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Human Serum Albumin. Tyrosyl Residues and Strongly Binding Sites*

K. Zakrzewski and Halina Goch

ABSTRACT: The binding of dodecanoate and of other fatty acids was found to affect the properties of some of the tyrosyl residues in human serum albumin. The binding gave rise to a long-wavelength shift of the tyrosyl absorption spectrum; no involvement of the tryptophyl residue was detected. The magnitude of the 290-mμ difference absorbancy increased with increasing binding ratio. Concurrently, the Moffit a_0 parameter was less negative but the b_0 parameter remained invariant. The accessibility of tyrosyl residues to methanol perturbation decreased linearly with increasing binding ratios. The saturation of all seven strongly binding sites resulted in the masking of approximately five tyrosyl residues. The ionization of tyrosyl residues could be hindered as well as reverted by the binding of long-chain fatty acids; the magnitude of this effect depended also on the binding ratio. Difference titration analysis showed that a total of about six tyrosyl residues are affected in the alkaline solution by the binding of dodecanoate. The results are interpreted in terms of two sets of strongly binding sites, consisting of two and of five sites each. It is concluded that three tyrosyl residues are located in, or closely associated with, each set of the strongly binding sites in human serum albumin.

A wealth of information has been accumulated in the past concerning the binding properties of serum albumins. Since the matter has been comprehensively reviewed in recent years (Foster, 1960; Putnam, 1965), no attempt will be made here to treat it in detail. Based mainly on studies with detergents, it is considered that there are two major sets of the binding sites: a small number of preexisting and strongly binding sites, and a much larger number of weaker sites which are formed as a result of a cooperative alteration of albumin conformation following the saturation of the strongly binding sites. Thus the interactions taking place at the preexisting sites appear to prepare the molecule of serum albumin for the subsequent conformational transition.

Estimates of the number of strongly binding sites are somewhat variable, possibly depending on the nature of the albumin preparation and/or of the ligand employed. For anionic detergents there are 10–12 such sites (Yang and Foster, 1953; Pallansch and Briggs 1954; Decker and Foster, 1966); they appear to be essentially equivalent, although this has been questioned recently by Reynolds *et al.* (1967). For the long-chain fatty acids the heterogeneity of the strongly binding sites has been rather convincingly documented. Goodman (1958), who was the first to study the binding properties of human serum albumin, carefully freed of residual fatty acids, found that his experimental data could be best accounted for by assuming the existence of three classes of binding sites. The strongest class consisted of two sites, and the intermediate class of five sites; in addition there were more than twenty weaker sites. In an earlier study, Teresi and Luck (1952) found five strongly bind-

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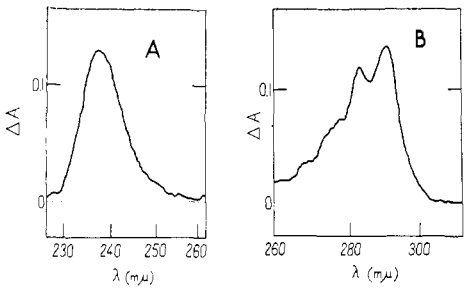

FIGURE 1: Difference absorption spectra generated by dodecanoate in a solution of human serum albumin. Protein concentration 0.58×10^{-4} M in 0.15 M sodium chloride (pH 7). (A) 2-mm light-path cell. (B) 10-mm light-path cell.

ing sites and about thirty weaker ones. As Teresi and Luck (1952) used an albumin preparation which presumably was contaminated with residual fatty acids, and thus had the two strongest sites saturated, the agreement between their estimate and that of Goodman was quite satisfactory. A very similar pattern of distribution of the binding sites for thyroxine has been reported by Tabachnik (1967) who found one to two strongly binding sites and five to six weaker ones in human serum albumin; long-chain fatty acids appear to compete with thyroxine for the binding sites (Tritsch and Tritsch, 1963). Although it is uncertain whether the same sites bind fatty acids and inorganic anions, it is of interest to note that Scatchard *et al.* (1957, 1959) obtained the best fit for his data on inorganic anions by assuming the existence of one strongest site, eight intermediate sites, and eighteen weaker sites.

Little is known about the nature of the strongly binding sites in serum albumins. Long-chain anions are bound very tightly, but their polar group appears not to be a major contributor to the total energy of binding (Decker and Foster, 1967; Ray *et al.*, 1966). Nevertheless, the participation of a cationic group of serum albumin in the binding was postulated by several authors (*cf.* Foster, 1960); it has also been shown that the binding of long-chain fatty acids blocks the availability of certain ε-amino groups of lysine to dinitrofluorobenzene (Green, 1963). In this connection it is worthy of note that a tyrosine residue in serum albumin appears to be made inaccessible to fluorophosphate by long-chain fatty acids (D. C. Shaw quoted by Glazer and Sanger, 1963).

Whatever the participation of the polar group of the ligand, little doubt remains that a prominent role is played in the binding of long-chain ligands by hydrophobic interactions. Serum albumins not only have the propensity for binding of apolar hydrocarbons (*cf.* Foster, 1960; Wishnia and Pinder, 1964), but the energy of binding of long-chain fatty acids sharply increases with increasing length of their hydrocarbon chain (Teresi and Luck, 1952; Goodman, 1958). Thus, the hydrophobic interactions are directly involved in the binding, and possibly exhibit some specificity. The properties of the binding sites may be expected to reflect a definite distribution of hydrophobic amino acid side chains within the binding area.

A novel approach to the study of the nature of the strongly binding sites was made possible by the observation that the interaction of long-chain fatty acids with human serum albumin is followed by a perturbation of the absorption spectrum of tyrosyl residues (Zakrzewski and Goch, 1964; Goch and Zakrzewski, 1965). These observations are further extended in the present paper, and evidence is adduced to show that several tyrosyl residues form a part of, or are closely associated with, the fatty acid strongly binding sites in human serum albumin.

Materials and Methods

Human serum albumin (HSA[1]) was obtained from Messrs. Biomed, Warsaw. It was usually supplied as a solution of Cohn's fraction V just prior to lyophilization, *i.e.*, as a solution containing about 10% w/v protein in about 10% v/v ethanol. Residual fatty acids were removed by charcoal adsorption, a procedure which has been in use in this laboratory for several years.[2] The ethanol solution of fraction V was acidified with 1 N hydrochloric acid to pH 2, and 30 g of charcoal (medicinal grade) was added/l. After being stirred for 1 hr, charcoal was filtered on Seitz filter pads, and the albumin solution was brought to pH 7 with 1 N sodium hydroxide (pH was always measured on samples diluted to 4% v/v ethanol with 0.2% sodium chloride). The solution was then dialyzed at 0° against distilled water, and stored in small aliquots at −25° until used. HSA thus prepared contained less than 0.1 mole of fatty acid/mole of HSA, as determined by titration according to Dole (1956). The electrophoretic purity of HSA in pH 8.6 Veronal buffer was at least 99%. Extensive immunological testing *in vitro* and *in vivo*, including in human volunteers, showed that no alteration of native properties of albumin has resulted from the exposure to low pH in dilute ethanol. Concentrations of HSA were determined from optical density measurements at 279–280 mμ, using the $OD_{1\%}^{1\,cm}$ 5.3 (Clark *et al.*, 1962). All calculations have been based on mol wt 69,000 and on 18 tyrosyl residues/HSA.

HSA complexes with fatty acids were prepared by mixing stock solutions of HSA (about 10^{-3} M in water) with stock solutions of fatty acid sodium salts (about 10^{-2} M in water). The mixture was then diluted with a concentrated solution of sodium chloride so as to yield the desired final concentration in 0.15 M sodium chloride. The pH was adjusted either prior to mixing of reagents, or afterwards, as described under Results. The relatively concentrated solutions of fatty acid sodium salts were strongly opalescent, but sufficiently stable to permit precise pipetting. On mixing the solutions of fatty acids with HSA, the opalescence immedi-

[1] Abbreviation used that is not listed in *Biochemistry* 5, 1445 (1966), is: HSA, human serum albumin.

[2] Shortly after this study was completed, a paper appeared by Chen (1967) describing in detail the removal of fatty acids from serum albumin by adsorption on charcoal. The procedure elaborated by Chen is essentially similar to that employed in this work except that we have found it advantageous to carry out the charcoal adsorption at a slightly lower pH and in the presence of ethanol.

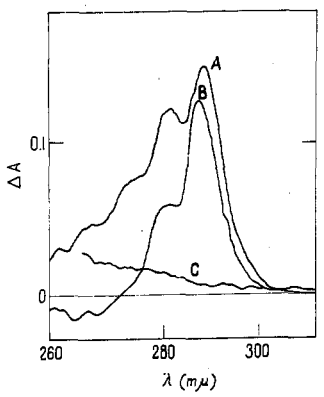

FIGURE 2: Effect of urea and of disulfide-bond cleavage on the difference spectra generated by dodecanoate in human serum albumin solutions. (A) 0.58 × 10^{-4} M albumin in 0.15 M sodium chloride (pH 7). (B) 0.58 × 10^{-4} M albumin in 8 M urea. (C) 0.61 × 10^{-4} M albumin reduced by thioglycollate, in 8 M urea.

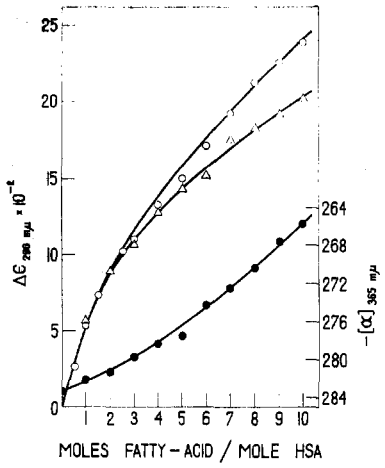

FIGURE 3: Effect of fatty acids on the optical properties of human serum albumin. Solvent, 0.15 M sodium chloride (pH 7). (—△—) Perturbation of tyrosyl spectrum by octanoate. (—○—) Perturbation of tyrosyl spectrum by dodecanoate. (—●—) Rotatory power.

ately disappeared, providing the molal ratio of fatty acids to HSA was not in excess of 15 for octanoate, and 10 for dodecanoate.

Denatured HSA was prepared by cleavage of disulfide bonds with thioglycollate in 8 M urea, as described by Herskovits and Laskowski (1962).

Fatty acids, reagent grade (Schuchardt, Berlin; and POCH, Gliwice, Poland), were used without further purification.

Spectrophotometric measurements were carried out on UNICAM SP 500 and UNICAM SP 700 instruments. The difference spectra were recorded on the SP 700 at ambient temperature, using two matched rectangular 1-cm cells, set closely together in each beam. The two pairs were carefully compensated, using the "*multipot*" arrangement, over the whole range of wavelengths employed. The effects of solvents and of solutes were compensated for by appropriate arrangement of solvents and solutes in the cells. The difference spectra were recorded at a scan speed of 2200 cm^{-1} min^{-1} and at a chart speed of 30 in./hr. The concentration of HSA for the difference spectra at neutral pH was about 0.06 × 10^{-3} M; the resulting slit width was about 0.76 and 0.2 mm around the 280- and 290-mμ peaks, respectively. The magnitude of the difference absorbancy obeyed the Lambert–Beer law within the range employed.

Optical rotatory dispersion measurements were carried out on a Rudolph Model 80 spectropolarimeter equipped with a rocking polarizer, set at approximately 1°. The light source was a mercury arc lamp AH-4; readings were taken at the 365, 405, 435, 546, and 578 bands, using the filters supplied with the instrument. Approximately 0.06 × 10^{-3} M solutions of HSA were placed in a 1-dm polarimeter tube; the temperature was maintained during measurements at 25° by circulation of thermostatically controlled water through the jacketed polarimeter compartment. The optical rotatory dispersion parameters, a_0 and b_0, were computed from the Moffit–Yang equation (Moffit and Yang, 1956) using the least-squares method and assuming λ_0 218 mμ (Sogami *et al.*, 1963).

pH was measured with a glass electrode using the TTT1 titrator (Radiometer, Copenhagen). The readings were corrected, whenever necessary, for sodium ion error using the Radiometer nomograms. pH measurements were standardized using the Radiometer pH 6.5 buffer.

Results

Perturbation of Tyrosyl Spectrum by Fatty Acids. The difference spectra generated by solutions of HSA to which fatty acids have been added showed three peaks of positive absorption: at 237–239 (Figure 1A), 282–284, and 289–291 mμ (Figure 1B). The spectrum in the 282–291 mμ can be interpreted as showing a red shift of the tyrosyl absorption band. This effect could be observed even at very low fatty acid to HSA molal ratios, providing the chain length of the ligand was no less than eight carbon atoms. With shorter chains, the effect was still observable but considerably reduced in magnitude.

The difference absorption spectrum generated by hexanoate was 30%, and by acetate about 5%, of that due to octanoate at equal molal ratios. With increasing molal ratios of the long-chain ligand to HSA, the magnitude of the difference absorbance increased (see below) with no evidence of other spectral changes than a slight shift (by about 2 mμ) of the 282–284- and 289–291-mμ peaks, which tended to be close to the lower limits of their respective wavelength ranges at the low molal ratios. The position of the 237–239-mμ peak has not been investigated in this respect.

The perturbation of the tyrosyl spectrum by fatty acids occurs not only in the dilute salt solution but also in a strong urea solution (curve B, Figure 2). It is only after HSA has been exhaustively cleaved by thioglycollate in urea, that no difference spectrum could be detected following addition of fatty acids (curve C, Figure 2).

The magnitude of the 289–291-mμ difference absorption peak depends on the degree of saturation of

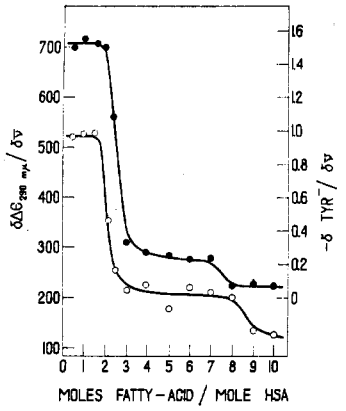

FIGURE 4: Increments in tyrosyl perturbation per mole of dodecanoate added, pH 7 (—○—). Increments in the suppression of tyrosyl ionization per mole of dodecanoate added, pH 11.5 (—●—).

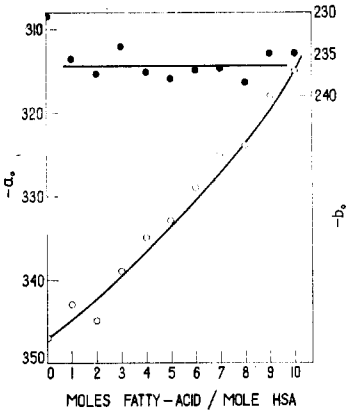

FIGURE 5: Optical rotatory dispersion of human serum albumin in the presence of dodecanoate. Solvent, sodium chloride 0.15 M, pH 7. (—●—) b_0; (—○—) a_0.

HSA with ligand. It will be seen from Figure 3 that the plot of $\Delta\epsilon_{289-291\ m\mu}$ *vs.* ligand:HSA molal ratio deviates from the straight line. In order to investigate this relationship in more detail, the increments in $\Delta\epsilon_{290\ m\mu}$ due to each successively bound mole of dodecanoate per mole of protein have been plotted against $\bar{\nu}$, the number of moles of ligand bound per mole of protein.[3] The differential curve so obtained (Figure 4) shows three distinct sections, each of zero slope. The binding of the first 2 moles of dodecanoate gives rise to the increments in perturbation which are equal but much higher than those due to the binding of the subsequent 5 moles of the ligand. This second plateau is followed by a third plateau which has not been investigated beyond $\bar{\nu}$ = 10. The increments due to octanoate and to decanoate were equal to those due to dodecanoate, at lower range of the binding ratios, and somewhat lower at the higher binding ratios. The pattern shown in Figure 4 satisfactorily corresponds with the distribution of the seven strongly binding sites among the two classes differing in association constants and described by Goodman (1958). It was of interest to note that the rise of $\Delta\epsilon_{290\ m\mu}$ on going from $\bar{\nu}$ = 0 to 2 is approximately equal to the rise between $\bar{\nu}$ = 2 and 7 (Figure 3).

The increase in the magnitude of $\Delta\epsilon_{290\ m\mu}$ is accompanied by a decrease in levorotation, as represented in Figure 3. Optical rotatory dispersion measurements revealed that this change in levorotation was reflected only in a change in the a_0 parameter; the b_0 parameter remained constant throughout the investigated range of binding ratios (Figure 5).

Suppression of Tyrosyl Ionization. It was observed by Ott in 1962 that the addition of 6 moles of various long-chain fatty acids to HSA in a pH 11.55 buffer resulted in a rapid decrease of the 295-mμ absorbancy. We have confirmed this observation, and found that a similar effect took place when the order of addition of reagents was reversed, *i.e.*, if fatty acids were added to a neutral solution of HSA first, and the pH then shifted to 11.5. A number of such complexes of HSA with octanoate and with dodecanoate have been prepared at various molal ratios, all at pH 11.5, in 0.15 M sodium chloride. Their difference spectra were recorded against the neutral solution of defatted HSA in 0.15 M sodium chloride. The number of dissociated tyrosyl residues was calculated, assuming $\Delta\epsilon_{295\ m\mu}$ 2350/residue, for $\bar{\nu}$ values[3] ranging from 0 to 10. From these, the increments have been computed in the number of tyrosyl residues which failed to ionize at pH 11.5 as a result of each successively bound mole of the ligand. The results for the dodecanoate effect are summarized in Figure 4. The plot evidently closely parallels the plot of the increments in dodecanoate perturbation of HSA–tyrosyls in the neutral solution. However, the suppression of tyrosyl ionization by octanoate was only 30%, and by decanoate 60%, of that due to dodecanoate. The increments in tyrosyl ionization suppression resulting from the binding of octanoate and of decanoate yielded the plot which was parallell to that for dodecanoate.

The tyrosyl residues in HSA and its complexes with fatty acids were also titrated spectrophotometrically over the whole range of alkaline pH. The titrations were performed on the complexes formed at neutral pH to which sodium hydroxide was gradually added, as well as on the complexes which were prepared by mixing HSA and fatty acid sodium salts brought to the desired pH prior to mixing. The results were essentially the same.

A family of the spectrophotometric titration curves, represented in Figure 6, was obtained from complexes formed by mixing the preadjusted reagents; pH shown on the axis of abscissae is the final pH. The difference spectra were recorded against the defatted HSA in pH 7.0 sodium chloride (0.15 M), *i.e.*, at approximately the

[3] In the present studies the binding ratios have not been determined. However, with the association constants for dodecanoate of the order of 10^6–10^5 (Goodman, 1958), the ligand:protein molal ratio in solution is not materially different from $\bar{\nu}$, the number of moles of the ligand bound per mole of protein. The same approximation has been used for the experiments carried out in alkaline solution. Although the binding ratios for fatty acids have not, to our knowledge, been studied in alkaline solutions of HSA, in the case of detergent binding Decker and Foster (1967) demonstrated that the stoichiometry of bovine serum albumin complexes with detergents at pH 11.5–11.6 remained essentially the same as in neutral solutions.

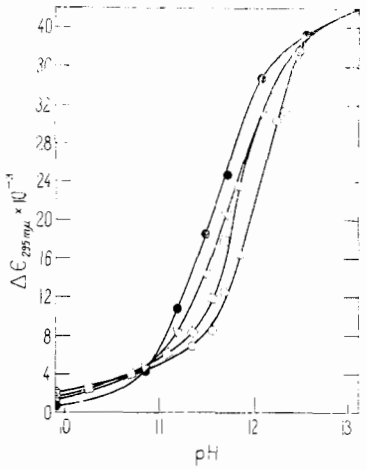

FIGURE 6: Effect of dodecanoate on spectrophotometric titration of tyrosyl residues in human serum albumin. (—●—) Fatty acid free albumin; (—△—) dodecanoate:albumin, molal ratio 1; (—□—) dodecanoate:albumin, molal ratio 2; (—○—) dodecanoate:albumin, molal ratio 7.

same ionic strength as the alkaline samples. It will be seen from Figure 6 that the midpoint for the tyrosyl titration in the defatted HSA was found at approximately pH 11.6, in satisfactory agreement with the value of pH 11.7–11.9 reported by Eisenberg and Edsall (1963) for human mercaptoalbumin. With increasing saturation of the strongly binding sites, the midpoints were shifted toward higher pH. A similar behavior of the tyrosyl titration curves has been reported by Lovrien (1963) and by Decker and Foster (1967) for bovine serum albumin complexes with detergents.

It is apparent from Figure 6 that a smooth sigmoid plot of $\Delta\epsilon_{295\ m\mu}$ *vs.* pH for defatted HSA is shifted to the right after the first mole of dodecanoate has been bound. The saturation of the first two binding sites resulted not only in the shift of the curve but also in a significant distortion of its lower part. The saturation of the next five sites further distorted the curve, especially in its upper part. In order to investigate the titration profiles in greater detail, the difference titration curves between defatted HSA and various HSA complexes have been constructed, using the data from spectrophotometric tyrosyl titration, such as shown in Figure 6. Two such difference–titration curves are represented in Figure 7. Curve A shows the difference between the number of phenolate ions in defatted HSA and in HSA at the dodecanoate:protein molal ratio 2; curve B shows the difference between the number of phenolate ions in HSA at the dodecanoate:protein ratio 2 and 7. The difference titration curve for defatted HSA and HSA with all seven strongly binding saturated (not shown in Figure 7) was a sum of both curves represented in Figure 7, clearly exhibiting both peaks.

Accessibility of Tyrosyl Residues. Solvent perturbation technique elaborated by Herskovits and Laskowski (1962) offered convenient means for studying the changes in the immediate environment of tyrosyl residues, which were likely to result from the interaction of fatty acids with the strongly binding sites in HSA. Since it has been found (Herskovits and Laskowski, 1962)

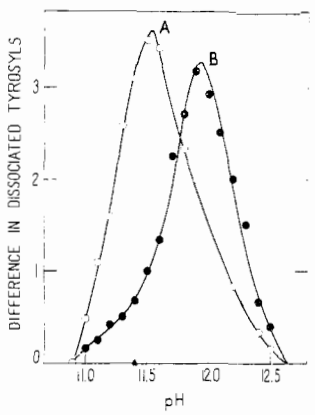

FIGURE 7: Difference–titration curves of tyrosyl residues in the complexes of human serum albumin with various amounts of dodecanoate. (A) (—○—) Difference between fatty acid free albumin and dodecanoate–albumin complex at molal ratio 2. (—●—) Difference between the dodecanoate–albumin complexes at molal ratios of 2 and 7.

that the estimate of tyrosyl accessibility depended on the nature of the perturbant employed, it was considered expedient to probe the surface of HSA with two different perturbants, methanol and glycerol. Optical rotatory dispersion measurements (Table I) showed that methanol, despite its known denaturing properties, does not affect the gross conformation of HSA at the concentration employed in this work, *i.e.*, at 30% v/v. No change of conformation has been found by optical rotatory dispersion measurements in glycerol.

Difference absorption spectra generated by solutions of HSA to which methanol has been added are shown in Figure 8. The thioglycollate-cleaved HSA in 8 M urea exhibits a major peak at about 286 mμ, with $\Delta\epsilon_{280\text{–}288\ m\mu}/\epsilon_{277\ m\mu}$ 0.077; this value was used in computing the accessibility of tyrosyl residues in HSA to methanol as the "100% exposed," following the usage suggested by Herskovits and Laskowski (1962). Native, defatted HSA when perturbed with methanol exhibited the peak of the difference absorbancy shifted toward longer wavelength by about 2 mμ (curve B, Figure 8). Methanol perturbation spectra of HSA–dodecanoate complexes are somewhat different (*cf.* curves C and D, Figure 8) but show the red shift of the tyrosyl absorption spectrum.

In Figure 9 the relationship is shown between the number of moles of dodecanoate bound and the accessibility of tyrosyl residues in HSA. It will be seen that approximately 50% of all tyrosyl residues are accessible to methanol in the defatted HSA. In HSA–dodecanoate complexes the accessibility progressively de-

TABLE I: Effect of 30% v/v Methanol on the Optical Rotatory Dispersion of Human Serum Albumin.

Solvent	a_0	b_0
0.15 M NaCl	−354	−259
30% methanol	−314	−256

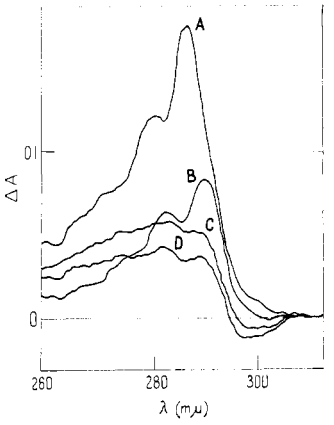

FIGURE 8: Perturbation of tyrosyl residues in human serum albumin by 30% methanol. (A) Thioglycollate reduced albumin, 0.61 × 10^{-4} M, in 8 M urea. (B) Fatty acid free albumin, 0.58 × 10^{-4} M. (C) Dodecanoate-albumin complex, 0.58 × 10^{-4} M, molal ratio 5. (D) Dodecanoate-albumin complex, 0.58 × 10^{-4} M, molal ratio 10.

creased with the increasing binding ratios, and reached a value of about 30% at $\bar{\nu} = 7$. At around that point, the curve (*cf.* Figure 9) turns down sharply and levels off at about 20% accessibility.

An entirely different pattern was observed with glycerol as the perturbant (Figure 9). Independent of the degree of saturation of HSA with ligand, the accessibility of tyrosyl residues in HSA remains invariant throughout the investigated range of the binding ratios.

Discussion

The results reported in the present paper demonstrate that the binding of fatty acids with the highest affinity sites in HSA is followed by a red shift of the tyrosyl absorption spectrum. This is in contrast to the findings of other authors who studied the binding of dodecylsulfate (and some other detergents), and observed a blue shift in the same spectral range and at comparable binding ratios (Bigelow and Sonenberg, 1962; Ray *et al.*, 1966). The source of the discrepancy is not clear, but it must reside in the polar group and not in the paraffin chain of the ligand, because the effects are opposed also in the case of dodecyl sulfate and dodecanoate. Sulfates, of course, differ in their ionization properties from carboxylates, but the charge effect alone is not sufficient to account for the observed difference, because the uncharged ligand, octanol, was found to give rise to the blue shift (Ray *et al.*, 1966). Thus the observed difference between the spectral perturbations appears to reflect the configurational dissimilarity between various ligands. It may be that strongly binding sites in serum albumins are specifically adapted to accepting the carboxyl of fatty acids, which, after all, are physiological ligands.

The difference spectrum arising from the binding of fatty acids exhibited absorption peaks at 282–284 and 289–291 mμ, *i.e.*, the peaks which were displaced by about 2 mμ toward the longer wavelengths from what is the position usually assigned to the tyrosyl absorption band in proteins (Yanari and Bovey, 1960). The peak ratio is about two-thirds, as often found in tyrosyl perturbation (Wetlaufer, 1962), and the curve descends smoothly on the long-wavelength side. Thus there is no evidence of the perturbation of the tryptophyl residue, such as has been observed in case of binding of detergents (Bigelow and Sonenberg, 1962; Reynolds *et al.*, 1967). This appears to be indicative of another subtle difference between the interaction of fatty acids and of detergents with serum albumin molecule.

The red shift of the tyrosyl spectrum was accompanied by a small but significant decrease of levorotation, similar to that reported by Markus *et al.* (1964) and by Reynolds *et al.* (1967) for binding of detergents. The decrease of levorotation without a concomitant change in the b_0 parameter may be interpreted as showing the formation of new hydrophobic surfaces (Tanford *et al.*, 1960) which increase the refractive index in the vicinity of chromophores. However, an alternative explanation of the same phenomenon has been offered by Leonard *et al.* (1963), who suggested that the unmasking of side-chain groups causes a decrease of levorotation as a result of an increased rotational freedom of side chains. The coincidence of the red shift of the tyrosyl spectrum and of the decrease in levorotation makes the latter explanation less applicable to the present studies, although it should be remarked that the possibility cannot be ruled out of an unfolding reaction taking place simultaneously with the masking of tyrosyl residues, but in another segment of the polypeptide chain.

The results of probing the surface of the HSA molecule with methanol showed that the exposure of tyrosyls in the defatted HSA is approximately twice as great as in HSA in which all seven strongly binding sites have been saturated with dodecanoate. Assuming simply that the chromophore may either be freely accessible to methanol or else completely masked, it can be calculated that the saturation of seven strongly binding sites brings about the masking of about five tyrosyl residues[4] in HSA. (Approximately nine tyrosyls are exposed in the defatted HSA, and between four and five tyrosyls in dodecanoate: HSA at $\bar{\nu} = 7$, *cf.* Figure 9.) One additional tyrosyl residue was masked after all seven strongly binding sites had been saturated. A very sharp drop in the accessibility between $\bar{\nu} = 7$ and $\bar{\nu} = 8$, quite distinct from the linear decrease accompanying the saturation of seven strongly binding sites, may indicate the onset of structural alteration preceding the appearance of weakly binding sites.

The assumption on which the calculation of tyrosyl accessibility to methanol has been based, simplified as it undoubtedly was, appeared justified by the "short-range" character of methanol as perturbant (Herskovits and Laskowski, 1962). A short-range perturbant is considered to act by forming a definite molecular complex with the chromophore, and thus the perturbation

[4] The error in the estimation of the number of tyrosyls perturbed by methanol and by glycerol is approximately ±0.5 to ±1.0 residue per HSA, as computed from the precision of spectrophotometric readings and the protein concentration employed.

may be regarded as an all-or-none phenomenon, with no possibility of a partial perturbation.

The extent of exposure of tyrosyl residues, as measured by glycerol perturbation has been found constant throughout the investigated range of the binding ratios. It might be argued that this finding, as well as a similar result reported for sucrose perturbation of other ligand: albumin system (Ray *et al.*, 1967), invalidate the interpretation offered for the methanol perturbation. However, glycerol (and sucrose) belongs to "long-range" perturbants and therefore cannot distinguish between the partial and the total exposure of chromophores. Indeed, Herskovits and Laskowski (1962) concluded from their experiments, in which a large variety of perturbants was employed, that "very few if any tyrosyls in neutral solutions of serum albumin are fully exposed to the solvent." It may be inferred, from the present solvent perturbation experiments, that tyrosyl residues in HSA are located close to the surface of the molecule but inside crevices to which access is limited by factors such as the size of the opening or hydrophobicity so that methanol can penetrate but not the more bulky glycerol. The interaction between the ligand and the binding region would preclude contact between a tyrosyl residue and the solvent with the result that the effect of glycerol would perhaps be somewhat decreased, but methanol would be unable to form a complex with the chromophore. (In the present discussion of the results of the solvent perturbation experiments, the possibility has not been included that the binding of fatty acids was markedly altered by the presence of the solvent. A major decrease of the affinity of the binding sites toward the ligands employed is perhaps not likely in view of the extremely high association constants in water and at low ligand:protein ratios studied. Nevertheless, until this point be clarified, the interpretation offered in this paper of the solvent perturbation experiments may be considered tentative.)

The data for tyrosyl ionization reported in the present paper have not been corrected for electrostatic interactions, because such a correction would involve a number of *a priori* assumptions such as a choice between a generalized and a local swelling of the molecule, and between a discrete and a statistical distribution of charges. Thus no detailed interpretation of the results is feasible at the moment, but some conclusions can be drawn from the observed state of ionization of tyrosyl residues in HSA and in its complexes with various amounts of the ligand. The maximum difference of about three tyrosylates has been found between the defatted HSA and HSA in which the first two sites have been saturated with the ligand. Also approximately three tyrosylates less have been found in HSA with all seven strongly binding sites saturated than in the HSA in which only the first two sites were saturated. A total of six tyrosyls, the ionization of which appears to be interfered with by the saturation of all seven strongly binding sites, is in agreement with the number of tyrosyl residues which are blocked against methanol perturbation at a comparable binding ratio.

Decker and Foster (1967) suggested that the sharpening of the tyrosyl titration curve which followed the binding of detergents with bovine serum albumin may be brought about by a shift in the alkaline isomerization of the protein toward higher pH. This supposition could well account for the effect of fatty acids on the ionization properties of tyrosyl residues alone, but would not be applicable to the other phenomena reported in this paper. That it is the same tyrosyl residues which are affected in the ionized as well as in the unionized state by the long-chain fatty acids, may be inferred from (a) the coincidence of the increments plots for ionization suppression and for fatty acid perturbation (*cf.* Figure 4), and (b) the conformity of the estimates of the number of tyrosyls interfered with as computed from methanol perturbation experiments and from titration. It appears, therefore, as if all these tyrosyl residues were located close to the surface of the molecule, rather than deep among the folds of the polypeptide chain. Consequently, it might be postulated that the observed effects of the interaction with long-chain fatty acids resulted from a direct interaction of the ligand with the environment of the tyrosyl residues, although the possibility cannot at the moment be ruled out of a more or less local change in protein conformation induced by the ligand.

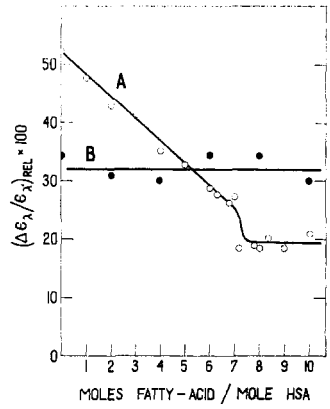

FIGURE 9: Effect of dodecanoate on the perturbation of tyrosyl chromophores in human serum albumin. (A) Perturbation with 30% v/v methanol. (B) Perturbation with 20% v/v glycerol.

The strongly binding sites, or at least that part of the sites with which the tyrosyl residues are associated, appear to be located in a segment of the polypeptide chain which is constrained by disulfide bond(s). The resulting inflexibility may underlie the remarkable ability of serum albumin to bind ligands not only in neutral aqueous solutions but also in strongly alkaline medium and in concentrated urea. It would be interesting to see whether the association of three tyrosyl aromatic rings with each of the sets of strongly binding sites is relevant in this respect.

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