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Physical and Binding Properties of Large Fragments of Human Serum Albumin

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Three large fragments of human serum albumin were produced by peptic digestion of the native protein [Geisow & Beaven (1977) *Biochem. J.* **161**, 619–625]. Fragment P44 represents residues 1–386 and fragments P29 and P31 represent residues 49–307 and residues 308–584 respectively of the albumin molecule. The large *N*-terminal fragment P44 has a similar percentage of α-helix to stored defatted albumin, although the α-helix content of all the fragments is significantly less than that of freshly prepared albumin. The fragment P44 appears to account for all the binding of the hydrophobic probe 8-anilinonaphthalene-1-sulphonate to albumin. *N*-Acetyl-L-tryptophan binds to this fragment and displaces one of the bound molecules of 8-anilinonaphthalene-1-sulphonate. Bilirubin binds to fragments P44 and P29, and the complexes show similar circular-dichroism spectra to that of the complex between bilirubin and whole albumin. These results are in agreement with affinity-labelling work on albumin with reactive ligands where substitution occurs in the *N*-terminal region of the molecule. The sharp conformational transition in albumin which is observed between pH4 and 3.5 was absent from the fragments. This isomerization, usually called the N–F transition, probably occurs in intact albumin as a result of the unfolding or separation of the *C*-terminal third of the protein from the remainder of the molecule.

Reed *et al.* (1975) found that fragments of bovine serum albumin produced by proteolysis retained secondary structure and ligand-binding properties associated with the native protein. It is likely that the extensive disulphide cross-linking of the molecule (Brown, 1975) provides sufficient constraints on the folding of the polypeptide chain to allow large fragments to retain their original conformation. The pattern of disulphide links forms a structural unit of one small and two large disulphide-linked double loops (Fig. 8), which repeats three times in the proposed primary structure (Brown, 1975). This pattern is broken at the *N*-terminus, but the presence of an unpaired cysteine suggests that the first loop may have been lost through a deletion during the evolution of albumin. There are strong amino acid homologies between these groupings of double loops and between the long double loops themselves (Meloun *et al.*, 1975). It has been suggested that these regions of polypeptide chain may have closely related folding and may represent domains in the protein tertiary structure (Brown, 1975). In the latter instance, the cleavage of connecting peptides between domains would not be expected to cause unfolding of the domains.

Three large peptic fragments of human albumin could be isolated from a digest of the low-pH or F form of albumin. These have been located in the amino acid sequences published by Behrens *et al.* (1975) and Meloun *et al.* (1975). The present paper reports measurements of the secondary structure of the fragments and their interaction with some representative ligands of native human albumin, namely tryptophan, bilirubin and 8-anilinonaphthalene-1-sulphonate. The fragments were examined spectroscopically to determine whether the characteristic N–F transition of albumin (Foster, 1960) could be observed.

Materials and Methods

Large fragments of albumin (P44, P31 and P29, representing residues 1–386, 308–584 and 49–307 of human albumin) were produced as previously described (Geisow & Beaven, 1977). Bilirubin (lot no. 99B-8110) and *N*-acetyl-L-tryptophan were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Human albumin, prepared as described previously (Geisow & Beaven, 1977), was defatted by the method of Chen (1967).

Circular-dichroism spectra were measured with a Jouan CD 185 Dichrograph (Instruments S.A., Division Jobin-Yven, 16–18 Rue du Canal, 91150 Longjumeau, France). Peptides were made up to approx. 0.2mg/ml in 0.003M-sodium phosphate, pH7.2. Percentages of α, β and aperiodic peptide

structures were calculated from the observed spectra in the 250–200nm region by comparison with the ellipticities published by Chen *et al.* (1972). A least-squares regression programme written by Dr. M. T. Flanagan (National Institute of Medical Research) was used to obtain a best fit of the three structural components at 13 wavelengths in this interval. The fitting procedure also calculated the mean molar residue concentration, which could be compared with the known value of this parameter as a check on consistency.

Circular-dichroism spectra of bilirubin-fragment conjugates were measured as described by Beaven *et al.* (1973). Stock bilirubin solution was prepared immediately before use by vortexing 5mg of bilirubin in 50μl of 1M-NaOH. The volume was adjusted to 10ml with 0.05M-Tris/HCl, pH8.5, and the solution was centrifuged at low speed to remove undissolved bilirubin. This solution was diluted with 0.05M-Tris/HCl, pH8.5, to give a bilirubin concentration of between 0.1 and 0.19mM. Solutions of peptides in the same buffer were mixed with bilirubin solution to give peptide/bilirubin molar ratios between 0 and 10 at a final bilirubin concentration of 10μM. Bilirubin and peptide mixtures were allowed to equilibrate in the dark for 30min before measurement.

The effect of pH on the bilirubin–peptide complex was investigated at a bilirubin and peptide concentration of 0.01mM. Titration to the required pH was carried out in the cuvette by addition of 1M-HCl from a syringe micro-burette (Micro-Metric Instrument Co., Cleveland, OH, U.S.A.). Circular-dichroism measurements at fixed wavelengths were corrected for dilution. Back-titration of the solution with 1M-NaOH was carried out after the pH had reached its lowest value to check for irreversible changes in the bilirubin–peptide complex.

Intrinsic peptide fluorescence was measured with a Perkin–Elmer MPF4 fluorescence spectrophotometer equipped with a Grant thermostatic bath set at 25±0.5°C. The A_{280} of the peptide solution was never greater than 0.1. Peptides or albumin were dissolved in 0.1M-sodium phosphate, pH7.2, containing 0.8% NaCl. The titration of peptide solutions was performed as described for bilirubin–peptide complexes. Back-titration was used to check for hysteresis effects.

Solutions of peptide [2μM in 0.01M-sodium phosphate (pH7.2)/0.8% NaCl] were titrated with 2mM solutions of the magnesium salt of 8-anilinonaphthalene-1-sulphonate in the same buffer. All solutions were passed through a 0.22μm Millipore filter just before use. The A_{350} of the peptide solutions after additions of 8-anilinonaphthalene-1-sulphonate was used to correct the fluorescence intensity at 465nm where necessary. *N*-Acetyl-L-tryptophan (5mM) was made up in the same buffer as used for the peptides.

Results and Discussion

Fig. 1 shows the far-u.v. circular-dichroism spectra of human albumin and the three large peptide fragments. Table 1 gives the percentages of secondary and aperiodic structures calculated from the observed spectra. These values should be regarded as very approximate; in particular, the standard deviation given for each entry is an indication of the goodness of fit of the calculated spectrum to the observed spectrum, rather than the precision of the estimation of the true percentage of secondary structure. The inclusion of data for human albumin does, however, provide a comparative standard.

There was more aperiodic structure in the fragments at the expense of α-helix, when compared with freshly prepared human albumin. Stored defatted human albumin, which is probably closer to the condition of the fragments, showed less helical structure than did untreated human albumin and displayed a very similar far-u.v. circular-dichroism spectrum to that of the fragment P44. Fragments P29 and P31, which correspond approximately to the *N*- and *C*-terminal halves of human albumin, had significantly less secondary structure than that reported by Reed *et al.* (1975) for complementary fragments (1–306 and 307–581) of bovine albumin.

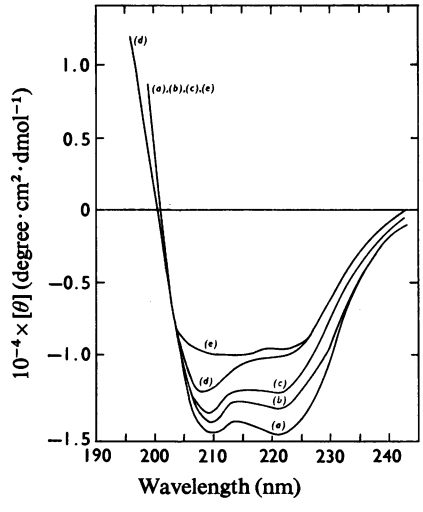

Fig. 1. *Circular-dichroism spectra of human serum albumin and fragments*

The peptides were dissolved in 0.003M-sodium phosphate, pH7. (*a*) Freshly prepared human albumin; (*b*) stored defatted human albumin; (*c*) fragment P44; (*d*) fragment P29; (*e*) fragment P31. [θ] is the mean residue ellipticity calculated from the actual peptide concentration and a mean residue weight of 110.

Table 1. *Secondary and aperiodic structure of human albumin and fragments determined by far-u.v. circular dichroism*
The secondary and aperiodic structure content of each fragment and human albumin is given as a percentage (±S.D.). The fitting procedure calculated a mean amino acid residue concentration after computing the amounts of each type of structure present. This value is shown, together with the actual concentration used in the experiment. Agreement between these two parameters places more confidence on the validity of the structure-content determinations.

	Content of structure (%)			Actual mean residue concentration (mM)	Calculated mean residue concentration (mM)
	α	β	Aperiodic		
Human albumin	44 (±2)	10 (±2)	45 (±2)	2.66	2.93
Human albumin (defatted)	40 (±1)	11 (±3)	49 (±3)	1.5	1.43
Fragment P44	35 (±1)	19 (±2)	46 (±2)	2.52	3.45
Fragment P31	24 (±2)	9 (±2)	67 (±15)	3.23	3.64
Fragment P29	28 (±1)	21 (±3)	50 (±3)	1.52	1.64

This may be due to the peptic cuts in the analogous human albumin fragments at residue 49 in fragment P29 and between residues 422 and 423 in fragment P31. Both of these peptic cuts are in areas of the polypeptide chain predicted to be predominantly α-helical in character (Brown, 1975). These cuts are not present in the analogous fragments described by Reed *et al.* (1975).

8-*Anilinonaphthalene*-1-*sulphonate binding by albumin fragments*

8-Anilinonaphthalene-1-sulphonate binds to human albumin with a blue shift in the maximum of the emission spectrum and a large increase in the quantum yield of fluorescence. Three strong binding sites for 8-anilinonaphthalene-1-sulphonate are reported, with an average binding constant of $10^6 M^{-1}$ (Ma *et al.*, 1973; Naik *et al.*, 1975). In the present work, 8-anilinonaphthalene-1-sulphonate has been used as a probe to compare the affinities of the fragments and human albumin itself for the dye. Fig. 2 shows the fluorescent titration of fragment P44 and human albumin with 8-anilinonaphthalene-1-sulphonate in buffered saline described in the Materials and Methods section. Fig. 2(*a*) shows the fluorescent emission spectra of equimolar amounts of human albumin and fragments, mixed with sufficient 8-anilinonaphthalene-1-sulphonate to saturate the binding sites on human albumin. Under these conditions, the emission spectra of 8-anilinonaphthalene-1-sulphonate bound to either human albumin or fragment P44 were indistinguishable, although the titration experiment indicates that below saturation with 8-anilinonaphthalene-1-sulphonate there are differences in the binding of the dye. The relative fluorescence of 8-anilinonaphthalene-1-sulphonate bound by fragment P29 was significantly lower than when 8-anilinonaphthalene-1-sulphonate was bound by equimolar amounts of fragment P44, although the sites were not saturated by the dye at this concentration. The relative fluorescence of 8-anilinonaphthalene-1-sulphonate bound to fragment P31 was very low and the emission maximum increased from 465nm to 472nm.

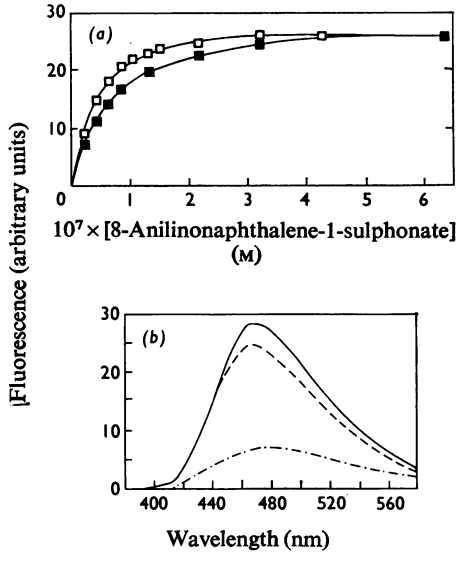

Fig. 2. *Fluorescence of 8-anilinonaphthalene-1-sulphonate bound to human albumin and fragments*
(*a*) The titration of human albumin (□) and fragment P44 (■) with 2mM-8-anilinonaphthalene-1-sulphonate in 0.01M-sodium phosphate (pH7.2)/0.8% NaCl was followed by the fluorescence of the bound dye at 465nm. (*b*) The uncorrected fluorescence emission spectra of 63μM-8-anilinonaphthalene-1-sulphonate in the presence of 3μM solutions of albumin and peptides. These conditions correspond to the plateau fluorescence emission of the dye shown in the titration experiment (*a*). ——, Human albumin; – – – –, fragment P29; –·–·, fragment P31. The emission spectrum of the dye bound to fragment P44 was the same as shown for human albumin.

Weber & Young (1964) reported that cleavage of an

average of only five peptide bonds/molecule of bovine albumin destroyed 75–80% of the fluorescence of bound 8-anilinonaphthalene-1-sulphonate. From the experiments reported here, however, it seems likely that the *N*-terminal two-thirds of human albumin contains all of the strong 8-anilinonaphthalene-1-sulphonate-binding sites and that the removal of the *C*-terminal region only minimally perturbs those sites. This conclusion is supported further by the relatively weak enhancement of 8-anilinonaphthalene 1-sulphonate fluorescence by the *C*-terminal fragment P31 and the strong enhancement of fluorescence by the *N*-terminal fragment P29.

Competition for binding sites between specific ligands and 8-anilinonaphthalene-1-sulphonate is a classical method of following the interaction of ligands with proteins. L-Tryptophan binds to albumin at one primary site with a binding constant $K_A = 1.6 \times 10^4 M^{-1}$ (McMenamy & Oncley, 1958). In experiments where *N*-acetyl-L-tryptophan was added to bovine albumin–8-anilinonaphthalene-1-sulphonate complexes, the fluorescence of bound 8-anilinonaphthalene-1-sulphonate could be decreased to approx. 70% of its initial value (M. T. Flanagan, personal communication). This observation suggests that one of the bound molecules of 8-anilinonaphthalene-1-sulphonate occupies all or part of the indole-binding site. In similar experiments with the *N*-terminal fragment P44 fully complexed with the fluorescent dye, *N*-acetyl-L-tryptophan displaced a maximum of 31% of the bound 8-anilinonaphthalene-1-sulphonate (Fig. 3). Assuming a similar quantum yield for 8-anilinonaphthalene-1-sulphonate in each of the strong binding sites occupied, this observation is consistent with the displacement of one of three bound molecules of 8-anilinonaphthalene-1-sulphonate. By using the statistical binding constant of $10^6 M^{-1}$ for 8-anilinonaphthalene-1-sulphonate binding to human albumin, the titration curve in Fig. 3 gives a value of $10^4 M^{-1}$ for the association constant of *N*-acetyl-L-tryptophan with fragment P44. The competition by *N*-acetyl-L-tryptophan for 8-anilinonaphthalene-1-sulphonate bound to fragment P44 places the indole-binding site in the *N*-terminal part of the molecule. This is in agreement with the affinity labelling of histidine-146 of human serum albumin by bromoacetyl-L-tryptophan (Gambhir *et al.*, 1975).

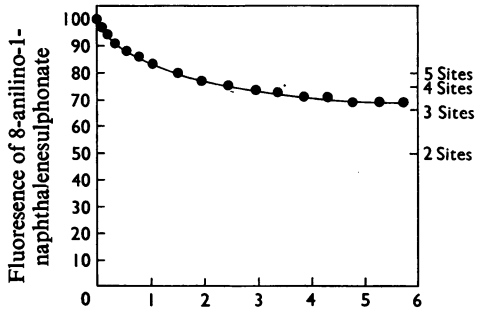

Fig. 3. *Displacement of bound 8-anilinonaphthalene-1-sulphonate by N-acetyl-L-tryptophan from fragment P44*
Fragment P44 was mixed with 8-anilinonaphthalene-1-sulphonate at final concentrations of 0.945 and 46.4 μM respectively in 0.01 M-sodium phosphate (pH 7.2)/0.8% NaCl. To this solution was added portions of 20 mM-*N*-acetyl-L-tryptophan, until the fluorescence at 465 nm was constant. On the right-hand side of the Figure the short lines indicate the expected fluorescence after the displacement of one bound molecule of 8-anilinonaphthalene-1-sulphonate from a total of two to five bound molecules. The ordinate expresses the fluorescence of the dye as a percentage of that measured in the absence of *N*-acetyl-L-tryptophan.

No convincing displacement by *N*-acetyl-L-tryptophan of 8-anilinonaphthalene-1-sulphonate bound to the smaller *N*-terminal fragment P29 could be obtained. This might be due to either the destruction of the indole-binding site or the inability of 8-anilinonaphthalene-1-sulphonate to bind in the indole-binding site of this fragment.

Bilirubin binding by albumin fragments

Bilirubin is bound by albumin at a single strong site, although the analysis of Beaven *et al.* (1973) suggests that up to two minor sites may be present. Fig. 4 shows the induced circular-dichroism spectra of bilirubin in the presence of stoicheiometric amounts of human albumin, fragments P44, P31 and P29. Fig. 5 shows the titration of bilirubin with human albumin and with fragments P44 and P29, followed by monitoring the ellipticity of the ligand at 460 and 407 nm.

It is clear that fragments P44, P29 and human albumin induce a qualitatively similar circular-dichroism spectrum in bound bilirubin. However, the magnitudes of the Cotton effects are less in the order human albumin > P44 > P29. Fragment P31 interacts only weakly with bilirubin, as judged by the induced circular-dichroism spectrum. The signs and magnitudes of the two Cotton effects are a sensitive indication of the conformation of the bound ligand: the signs of the two Cotton effects are reversed in the bovine albumin–bilirubin complex (Harmatz & Blauer, 1975). The close relationship of the bilirubin circular-dichroism spectra induced by human albumin and the two *N*-terminal fragments is an indication of the conformational similarity of the bilirubin-binding sites in the three molecules. From

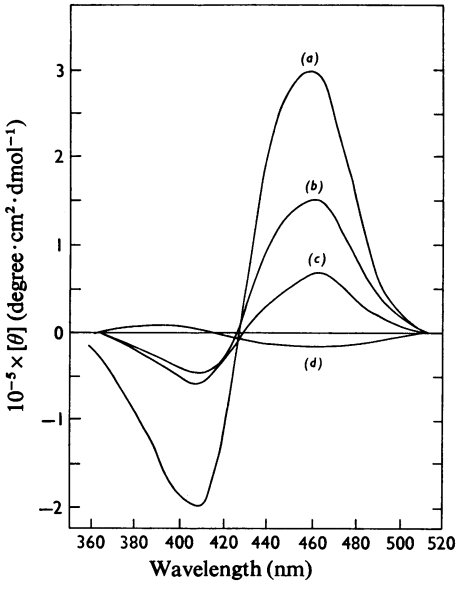

Fig. 4. *Circular dichroism of bilirubin bound by albumin and fragments*

Protein or peptides at a final concentration of 10μM were mixed with equimolar amounts of bilirubin in 0.05M-Tris/HCl, pH8.5. (*a*) Human albumin; (*b*) fragment P44; (*c*) fragment P29; (*d*) fragment P31.

these observations it seems certain that the primary binding site for bilirubin also resides in the *N*-terminal half of albumin.

From the appearance of an ellipticity maximum in the titration curve of bilirubin with human albumin, followed by a lower plateau region, Beaven *et al.* (1973) predict the presence of minor binding sites. These sites have a weaker affinity for bilirubin, but induce a larger dichroism than that of the strong binding site. Hence, as the protein/bilirubin ratio increases, only the strong binding sites are occupied and the initial maximum decays to a plateau region in the titration curve (Fig. 4). This effect is much less obvious in the large fragments of human albumin, which may reflect the loss of these weak sites.

N–F isomerization of albumin

As described previously (Geisow & Beaven, 1977), the three large human albumin fragments were produced from the F (low-pH) form of albumin (Foster, 1960). The reversible expansion of human albumin between pH4 and 3 has been associated with particular regions of the molecule (Geisow & Beaven, 1977). If the peptic cleavage has occurred in those regions of human albumin involved in the N–F isomerization, no similar transition would be expected in the fragments, although the effects of the subsequent acid-induced expansion (below pH3.0) may still be apparent.

Fig. 6 shows the fluorescence of human albumin and fragments at 300 and 350nm as a function of pH. Excitation wavelengths of 260 and 300nm respectively were chosen to separate the fluorescence of tyrosine and tryptophan as far as possible. The fluorescence of fragment P31 is not shown in Fig. 6, since it contained no tryptophan, and few of the total number of tyrosine residues and had a low fluorescence compared with the *N*-terminal fragments and human albumin.

The fluorescent emission of the human albumin chromophores showed a sharp inflexion with a midpoint at pH3.7. The intrinsic fluorescence of charcoal-defatted human albumin showed a similar pH-dependence, except that the midpoint of the inflexion moved to pH4. The tryptophan emission of defatted human albumin did not reflect any well-defined alteration with pH, suggesting that its environment had changed as a result of defatting.

The intrinsic fluorescence of fragments P44 and P29 showed no inflexion in the region of the N–F

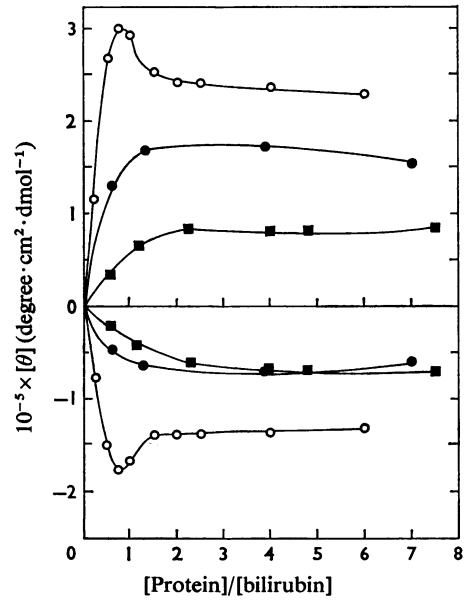

Fig. 5. *Ellipticity of bound bilirubin as a function of albumin or fragment concentration*

The titration of bilirubin (10μM) with protein or peptides was followed by measuring the ellipticity at 460 and 407nm at various protein/bilirubin molar ratios. ○, Human albumin; ●, fragment P44; ■, fragment P29.

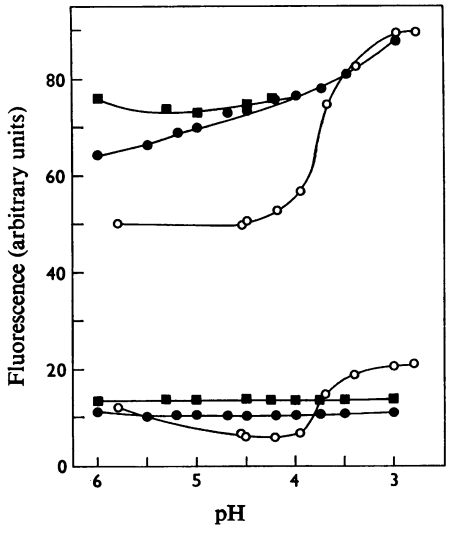

Fig. 6. *Intrinsic fluorescence of human albumin and fragments as a function of pH*
The upper set of curves was measured at 310nm with an excitation wavelength of 260nm, and the lower set at 350nm with an excitation wavelength of 280nm. The choice of these wavelengths was made to separate tyrosine (310nm) and tryptophan (350nm) as far as possible. ○, Human albumin; ●, fragment P44; ■, fragment P29.

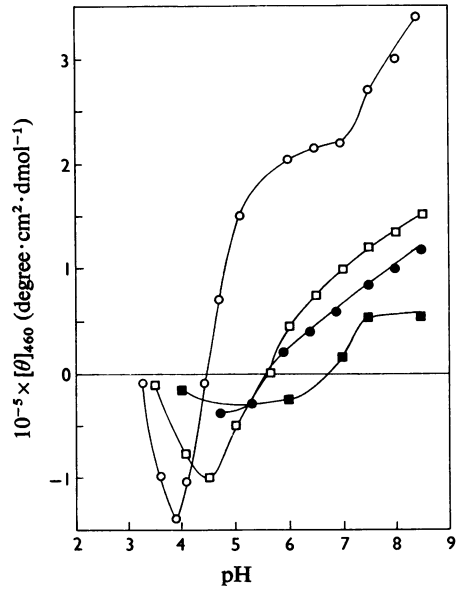

Fig. 7. *Ellipticity of bilirubin bound to human albumin and fragments as a function of pH*
The ellipticity of 0.01mM-bilirubin at 460nm in the presence of equimolar amounts of albumin or fragments is shown as a function of pH. ○, Human albumin; □, human albumin in 0.5M-NaCl; ●, fragment P44; ■, fragment P29.

transition. Instead, there were monotonic changes of the tyrosine fluorescence and little significant change in the tryptophan fluorescence as the pH was varied. All the alterations of fluorescence were reversible without hysteresis.

These observations are consistent with the idea that the N–F isomerization involves the unfolding or the separation of the *C*-terminal region of albumin (residues 387–584) from the remainder of the molecule. The weak fluorescence of the *C*-terminal fragment, P31, showed no inflexion in the region of the N–F isomerization. It is apparent, however, that some structural alterations of all the fragments occurred as the pH of their solutions was varied, since there was a strong dependence of the fluorescence of tyrosine on pH.

Blauer & Wagnière (1975) reported an inversion of the Cotton effects of bilirubin bound to bovine albumin at low pH. A similar inversion has been reported for the bilirubin–human albumin complex (Beaven *et al.*, 1973). This inversion occurred near, but not at, the pH of the N–F isomerization, and the cross-over point of the inversion was highly salt-dependent (Beaven *et al.*, 1973). The pH-dependence of bilirubin ellipticity in 1:1 molar complexes with human albumin, fragments P44 and P29 is shown in Fig. 7. In each case there is an inversion of the Cotton effects, which occurs in the order P29, P44, human albumin as the pH is lowered. This is further evidence that the binding of bilirubin by fragments is closely similar to the binding of the ligand by human albumin. The pH-dependence of the ellipticity of the bilirubin–fragment P44 complex is very similar, in both magnitude and point of inversion, to that of the bilirubin–human albumin complex in the presence of 0.5M-NaCl. The presence of NaCl is thought to decrease the binding of bilirubin to the high-ellipticity low-affinity sites (Beaven *et al.*, 1973).

It seems probable that one result of the removal of the *C*-terminal region of human albumin is the loss of one or more weak bilirubin-binding sites. In this respect, the salt-free fragment P44 then resembles human albumin in the presence of high salt concentrations. The pH effects shown in Fig. 7 were reversible on back-titration of complexes to high pH, but the degree of reversibility was dependent on the length of exposure of the complex to low pH. This presumably reflects the instability of bilirubin, rather than the loss of order in the fragment, since the intrinsic protein fluorescence of the *N*-terminal fragments was reversible over the same pH range.

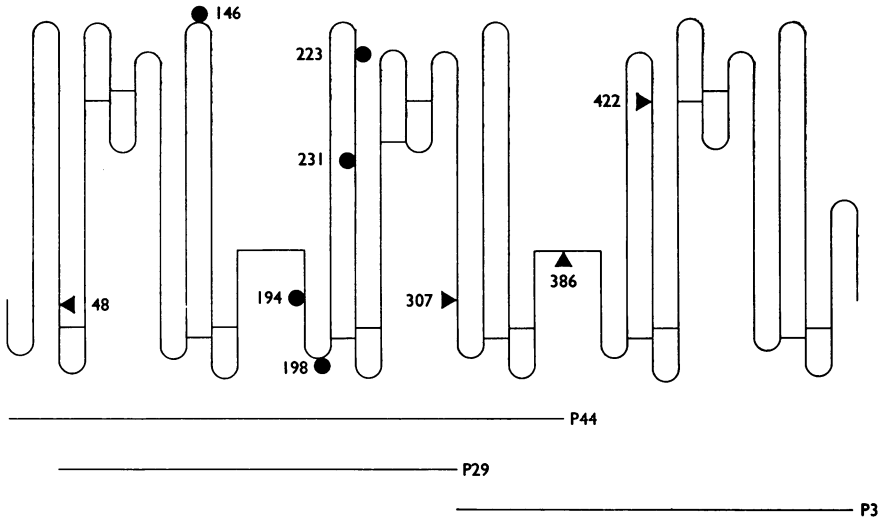

Fig. 8. *Location of ligand-binding sites on albumin*

The Figure represents the albumin molecule as the double-loop disulphide-linked structure proposed by Brown (1975) with the disulphide bridges represented by short lines joining the loops in the chain. The positions of peptic cleavage giving rise to the large fragments described are indicated by arrows. The number refers to the *C*-terminal residue of each peptic fragment. Under the chain, horizontal lines indicate the location of fragments P29, P31 and P44. The circles represent those residues which become covalently labelled as a result of the affinity techniques listed below. (1) Histidine-146 (human albumin) labelled with bromoacetyl-L-tryptophan (Gambhir *et al.*, 1975). (2) Lysine-194 (human albumin) labelled with 5-dimethylaminonaphthalenesulphonyl chloride or pyridoxal phosphate (Gambhir *et al.*, 1975). (3) Lysine-198 (human albumin) acetylated by aspirin (Walker, 1976). (4) Lysine-223 (bovine albumin) labelled by pyridoxal phosphate (Andersson *et al.*, 1971). (5) Lysine-231 (bovine albumin) labelled by fluorescein (Andersson *et al.*, 1971).

Location of binding sites in serum albumin

Fig. 8 shows the location of the two large *N*-terminal albumin fragments in the model of albumin proposed by Brown (1975). Also indicated in Fig. 8 are particular residues of human and bovine albumin which have been identified by affinity-labelling techniques. Gitzelmann-Cumarasamy *et al.* (1976) have identified two human albumin peptides which become covalently labelled with a reactive bilirubin analogue (residues 124–297 and residues 446–547). It seems clear from the weight of evidence that the two large disulphide-linked double loops (residues 124–252 in human albumin; residues 123–251 in bovine albumin) constitute the major binding sites for organic ligands of bovine and human albumin. Apart from the description of a fatty acid-binding site (Reed *et al.*, 1975; King, 1973), no strong binding sites for any other ligands in the *C*-terminal portion of the molecule appear to have been reported. The labelling of residues 446–547 by bilirubin may be an exception, but on the present evidence this region could represent one of the weaker binding sites which are labile to salt.

The clustering of most of the ligand-binding sites in the *N*-terminal part of the molecule, probably also in a restricted region of the tertiary structure, certainly has important consequences *in vivo*. Competition between drugs and other molecules transported by albumin in plasma is known to occur, and the common location of the important ligands of albumin probably means that this will be the rule rather than the exception.

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