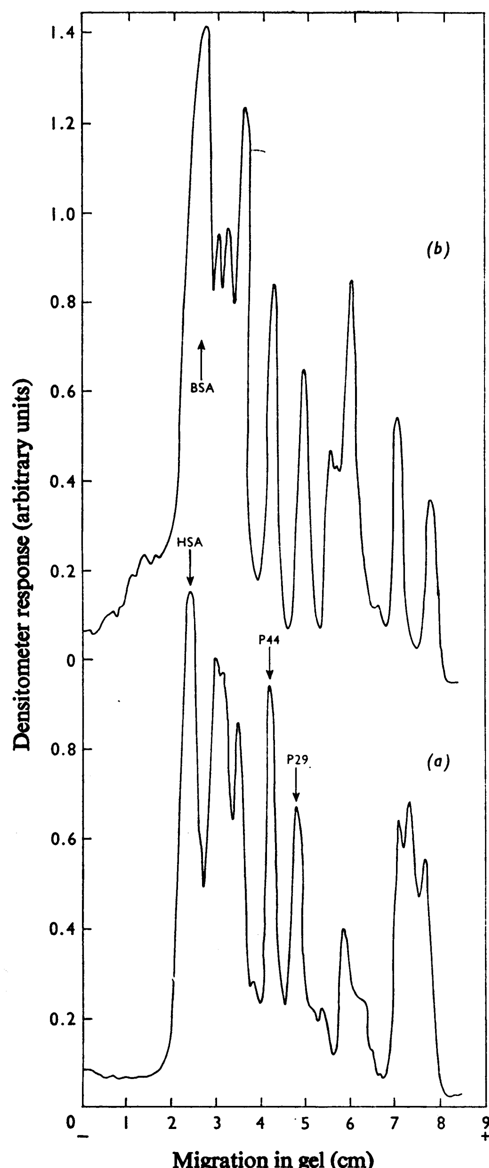

Fig. 1. *Polyacrylamide-gel densitometry of albumin digests*
A comparison of (*a*) human and (*b*) bovine serum albumin digested at pH3.6 in 0.02M-KSCN with pepsin at a pepsin/albumin ratio of 1:3000 (w/w) for 30min. Some components of the human albumin digest are identified with fragments described in the text. BSA, HSA, bovine and human serum albumin respectively.

Digestion of albumin with pepsin

Half-cystinyl human albumin (1g) was dissolved in 30ml of 0.15M-ammonium formate/formic acid buffer, pH3.5, and the pH was readjusted to 3.5 with 2M-formic acid. Digestion was carried out for 30min at 25±0.5°C by the addition of 1ml of pepsin solution (final pepsin/human albumin ratio was 1:1000, w/w). The reaction was stopped and the pepsin denatured by the addition of 2M-Tris until the pH was 8.0. The solution was stirred for 1h at 25°C and then stored at 0°C for 16h.

Pre-fractionation of the human serum albumin digest

The human albumin digest was concentrated by using N_2 pressure in an ultrafilter with an Amicon PM10 membrane. The concentrate was adjusted to pH3.0 with 2M-formic acid and the solution (10ml) was applied to a column (95cm×3.5cm) of Sephadex G-100 pre-equilibrated with 0.2M-ammonium formate buffer, pH3.0 (Fig. 3*a*). The column was eluted at 30ml/h, 10ml fractions were collected and peptides with mol.wt. between 48000 and 25000 were pooled. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was used as an assay for the molecular weight of peptides. Electrophoresis of a sample of the pooled material contained three principal components (mol.wt. 44000, 31000 and 29000 approx.). The pooled material was dialysed against 0.05M-sodium acetate buffer, pH5.0, at 4°C. Attempts to fractionate components on Sephadex G-100 under less acidic conditions failed to separate low-mol.wt. peptides (10000–15000) from the high-molecular-weight fraction. The most likely explanation for this is association of fragments. This association is evidently suppressed under acidic conditions.

Ion-exchange chromatography of the pooled Sephadex fraction

The dialysed pool (80ml) was applied to a column (30cm×1.5cm) of Whatman CM-cellulose CM-52 equilibrated with 0.05M-sodium acetate buffer, pH5.0. After washing with 300ml of the same buffer, the column was eluted with a linear salt gradient formed from 300ml of 0.05M-sodium acetate, pH5.0, and 300ml of 0.05M-sodium acetate/0.2M-NaCl, pH5.0. Fig. 3(*b*) shows the elution profile. The first u.v.-absorbing peak contained a single peptide of mol.wt. 44000.

The remaining u.v.-absorbing material was pooled, concentrated to 10ml in an ultrafilter and diluted to 50ml with 0.1M-Tris/HCl, pH7.6. This solution was applied to a column (14cm×4cm) of Whatman DEAE-cellulose DE-52, equilibrated with 0.1M-Tris/HCl, pH7.6. The column was washed free of unabsorbed peptides, then was eluted with a linear salt gradient formed from 200ml of 0.1M-Tris/HCl, pH7.6, and 200ml of 0.1M-Tris/HCl/0.2M-NaCl, pH7.6 (Fig. 3*c*). Two well-resolved peptides were obtained from this chromatographic step and these

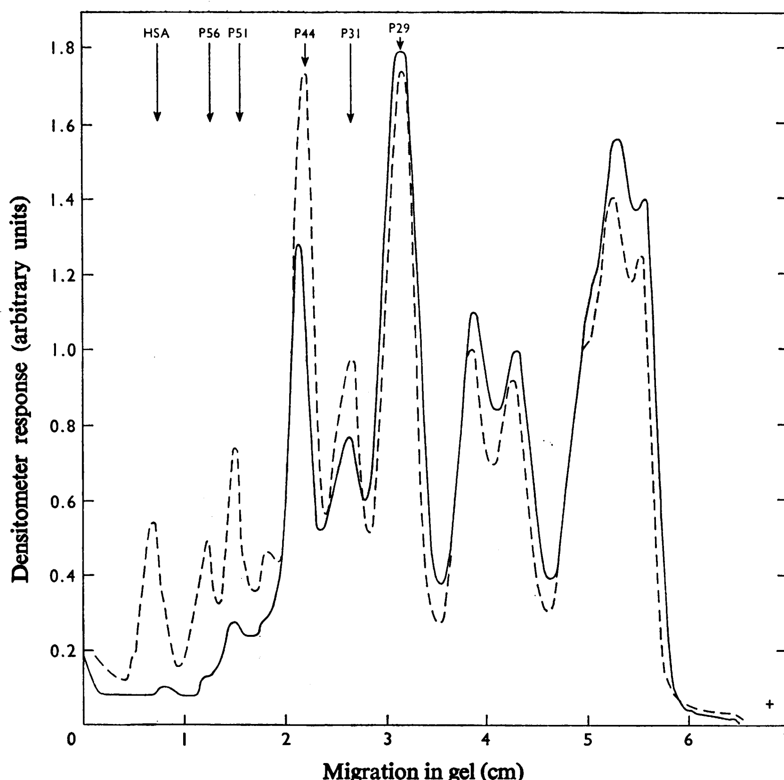

Fig. 2. *Distribution of human albumin fragments at pH 3.7 and 3.5*
The distribution of components of human albumin digested by pepsin in 0.15M-ammonium formate at pH 3.7 (----) and pH 3.5 (——) at a pepsin/albumin ratio of 1:1000 (w/w) for 30min. The fragments identified in the diagram are described in the text. HSA, human serum albumin.

were dialysed against water and stored in batches at −20°C.

Homogeneity of the fragments

All of the peptide components from the ion-exchange steps were homogeneous on polyacrylamide gels in the presence of sodium dodecyl sulphate, except for the 31000-mol.wt. fragment, which split into two components (17500 and 12000) on reduction with 2-mercaptoethanol. The possibility of reductive release of very small peptides, which would have been lost during the staining procedure, cannot, of course, be excluded. *N*-Terminal analyses showed aspartic acid and phenylalanine for the 44000- (P44) and 29000-mol.wt. (P29) fragments respectively, and both phenylalanine and valine in approximately equal amounts for the 31000-mol.wt. (P31) fragment. The presence of two *N*-termini in the last fragment is consistent with the results of the gel electrophoresis of the reduced fragment. Specific absorbance values were obtained for the fragments by measuring the absorption of solutions of weighed portions of dialysed freeze-dried protein.

Location of the peptides in the albumin sequence

Human albumin is provided with two unique amino acid markers, the single tryptophan residue at position 213 and the free thiol group at position 34 in the sequence. These were used in a preliminary survey of the location of fragments in the human albumin sequence.

The presence of tryptophan was detected fluorimetrically in peptides P44 and P29 by scanning the emission spectrum of the peptide produced by an excitation wavelength of 300nm in a Perkin–Elmer MPF-4 fluorescence spectrophotometer. There was no detectable tryptophan in the P31 fragment. Fig. 4 shows the distribution of ^{14}C radioactivity in a peptic digest of ^{14}C-labelled human albumin produced as described in the Experimental section. The labelling

pattern clearly shows that peptides P29 and P31 cannot carry the free thiol group, which is present in fragment P44 and several other fragments.

Table 1 summarizes various data for the fragments, and Table 2 sets out their amino acid compositions. From the information in these Tables it was possible to assign fragments P44 and P29 to residues 1–386 and 49–307 of the human albumin sequence. There is a good correspondence of observed fragment molecular weights with those calculated from the sequence, as well as close agreement of the composition data (Table 2). This agreement is good enough to prefer the assignment by Meloun *et al.* (1975) of the serine and threonine residues in the human albumin sequence to that of Behrens *et al.* (1975). Fragment P29 is entirely contained in fragments P44 and the observed threonine and serine contents of both these fragments are in closer agreement with the sequence data of Meloun *et al.* (1975).

The assignment of fragment P31 was more difficult, since it contains two disulphide-linked peptides and might have lost some internal residues. The assignment was further aggravated by the extreme insolubility of the component peptides after reduction and *S*-carboxymethylation. The performic acid-oxidized fragment was more soluble, but only the peptide with *N*-terminal valine could be isolated in sufficient amounts uncontaminated by the peptide with *N*-terminal phenylalanine. The composition of

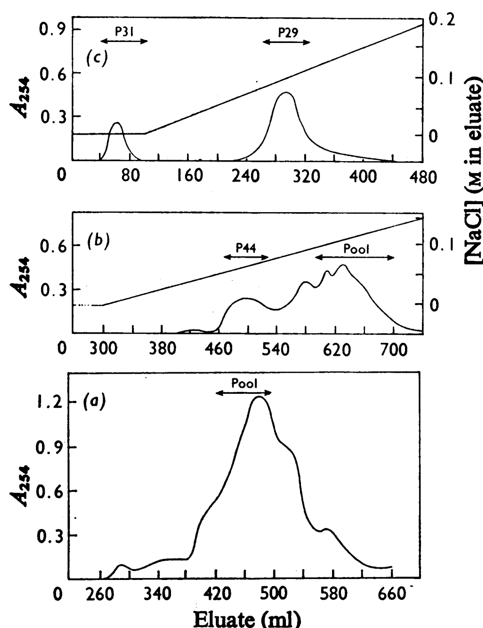

Fig. 3. *Chromatography of the human albumin fragments*
(*a*) Pre-fractionation of the peptic digest on Sephadex G-100 eluted with 0.2M-ammonium formate, pH 3.0. (*b*) Ion-exchange chromatography of the Sephadex G-100 pool (*a*) on CM-cellulose equilibrated with 0.05M-sodium acetate, pH 5.0. (*c*) Ion-exchange chromatography of the CM-cellulose pool (*b*) on DEAE-cellulose equilibrated with 0.1M-Tris/HCl, pH 7.6.

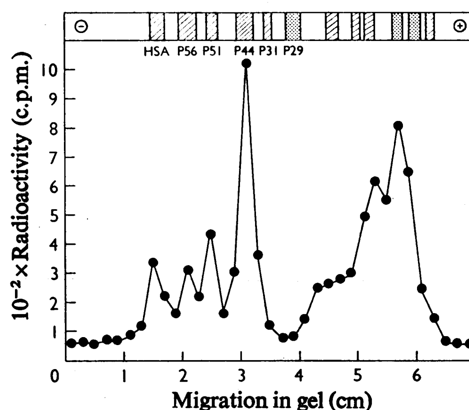

Fig. 4. *Distribution of the albumin thiol group among fragments*
The total count rate (a mean determination from two gels) is plotted against the migration distance in the gel of ^{14}C-labelled components of a peptic digest of human albumin. An identical gel stained for protein is represented for comparison. HSA, human serum albumin.

Table 1. *Fragment data*
The methods of obtaining the data in this Table are shown in the text. n.d., Not determined.

Fragment	Approx. mol.wt.	*N*-Terminal residue or sequence	*C*-Terminal residues or sequence	$A_{1cm}^{1\%}$ at 279 nm
P44	44000	Asx	Asn-Leu	6.0
P31	31000	Val and Phe	(Leu,Ala,Thr)	4.5
P31 (reduced)	12000	Phe-Val	n.d.	n.d.
	17500	Val-Glx	n.d.	n.d.
P29	29000	Phe	(Asp,Ala,Leu)	5.6

Table 2. *Amino acid composition of peptic fragments of human serum albumin*
The compositions observed for each fragment are scaled so that the sum of their constituent amino acids is equal to the number of residues in the corresponding segment of human serum albumin. The standard deviation (three experiments) of each amino acid component is shown, except for those (Ser, Thr, Tyr) that have been estimated by extrapolation to zero hydrolysis time. Fragment P31 (Val) corresponds to the peptide with *N*-terminal valine isolated from performic acid-oxidized fragment P31. No error estimates appear for the composition data, since only 24h hydrolyses were carried out on it. For comparison, the appropriate compositions of Behrens *et al.* (1975) (B) and Meloun *et al.* (1975) (M) are shown for each fragment.

		Residues 1–386			Residues 308–584		Fragment P31	Residues 423–584			Residues 49–307	
Amino acid	P44	B	M	P31	B	M	(Val)	B	M	P29	B	M
Asp	41.9±0.5	39	39	23.6±3	22	21	12.9	13	12	27.2±0.2	27	27
Thr	15.3	19	15	14.0	17	15	10.2	11	10	10.8	15	11
Ser	14.5	11	15	10.2	9	11	7.8	7	8	10.9	9	12
Glu	53.1±0.4	54	53	38.8±1	40	39	21.0	21	22	33.1±0.3	34	34
Pro	15.6±0.4	16	16	10.9±0.5	13	12	6.5	7	6	11.0±0.2	11	11
Gly	7.8±0.2	7	7	6.0±3	6	6	4.5	4	4	5.3±0.2	5	5
Ala	45.8±0.2	46	45	28.0±1	26	26	15.8	16	16	32.0±0.2	32	32
½Cys	24.2±1.4	23	23	17.2±2	16	16	9.9*	11	11	16.8±0.3	18	18
Val	19.2±0.5	21	21	24.3±2	24	26	14.4	15	16	10.3±0.5	10	10
Met	2.7±0.2	4	4	2.8±1	3	3	1.5	2	2	2.6±0.2	3	3
Ile	5.6±0.2	5	5	3.0±1	3	3	1.9	2	2	3.6±0.2	4	4
Leu	41.5±2.7	42	42	27.1±1	28	28	14.0	15	14	28.4±0.2	28	28
Tyr	12.1	14	14	9.1±3	10	10	2.0	2	2	7.2	7	7
Phe	21.0±0.8	21	21	14.4±3	14	14	7.1	7	7	13.0±0.2	13	13
Lys	35.2±0.6	34	35	31.7±1	31	31	19.5	20	20	24.0±0.2	23	23
His	12.3±0.4	12	12	6.0±1	6	6	3.7	4	4	7.4±0.3	7	7
Arg	16.9±0.2	17	17	9.6±2	9	9	6.0	5	6	13.1±0.2	13	13
Trp†	1	1	1	0	0	0	0	0	0	1	1	1
Total residues		386	386		277	277		162	162		259	259

* Determined as cysteic acid on the performic acid-oxidized fragment.
† Detected by fluorescence as described in the text.

the former is shown in Table 2. The data for fragment P31 were satisfied best by residues 308–584 with a split between residues 422 and 423 of the human albumin sequence of Behrens *et al.* (1975). This was confirmed by *N*- and *C*-terminal sequence analysis.

Albumin fragments and the tertiary structure

Fig. 5 shows the location of human albumin fragments with respect to the model of albumin proposed by Behrens *et al.* (1975). The approximate location of fragments P56 and P51 is based on the pattern of ^{14}C-labelling, molecular weight and *N*-terminal analyses.

All of these peptides are entirely consistent with the disulphide-linkage pattern proposed by Behrens *et al.* (1975) for human albumin, by analogy with that of bovine serum albumin. Fragment P44 is closely analogous to the fragment described by Feldhoff & Peters (1975) (residues 1–385 of bovine albumin). Fragment P29 is similar to their fragment P-B (residues 1–306 of bovine albumin), but lacks the first 49 residues. Fragment P31 is similar to their fragment P-A (residues 307–581 of bovine albumin but is 'nicked' between residues 422 and 423. Analogues of the other fragments that they describe seem quite likely on the basis of the comparison of the two digests (Fig. 1), although there are differences in detail. The close correspondence of the pattern of digestion of the two molecules suggests, not unexpectedly, a very similar overall folding of the polypeptide chains.

It is clear that the variation with pH of the peptide composition of the peptic digest of human albumin and bovine albumin is closely linked with the N–F transition, and probably associated with the ionization of β- and γ-carboxyl groups whose average pK is 3.7 (Foster & Clark, 1962). In human albumin, as the pH of the digestion mixture is lowered, the first major components to appear are fragments of 44000–66000 mol.wt. These are produced by cleavage in the *C*-terminal one-third of albumin (residues 386–584). Apart from the 'nicked' fragment P31, of which the yield was low, no large *C*-terminal fragments of human albumin were found at any pH. At pH 3.7, the fragment P44 (residues 1–386) and higher-molecular-weight components persist, but at pH 3.5 the yield of all these components decreases rapidly, and fragment

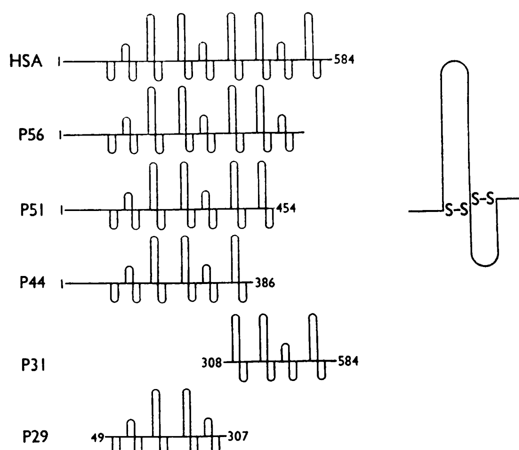

Fig. 5. *Location of large peptic fragments of albumin*
The 584-residue polypeptide chain of albumin is represented in the diagram as a series of loops separated by extended chain. Where each loop touches the horizontal chain, there is a disulphide link. The special feature of the albumin structure proposed by Brown (1975) is the double-loop, which is formed because two of the cysteine residues making disulphide bonds in each loop are adjacent in the linear sequence (see inset). The large albumin fragments described are shown below the representation of the intact molecule. The loops of albumin and its fragments are shown in register for comparison, and the sequence numbers, where known, of free *N*- and *C*-terminal residues are also indicated. The numbers refer to the sequence of Behrens *et al.* (1975). The preferential digestion of the *C*-terminus of albumin is apparent from the diagram. HSA, human serum albumin.

P29 (residues 49–307) is the major peptide present in the digest. This digestion pattern favours a two-stage unfolding process, whereby the *C*-terminal region, closely followed by a region represented by residues 307–386, change their conformation in such a way as to allow susceptible peptide links to become accessible to pepsin. From a study of the pH-dependence of physical parameters of the intact molecule, such as the intrinsic viscosity (Tanford *et al.*, 1955), just such a two-step process has been observed.

In the presence of octanoic acid, King (1973) obtained an intact *C*-terminal fragment of bovine albumin (residues 306–581) using pepsin, and Peters & Feldhoff (1975) obtained a fragment (residues 377–581) from a tryptic digestion of bovine albumin bound to palmitoyl-aminoethyl-agarose. It seems likely that the *C*-terminal one-third of albumin constitutes the principal fatty acid-binding site and that the presence of a fatty acid prevents or delays the low-pH transition of this region and so protects this region from proteolysis.

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References

Ambler, R. P. (1972) *Methods Enzymol.* **25**, 143–154
Andersson, L.-O. (1966) *Biochim. Biophys. Acta* **117**, 115–133
Behrens, P. Q., Spiekerman, A. M. & Brown, J. R. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 591
Braam, W. G. M., Harmsen, B. J. M. & Van Os, G. A. J. (1971) *Biochim. Biophys. Acta* **236**, 99–104
Braam, W. G. M., Hilak, M. C., Harmsen, B. J. M. & Van Os, G. A. J. (1974) *Int. J. Peptide Protein Res.* **6**, 21–29
Brown, J. R. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 1389
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
Feldhoff, R. C. & Peters, T., Jr. (1975) *Biochemistry* **14**, 4508–4514
Foster, J. F. (1960) in *The Plasma Proteins* (Putman, F. W., ed.), vol. 1, pp. 179–233, Academic Press, New York
Foster, J. F. & Clark, P. (1962) *J. Biol. Chem.* **237**, 3163–3170
Gray, W. R. (1972*a*) *Methods Enzymol.* **25**, 121–138
Gray, W. R. (1972*b*) *Methods Enzymol.* **25**, 333–344
Hilak, M. C., Harmsen, B. J. M., Braam, W. G. M., Joordens, J. J. M. & Van Os, G. A. J. (1974) *Int. J. Peptide Protein Res.* **6**, 95–101
Hilak, M. C., Harmsen, B. J. M., Joordens, J. J. M. & Van Os, G. A. J. (1975) *Int. J. Peptide Protein Res.* **7**, 411–416
King, T. P. (1973) *Arch. Biochem. Biophys.* **156**, 509–520
Meloun, B., Moravek, L. & Kostka, V. (1975) *FEBS Lett.* **58**, 134–137
Peters, T., Jr. & Feldhoff, R. C. (1975) *Biochemistry* **14**, 3384–3391
Patterson, M. S. & Green, R. C. (1965) *Anal. Chem.* **37**, 854–857
Reed, R. G., Feldhoff, R. C., Clark, O. L. & Peters, T., Jr. (1975) *Biochemistry* **14**, 4578–4583
Tanford, C., Buzzell, J., Rands, F. & Swanson, S. (1955) *J. Am. Chem. Soc.* **77**, 6421–6428
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412