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CHARACTERIZATION OF A SMALL APOLAR ANION
BINDING SITE ON HUMAN SERUM ALBUMIN.

THE OHIO STATE UNIVERSITY, PH.D., 1978

CHARACTERIZATION OF A SMALL APOLAR ANION BINDING SITE
ON
HUMAN SERUM ALBUMIN

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Degree
Doctor of Philosophy in the Graduate School of the
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By

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1978

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Although I strongly believe I shall remember my gratitude for every one I am indebted to, my experience tells me the opposite. Time, and something called "Encephalitis" which I encountered in October, 1977 take away memories with no mercy. When written in words, it shall always refresh my mind and bring back my memories.

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ABBREVIATIONS

1. HSA human serum albumin; while HSA' represents the binding site on HSA

2. K_d Observed value for the dissociation constant for the ligand

$$K_d (\text{obs}) = K_d \left(1 + \frac{[NphOAc]}{K_s} \right)$$
, however, since $[NphOAc] \ll K_s$ under all conditions, $K_d (\text{observed}) = K_d$

3. K_M Michaelis constant

4. K_s Dissociation constant for the reactant; in the present study K_s is presumably equal to K_M .

5. k Observed first order rate constant

6. k_o Observed first order rate constant; to distinguish it from k it is only limited to conditions in the absence of any ligand

7. k_ψ Unimolecular rate constant

8. NphOAc p-Nitrophenyl acetate

9. MphOAc p-Methoxyphenyl acetate

INTRODUCTION

A. General

Serum albumin is the most abundant protein in human blood. It is easily prepared in large quantities in highly purified and native form, and has been known for over a century (Peters,1975). It has been used extensively as a model protein to study the physico-chemical behavior of soluble proteins. Unlike other plasma proteins which have one single function, human serum albumin appears to play numerous physiological roles. It is the principle agent responsible for the colloid osmotic pressure of the blood, for the transport of free fatty acids, and for sequestering and transporting bilirubin. Its binding of L-tryptophan, the only amino acid bound to albumin, plays an important role in affecting the mental condition (Fernstrom and Wurtman,1974). Serum albumin also binds thyroxine and numerous steroid hormones, but with lower affinity than specific hormone binding proteins (Daughaday,1959; Burton and Westphal,1972). Its binding of cations is less dramatic. Only Cu(II) (Peters and Blumenstock, 1967), Ni(II) (Callan and Sunderman,1973), and Mg(II) (Mildvan and Cohn, 1963) appear to bind at specific sites. Various dyes and drugs also bind to serum albumin. The binding of drugs is of essential pharmacology interest, because it is probably the unbound form of drugs which are active. The rate at which a drug is released to sites of catabolism or pharmacological action is largely influenced by its affinity for serum albumin (Schuenemann et al.,1973), and by the presence of other ligands.

Human serum albumin is a single peptide chain of 583 residues. amino acid sequence has recently been elucidated (Brown, 1974, 1975; Brown et al., 1971; Behrens et al., 1975; and Meloun et al., 1975). The multiple adjacent double disulfide loop pattern of the primary structure (Fig 1.) defined by Brown (1974, 1975) may help explain some characteristics of serum albumin, i.e. flexibility, stability, the diversity of its ligand binding sites and the selective protease fragmentation patterns.

One amazing fact about this well studied protein is that even in the most carefully prepared samples, heterogeneity of the albumin is always found. Depending on the methods by which it is purified, there is normally 2-30% non-monomeric albumin present in the product (Franglen, 1974). Other forms of heterogeneity center around the presence of the cysteine residue, $\text{CySH}_{3/4}$. The commonly used Fraction V human serum albumin generally contains approximately 0.65 sulfhydryl groups per albumin (Peters, 1975). The remaining 35% of the protein consists of mixed disulfides of CyS and glutathione. Mercaptalbumin, which contains 1.0 sulfhydryl group per albumin molecule, can be generated from the Fraction V HSA (Hughes and Dintzis, 1964).

The sulfhydryl $\text{CySH}_{3/4}$ is reactive with L-cysteine in the presence of oxygen, or L-cystine to form mixed disulfides (Isles and Jocelyn, 1963; Edwards et al., 1969). Human serum albumin also links to a disulfide containing peptide, vasopressin but at a slower rate than its corresponding reaction with cysteine (Edwards et al., 1969). It has also been suggested that intramolecular exchange can occur between $\text{CySH}_{3/4}$ and one or more nearby S-S bonds. This intramolecular exchange has been proposed to be one source of "microheterogeneity" in albumin (Sogami et al., 1969).

Figure 1. Amino acid sequence of human serum displaced in a model showing the alignment of cystine bonds. (From Behrens et al., 1975)

The term "microheterogeneity" was first used by J. F. Foster to describe albumin species which differ with respect to the pH at which they undergo the N-F conformational transition (Foster et al., 1965). Microheterogeneity is observed even in the albumin of a single donor. The possible causes of this microheterogeneity, other than the factors described above, are (1) tightly bound fatty acids, although removal of such decreases but does not eliminate this heterogeneity (McMenamy, 1967; Wong and Foster, 1969), (2) differences in amide content or location (Spencer and King, 1971), (3) the protease degraded HSA (Wilson and Foster, 1971), or (4) various in vivo modified forms such as that formed by acetylation at a specific amino group by aspirin (Hawkins et al., 1969). The last possibility deserves special consideration in light of the observation of the present study that only approximately 0.7 equivalents of inhibitor to HSA is sufficient to cause full loss of activity at the site under study.

B. Acetylation of HSA by p-Nitrophenyl Acetate

Human serum albumin reacts very rapidly with p-nitrophenyl acetate, (NphOAc) (Huggins and Lapidus, 1947; Dirks and Boyer, 1951). The reaction, resulting in the formation of p-nitrophenolate ion, is biphasic (Bruno and Ringold, 1969; Tildon, 1969, 1972). Rapid formation of approximately one equivalent of p-nitrophenol per molecule of albumin is followed by slower formation of additional p-nitrophenol. With low mole ratios of NphOAc to HSA, acetylation of a specific tyrosine residue largely accounts for the initial rapid formation of p-nitrophenolate ion (Means and Bender, 1975; Means and Bender, unpublished results). The reactive tyrosine appears to be located in an apolar cavity of the protein, and strong reversible binding of NphOAc appears to be a major contributor to the high reaction velocity. After removal of bound lipids and at slightly alkaline pH, the rate

of acetylation by NphOAc exceeds that observed in the presumably 5
"catalytically" acetylation of chymotrypsin.

Small fatty acid anions appear to bind at the same site as NphOAc and compete with it, resulting in a decrease in the rate of its reaction with serum albumin. The extent of this competition thus affords a specific and extremely sensitive basis to study the interaction of compounds with serum albumin at this particular site. Furthermore, under stoichiometric conditions, strong inhibitors may be used to titrate the reactive site.

Rates of reaction of human serum albumin with p-nitrophenyl esters of various acyl chain length are highly variable, suggesting a definite specificity resembling the specificity of an enzyme for its substrate (Means and Bender, unpublished). In an attempt to gain further insight into the dimensions and characters of the reaction site, the reaction of HSA with a homologous series of p-nitrophenyl esters was studied.

The reaction of NphOAc with HSA is mechanistically analogous, and comparable in rate to those of some enzymes. Nevertheless, unlike most enzymes with which NphOAc is known to react, the reaction is not truly catalytic, as there is no subsequent rapid transfer of the acetyl group to another nucleophilic acceptor. Instead, acetyl-HSA is relatively stable and can be readily isolated. The reactivity of acetyl-HSA with various nucleophiles was studied to help characterize this unusually reactive site of human serum albumin.

A specific tyrosine of human serum albumin has been shown to be acetylated upon reaction with NphOAc (Means and Bender, unpublished). The U.V. spectrum of this particular tyrosine, however, is overshadowed by 17 other tyrosine residues and one tryptophan. Comparisons of spectra of HSA and acetyl HSA, however, should afford a means for its specific observation.

Thus, studying difference spectra of ligand bound serum albumin and free serum albumin, in conjunction with those of ligand bound acetyl-HSA and free acetyl-HSA, has been used to characterize the binding site, its reactive tyrosine, and their interactions with various ligands. The results are compared with that of organic solvent perturbation of model compounds (Herskovits and Sørensen, 1968).

C. Hydrophobic and Electrostatic Interactions Between Ligands and HSA.

The high reactivity of NphOAc and similar esters with serum albumin appears to be the result of their rapid reversible binding (Means and Bender, 1975), in close proximity to the reactive tyrosine residue and the existence of a high cationic charge density close to the reactive tyrosine (Means and Bender, unpublished). The latter influence is responsible for the low pK of the reactive tyrosine residue and its shift to higher values at higher ionic strength, the direction of that shift being opposite to what might normally be expected for a simple phenol-phenolate dissociation. Modification of arginines with 2, 3 butanedione results in a great loss in reactivity, suggesting the cationic groups are arginine residues (Means and Bender, unpublished results). It has been suggested (Jonas and Weber, 1971_{a,b}) that the binding sites on serum albumin for organic anions contain hydrophobic and positively charged amino acid residues, the latter being primarily arginine residues. Chemical modification of arginine residues but not lysine residues, resulted in a marked decrease in the affinity of HSA for anions (Jonas and Weber, 1971_b).

The observed inhibitory effects of fatty acid anions on the reaction of NphOAc with HSA is assumed to be due to its competitive binding at the NphOAc binding site (Means and Bender, 1975), and thus is consistent with a positively charged hydrophobic binding site. As the inhibition obeys

1 to 1 stoichiometry, a single site appears to be involved.

7

The interactions of a series of homologous saturated fatty acid anions from butyrate through laurate with the reactive site have been examined to help determine its size and shape. The relative free energy change upon binding increases with increasing acyl chain length, up to a limit of about ten carbons (Means and Bender, 1975). Larger fatty acid anions, i.e. palmitate inhibit the reaction only when present in excess (Means and Bender, 1975). Binding studies for hexanoate, octanoate and decanoate have been done with equilibrium dialysis (Ashbrook et al., 1972). These fatty acids are constituents of tryglycerides used clinically in treating patients with impaired utilization of ordinary dietary fat, i.e. in cystic fibrosis (Kuo and Huang, 1965), tropical sprue (Cancio and Menéndez-Corrada, 1964), and type I hyperlipo proteinemia (Levy and Fredrickson, 1968; Lees and Wilson, 1971).

D. Binding of L-tryptophan and Drugs by the NphOAc Binding Site.

L-tryptophan was first found to bind to a nondialyzable plasma component twenty years ago (McMenamy et al., 1957). This plasma component was later identified as serum albumin (McMenamy & Oncley, 1958). Binding of tryptophan and other indoles is specific and occurs mainly at one site (McMenamy, 1958, 1963). The anionic form of L-tryptophan is the principal form involved in its binding (McMenamy, 1963), and is competitively inhibited by Cl^- and SCN^- (McMenamy, 1964).

"Affinity labeling" of serum albumin with N-bromoacetyl-L-tryptophan largely blocked the binding of N-acetyl tryptophan (Gambhir and McMenamy, 1973). In the presence of indolepropionate, the labeling was much reduced. Cyanogen Bromide fragmentation of the labeled HSA lead to the isolation of a peptide fragment of molecular weight 18,000 (Fragment C) containing the

major locus for N-bromoacetyl tryptophan reaction. Sjöholm and Ljungstedt (1973) have shown that only fragment C among the three CNBr fragments of HSA showed affinity to the L-tryptophan-sepharose column with the carboxyl group of tryptophan free, while the column having the amino group free did not extract any fragment from the cyanogen bromide digest.

The binding of L-tryptophan to defatted HSA has been shown to be inhibited by palmitate only when the latter is present in excess (Cunningham et al., 1975). In contrast, laurate caused a marked decrease in tryptophan binding at molar ratios as low as 0.5.

Studies of tryptophan binding to serum albumin in the presence of various drugs, or vice versa, have been carried out vigorously. The goals of these studies have been set to find the most effective drug delivery condition (Martin 1965, Levy, & Yacobi, 1974), to determine the effects of drugs on free L-tryptophan levels in serum and thus on the synthesis of serotonin in the brain (Gessa and Tagliamonte, 1974). Drugs with very different molecular structures as well as pharmacological properties, are able to displace L-tryptophan from its single binding site on the HSA molecule (Muller and Wollert, 1975). Among the highly displacing ligands are chlorpromazine, dicoumarol, flufenamic acid, sodium benzoate, suramin, tolbutamide, and warfarin. The benzodiazepine derivatives bind essentially to one site (Muller, and Wollert, 1974; Sjödin et al., 1976), which is also the tryptophan binding site (Muller and Wollert, 1975).

In the present study, L-tryptophan is found to inhibit the reaction of p-nitrophenyl acetate with human serum albumin. The reaction therefore affords a sensitive and specific method to study the binding of L-tryptophan. And thus it can also be used to screen a broad spectrum of drugs and other metabolites which also bind at the L-tryptophan binding site.

E. P-Nitrophenol as a Probe to Study Ligand-HSA Interaction.

Binding of many dyes to serum albumin cause the color either to intensify, or to change in hue. A number of these dyes have been used for albumin assay (Arvan and Ritz, 1969, Doumas and Biggs, 1972).

Some experimental approaches used to study the binding of small molecules to HSA, involve optically detecting the changes in the conformation of the small molecules or protein. Detection techniques commonly used are circular dichroism (e.g. Woolley and Hunter, 1970), fluorescence (e.g. Rippie, 1976), spin labelling (e.g. Soltys and Hsia, 1977; Lagercrantz *et al.*, 1978), or by monitoring a shift of the absorption peak (Herskovits, 1967). These small molecules can then be used as probes to study binding of those which do not have detectable conformational changes upon binding to serum albumin (e.g. Woolley and Hunter, 1970).

P-Nitrophenolate ion inhibits the reaction of p-nitrophenyl acetate with human serum albumin, and thus appears to bind to the same site as L-tryptophan, small fatty acid anions and many drugs. Its absorption spectrum is blue shifted upon binding. Assuming that binding of the p-nitrophenolate to the reactive site results in the inhibition and the observed blue shift of the spectrum, p-nitrophenolate ion is thus a new probe to study the small molecule and HSA interactions.

MATERIALS

Human serum albumin (fraction V) from Sigma Chemical Company (Lot 24C-1632-8), human serum albumin (Lot 32), and human mercapalbumin (Lot 7) from Miles Laboratories were defatted according to Chen (1967). Fatty acid-free HSA purchased from Sigma Chemical Co. (fraction V, "essentially fatty acid-free", Lot 16C-7280) was used without further treatment.

Radioactive ^{14}C -p-nitrophenyl acetate, 8.2×10^8 cpm/mole was prepared previously (Means and Bender, 1975). p-Methoxyphenol and p-nitrophenol obtained from Eastman Organic Chemicals were recrystallized from chloroform. p-Chlorophenol from Eastman Organic Chemicals was recrystallized from carbon tetrachloride before use. Phenol and hydroxylamine hydrochloride, analytical reagent grade, from Mallinckrodt were used without further purification. Aniline from J.T. Baker Chemical Co. was redistilled before use. Water solutions of each nucleophile were adjusted to pH 7.5 and an ionic strength of 0.02 M by addition of triethanolamine, HCl or KOH.

p-Methoxyphenyl acetate was prepared by reacting p-methoxyphenol with an excess of acetic anhydride. The crystalline product obtained from chloroform melted at $33-34^\circ$.

NphOAc, obtained from Eastman Kodak Co., was recrystallized before use. Other p-nitrophenyl esters were purchased from Sigma Chemical Co.: p-nitrophenyl propionate, Lot 66B-5290; p-nitrophenyl butyrate, Lot 93C-5080-2; p-nitrophenyl valerate, Lot 77B-5180; p-nitrophenyl caproate

Lot 72c-511Q The concentrations were determined by alkaline hydrolysis and taking molar extinction coefficient of p-nitrophenol as $18,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 400 nm.

Fatty acids were obtained from various places: butyric acid, Fisher Scientific Co.; valeric, hexanoic, heptanoic, nonanoic, dodecanoic, and undecanoic acids, Sigma Chemical Co.; octanoic acid, Nutritional Biochemical Co.; and decanoic acid, Matheson, Coleman and Bell. The purity of decanoic acid was determined by titration to be $> 98\%$.

L-Tryptophan was purchased from Sigma Chemical Co. (Lot 90C-3310). N-Acetyl-L-tryptophan was purchased from Cyclo Chemical Co. (Lot #10625). Indolepropionic acid was purchased from Sigma Chemical Co. (Lot 85C-0349) and recrystallized in chloroform (M.P. $134-135^{\circ}\text{C}$, molar absorbancy of $5300 \text{ M}^{-1} \text{ cm}^{-1}$ at 279 nm in H_2O).

METHODS

All spectrophotometric measurements were made with a Cary 118C spectrophotometer with a thermostated cell holder at 25°. An extinction coefficient of 18,000 M⁻¹ cm⁻¹ at 400 nm is used to quantitate p-nitrophenolate anion in aqueous solutions, and the pK of p-nitrophenol, at 0.02 M ionic strength and 25° was taken as 7.14 (see Fernandez and Hepler, 1959 at 0 M ionic strength and 25°). Concentrations of L-tryptophan and indolepropionate in aqueous solutions were determined using molar extinction coefficients of 5250 and 5300 M⁻¹ cm⁻¹ at 279 nm respectively. An extinction coefficient $\epsilon_{278}^{0.1\%}$ of 0.531 (Cohn *et al.*, 1947) and a molecular weight of 66,400 were used to determine human serum albumin concentrations.

Counting of radioactive samples was done with a Beckman liquid scintillation counter, Model LS-133 in 5 ml of scintillation fluid (5g/L of Research Products International Preblend 2a60 in toluene or for aqueous samples, 16.5g R.P.I. preblend 2a60 in 2L of toluene plus one liter of triton X-100).

A. Acylation of HSA by Homologous Fatty Acids p-Nitrophenyl Esters.

Reactions of p-nitrophenyl esters with HSA, unless noted otherwise, were followed by monitoring the appearance of absorption of p-nitrophenolate anion at 373 nm in the presence of at least an eleven fold excess of HSA as described in detail by Means and Bender (1975). Nitrophenyl ester at approximately 10⁻³ M in spectrograde acetonitrile was added directly into a cuvette and rapidly mixed to initiate the reactions.

Concentration of acetonitrile never exceeds 0.5%. Pseudo-first order¹³ kinetics were observed from which first order rate constants were obtained from the slopes of plots of $\log (A_{\infty} - A_t)$ versus time using a linear least squares program and a Hewlett-Packard HP-55 calculator. Only rate constants with coefficient of determination of 0.999 or greater were used. Data obtained over a range of HSA concentrations are plotted in Lineweaver-Burk type plots, and intercepts and slopes are determined from the calculated best fit using a weighted linear least squares program (Wilkinson, 1961).

B. Perturbation of HSA Spectra.

Two equally concentrated HSA solutions of 2.6 ml in capped quartz cuvettes are each placed in the sample and reference compartments, and with adjustments to the multipots on the spectrophotometer a difference spectrum close to being flat between 350 and 270 nm is obtained. Subsequently, a small amount of either decanoate (i.e. neutralized to potassium salt prior to dissolving in triethanolamine buffer to give a solution of 6.08×10^{-3} M at pH 7.5 and final ionic strength of 0.022 M) or palmitate (i.e. 4.22×10^{-3} M in triethanolamine buffer pH 7.5, as sodium salt) is added to the sample cell, and an equal volume of triethanolamine HCl pH 7.5 buffer is added to the reference cell at the same time. After an incubation period of about five minutes, a spectrum scanning through 350 and 270 nm is taken. The procedure is repeated until the desired ratio of fatty acid anion to serum albumin is reached, while the total volume of fatty acid solution added does not exceed 2.6% of the starting volume.

The acetyl-HSA solutions are made by reacting HSA with 1.1 equivalents of NphOAc followed by extensive dialysis against 1 mM acetate

buffer pH 4.0 at 3° for three days. To remove the acetate the protein¹⁴ is dialyzed against H₂O for another day, and finally, against the triethanolamine HCl buffer at pH 7.5 and ionic strength of 0.02 M for several hours.

Spectral measurements are performed with the Cary 118C spectrophotometer set at the following conditions:

- 1) Absorption range of 0.1 Å.
- 2) Auto gain and slit width of 0.3 mm.
- 3) Pen period of 5 seconds.
- 4) Scan speed of 0.02 to 0.1 nm per second.
- 5) 25°

C. Deacylation of Acetyl-HSA.

Acetyl-HSA was prepared by essentially the same procedure as described in B, except HSA is reacted with one or less equivalent of NphOAc and the dialysis period is cut approximately to 50%. In preparing ¹⁴C-acetyl-HSA, Nph[¹⁴C]OAc with ¹⁴C labeled at the acetyl group (see Materials) was used and approximately 90% of the label was attached to the protein. The two preparations of ¹⁴C-acetyl-HSA used in this work contained 0.53 and 0.73 ¹⁴C-acetyl group per molecule of HSA.

1. Determination of kinetic constants by dialysis techniques.

Solutions of ¹⁴C-labeled acetyl-HSA of approximately 3.1 mg/ml (0.8 ml) in triethanolamine buffer, $\mu = 0.02$ M were placed on one side of a carefully washed cellulose acetate membrane (Union Carbide 23DM00D646Y6C11) in small acrylic dialysis cells (Technilab Instruments, Model 260 Pequannock, New Jersey) and an equal volume of the same concentration ($\pm 1\%$) of HSA (i.e. not acetylated) was placed on the opposite side. The addition of HSA opposite the acetyl-HSA was intended to

approximately equalize the binding of reaction products on each side of¹⁵ the membrane. Reactions were started after brief preincubation in a 30.5°C water bath by the addition of 5 to 200 μ l of an aqueous solution of the test nucleophile to both sides of the membrane. Samples of 25 μ l were then taken from the continuously shaking cells at intervals of time and subjected to liquid scintillation counting.

Using large molar excesses of nucleophile under the indicated conditions, deacetylation appears to follow pseudo first-order kinetics. Because the reactions are very slow, however, initial, zero-order rates, k' , were determined from approximately the first 10% of reaction. First-order rate constants, k , were calculated by dividing the zero-order constants, k' , by the molar concentration of acetyl-HSA. Rate constants in the presence of added nucleophiles were corrected for the rates observed in their absence. Data obtained over a range of nucleophile concentrations were plotted in Lineweaver-Burk type plots, and intercepts and slopes were determined from the calculated best fit using a weighted linear least squares program (Wilkinson, 1961).

2. Spectrophotometric monitoring of deacylation.

A small but significant increase in absorption at 278 nm accompanies the deacylation of acetyl-HSA. Rates of deacylation were thus determined by continuous monitoring the absorption at 278 nm of samples in sealed, glass stoppered quartz cells of 10 mm path length using a Cary 118 C spectrophotometer in the 0.02 A absorption range. Reactions were followed for at least eight half-lives and rate constants determined from the slopes of plots of $\log (A_{\infty} - A_t)$ versus time.

3. Determination of kinetic constants by toluene extraction. 16

To a series of tubes containing ^{14}C -labeled acetyl-HSA ($6.94 \times 10^{-5} \text{ M}$) in pH 7.5, triethanolamine buffer, $\mu = 0.02$ at 29.8°C was added 3 to $50 \mu\text{l}$ of $4.18 \times 10^{-2} \text{ M}$ p-methoxyphenol in the same buffer. Samples of $200 \mu\text{l}$ were taken at various time intervals and rapidly agitated with 1 ml of toluene. After a brief, low speed centrifugation 0.7 ml of the clear toluene phase was taken for scintillation counting. Rate constants were calculated from the radioactivity detected during approximately the first 10% of the reaction assuming $dp/dt = k [\text{acetyl-HSA}]$. In the absence of added nucleophile negligible reaction rates were observed.

4. Thin-layer chromatography and identification of p-methoxyphenyl acetate.

Radioactive material remaining after evaporation of a toluene extract from the reaction of $6.5 \times 10^{-5} \text{ M}$ ^{14}C labeled acetyl-HSA with $3.5 \times 10^{-3} \text{ M}$ p-methoxyphenol for 0.5 hr at 29° was applied to the bottoms of a series of silica gel G coated plastic thin-layer chromatography sheets (Kodak # 13179) and eluted with hexane: benzene (4:1) or hexane: ethyl acetate (85:15). Visual detection of sample was obtained after exposure to I_2 vapor. Radioactive spots were located by carefully scraping small sections from the plates into scintillation vials, adding 10 ml of scintillation fluid and counting. All the observed radioactivity migrated as a single spot in both eluant systems with mobilities identical to authentic p-methoxyphenyl acetate.

D. Determinations of Free Binding Site Concentrations¹ of HSA and Dissociation Constants for Ligands².

Free binding site concentrations, $[HSA']^1$, in the presence of added ligands (inhibitors) were determined from observed reactivity with NphOAc according to the relationship:

$$[HSA']/[HSA']_0 = (k - k_b) / (k_0 - k_b) \quad (1)$$

where k and k_0 are first order rate constants in the presence and absence of ligands, respectively, and k_b is that in the presence of a large excess and due to other reactions of NphOAc (Means and Bender, 1975).

Plots of k/k_0 versus the concentrations of ligand added decline linearly in proportion to added ligand at HSA concentrations much higher than K_d , the apparent ligand: albumin dissociation constant (see Figs. 28, 29); i.e. stoichiometric binding is observed either with strong binding ligands or at high HSA concentrations. Binding site concentrations are determined from such plots by extrapolation of the initial linear decline in reactivity to the level due to other reactions as obtained in the presence of a large excess of ligand (Means and Bender, 1975; see also Figs. 28, 29).

At HSA concentrations comparable to K_d or lower, reactivity declines along a series of smooth curves resembling simple binding isotherms. Values of K_d are estimated for each ligand from calculated theoretical binding isotherms best fitting the experimental data, assuming the existence of an equilibrium,

where L stands for "Ligand", and

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$$K_d = \frac{[HSA^+][L]}{[HSA \cdot L]} \quad (3)$$

and the total ligand and binding site concentrations are

$$[L]_0 = [L] + [HSA \cdot L] \quad (4)$$

and

$$[HSA^+]_0 = [HSA^+] + [HSA \cdot L] \quad (5)$$

An alternative method to determine the binding site concentration and the dissociation constants used is that described by Bieth (1974), Webb(1963), and Easson and Stedman (1936). With a modified equation 1 ;

$$1 = \frac{[HSA \cdot L]}{[HSA^+]_0} = \frac{(k - k_b)}{(k_0 - k_b)} \quad (1a)$$

and combination of it with equations (3) and (4), a new expression for the binding site concentration and the dissociation constant is obtained:

$$\frac{[L]_0}{1 - \frac{(k - k_b)}{(k_0 - k_b)}} = \frac{K_d}{\frac{(k - k_b)}{(k - k_b)}} + [HSA^+]_0 \quad (6)$$

RESULTS

A. Acylation of HSA by Homologous Fatty Acid p-Nitrophenyl Esters.

Similar to its reaction with p-nitrophenyl acetate, HSA also reacts rapidly with other straight chain fatty acyl p-nitrophenyl esters. Nevertheless, the reactivity varies significantly. Lineweaver-Burk plots for a series of p-nitrophenyl esters of straight chain fatty acid, from acetate through caproate, are shown in Figure 2 through Figure 6. Kinetic parameters calculated from these plots are shown in Table 1. It appears that the interaction (K_M) of esters with HSA steadily increases as the acyl carbon chain length increases. Reactivity, however, as measured by the bimolecular rate parameter k_p/K_M does not increase regularly as the unimolecular rate constant, k_p , varies irregularly and actually declines significantly for the longer chainlength. Thus greatest reactivity is obtained with p-nitrophenyl propionate which, as measured by the parameter k_p/K_M , is about 10 times more reactive than the acetate ester and about 8 times greater than the butyrate esters.

The reactive site specificity of HSA for these fatty acyl p-nitrophenyl esters is reflected by the corresponding second order rate parameter k_p/K_M as compared to that for simple hydrolysis of the same esters by dividing k_p/K_M by the second order rate constant for hydrolysis (k_{OH^-}) to give a dimensionless parameter, $k_p/K_M \cdot 1/k_{OH^-}$ (Marshall & Akgün, 1971). As shown in Figure 7, the optimum acyl carbon chain length is two methyl(ene) groups plus one carbonyl.

Figure 2. Double reciprocal plots for the reaction of NphOAc with HSA. HSA in excess at pH 7.5 in triethanolamine/HCl, 0.020 M ionic strength and 25°. Two parts are duplicates from which the calculated parameters for K_M are 1.97×10^{-4} M and 1.89×10^{-4} M; for k_y are 1.48×10^{-1} sec⁻¹ and 1.56×10^{-1} sec⁻¹ respectively.

Figure 2

Figure 3. Double reciprocal plot for the reaction of p-nitrophenyl propionate with HSA. Conditions are as in Figure 2. The calculated K_M and k_y are 5.83×10^{-5} and $2.45 \times 10^{-1} \text{ sec}^{-1}$ respectively.

Figure 3

Figure 4. Double reciprocal plot for the reaction of p-nitrophenyl butyrate with HSA. Conditions are as in Figure 2. The calculated K_M and k_{cat} are 4.69×10^{-5} M and 4.77×10^{-2} sec respectively.

Figure 4

Figure 5. Double reciprocal plot for the reaction of p-nitrophenyl valerate with HSA. Conditions are as in Figure 1. The calculated K_M and k_{cat} are 2.21×10^{-5} M and 9.54×10^{-3} sec⁻¹ respectively.

Figure 5

Figure 6. Double reciprocal plot for the reaction of p-nitrophenyl caproate with HSA. Conditions are as in Figure 2. The calculated K_M and k are 8.42×10^{-6} M and 1.14×10^{-2} sec⁻¹ respectively.

Figure 6

TABLE 1

KINETIC PARAMETERS OF P-NITROPHENYL ESTERS REACTION WITH HSA^a

P-Nitrophenyl esters	$k_p \times 10^2$ (sec ⁻¹)	$K_M \times 10^5$ (M)	k_{OH}^b (M ⁻¹ sec ⁻¹)	k_p/K_M (M ⁻¹ sec ⁻¹)	$k_p/K_M \times \frac{1}{K_{OH}}$
Acetate	15.6 14.8	18.9 19.7	10.3	825 750	80.13 72.79
Propionate	24.5	5.83	5.2	4204	808.5
Butyrate	4.77	4.69	9.8	1016	103.7
Valerate	0.954	2.21	4.4	432	98.38
Caproate	1.16 1.14	1.17 0.842	6.7	995 1367	148.6 204.1

a Experimental conditions same as in Figure 2.

b Values obtained from Marshall & Akgün, J. Biol. Chem. 246, 19, p. 6019-6023.

Figure 7. Reactive site specificity of HSA for fatty acyl p-nitrophenyl esters as a function of the number of methyl(ene) groups (n). The corrected specificity constants ($k_{\psi}/K_M \times 1/k_{\psi}(\text{OH}^-)$) are plotted against n. Values of k_{ψ} and K_M for the corresponding p-nitrophenyl esters are obtained from Figures 2 to 6. The second-order rate constants for OH^- hydrolysis of the esters ($k_{\psi}(\text{OH}^-)$) are obtained from Marshall & Akgün (1971).

u

Figure 7

Corrected Specificity Constant

Free energies of association increase as a function of the number³³ of methylene group in the acyl chain (Fig. 8). The steady increase corresponds to an average increase of 0.42 k cal/-CH₂-, while from the intercept at zero methylene groups one can estimate that the rest of the molecule (NO₂-) contributes 4.73 k cal/mole to the interaction.

Inhibition by small apolar anions of the reaction of p-nitrophenyl acetate with HSA is also observed in other p-nitrophenyl esters. Thus inhibition by L-tryptophan of the reactions with p-nitrophenyl propionate and p-nitrophenyl caproate are described in the section F on "Binding of L-tryptophan and Its Analogs", from which dissociation constants calculated for tryptophan are the same as that obtained with p-nitrophenyl acetate. Decanoate is also a strong inhibitor of these reactions giving inhibition similar to that with p-nitrophenyl acetate and p-nitrophenyl valerate. The apparent stoichiometry of the inhibition indicates the same number of reactive sites per HSA for both esters (Table 2). In other words, a discrepancy amounting to about 30% between the HSA concentration determined spectrophotometrically, and the reactive site titration by decanoate is observed in both p-nitrophenyl acetate (see the section on "Binding of Small Fatty Acid Anions") and p-nitrophenyl valerate reactions. The residual activities, the activities remaining in the presence of large excess of strong inhibitors, while reflecting background reactions of the p-nitrophenyl esters with HSA, however are different for p-nitrophenyl acetate and p-nitrophenyl valerate (Table 2).

B. Perturbation of the Reactive Tyrosyl and Tyrosyl Residues on HSA

The reactive nucleophile of HSA appears to be located in the high affinity binding site for small fatty acid anions, as its reaction with

Figure 8. Variation in free energy of association for the homologous fatty acyl p-nitrophenyl esters. Dissociation constants K_M obtained from Figure 2 through 6 are converted to free energies of association and plotted versus the number of methyl(ene) groups (n). Conditions are as Figure 2.

Figure 8

TABLE 2

INHIBITION OF THE REACTIONS BETWEEN HSA AND *p*-NITROPHENYL ESTERS BY DECANOATE^a.

[Decanoate] $\times 10^5$ (M)	<u>[Decanoate]₀</u> [HSA] ₀	<u>NphOAc</u> ($k_0 = 4.12 \pm 0.06 \times 10^{-2} \text{ sec}^{-1}$) k/k_0		<u>NphOVal</u> ($k_0 = 6.06 \pm 0.15 \times 10^{-2} \text{ sec}^{-1}$) k/k_0	
0	0	1.00		1.00	
6.08	0.076	0.87		0.89	
12.16	0.151	0.80		0.83	
18.24	0.228	0.71		0.72	
24.32	0.304	0.63		0.64	
304	3.78	0.065		0.025	
608	7.55	0.049		0.019	

^a[HSA]₀ = 8.05×10^{-5} M as determined spectrophotometrically; in triethanolamine/HCl buffer pH 7.5, 0.02 M ionic strength and 25°.

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NphOAc is strongly inhibited by their presence (Means and Bender, 1975).

As shown, small amounts of added decanoate ion alter the ultraviolet absorption of HSA (Fig. 9.). The resulting difference spectra, with maxima at 281 and 291 nm are similar to those generated upon the binding of dodecanoate (Steinhardt et al., 1972) and appear to reflect the transfer of several tyrosine residues into less polar environments (Herskovitz and Sorensen, 1968). Similarly, addition of decanoate ion to acetyl-HSA (i.e. as obtained from the reaction of HSA with NphOAc and thoroughly dialyzed to remove p-nitrophenolate ion: See Methods) gives similar, but seemingly more complex, difference spectra (Fig. 9.), but again also apparently reflecting the transfer of tyrosine residues into less polar media.

The difference between the two sets of spectra presumably reflect the acetyl group by which HSA and acetyl-HSA differ. Thus, the absorption increase at 290 nm is noticeably greater with HSA than with acetyl-HSA (i.e. see Figure 9). At low mole ratios of decanoate to HSA the difference between the two as plotted in Figure 10 reveals a pair of peaks at approximately 282 and 290 nm separated by a trough at 285 nm which again resembles a difference spectrum like that expected upon the transfer of a tyrosine side chain into a less polar medium (Herskovitz and Sorensen, 1968). Association with decanoate thus appears to perturb a tyrosine residue in HSA which is not similarly perturbed in acetyl-HSA.

Palmitate anion also binds strongly to HSA but apparently at a different site than decanoate (Means and Bender, 1975; see also section D on "Binding of Fatty Acid Anions"). Addition of palmitate thus also perturbs the absorption of HSA and the resulting difference spectra,

Figure 9. Difference spectra of HSA and acetyl-HSA induced by decanoate. The upper half of the figure represents ultraviolet spectra of 3.17×10^{-5} M HSA with increasing amounts of decanoate at ratios of [decanoate]/[HSA] of (from bottom to top) 0, 0.29, 0.59, 0.81, 1.18, 1.77, 3.54, and 5.01. The lower half of the figure represents the U.V. spectra of 3.47×10^{-5} M acetyl-HSA with decanoate present in ratios of [decanoate]/[HSA] of, (from bottom to top) 0, 0.27, 0.54, 0.81, 1.01, 1.68, 2.36, 3.03, 3.71. All in triethanolamine/HCl, ionic strength 0.02 M pH 7.5 at 25°.

Figure 9

Figure 10.

Difference difference spectra for fatty acid binding to HSA and acetyl-HSA. The upper half of the figure represents differences in the molar absorption change of HSA and acetyl-HSA upon binding decanoate as determined from Figure 9 as the difference in the absorption change upon their binding 0.59 and 0.54 equivalents of decanoate, respectively. The lower half of the figure represents differences in molar absorption change of HSA and acetyl-HSA upon binding palmitate as determined from Figure 11 as the difference in the absorption change upon their binding 0.58 and 0.54 equivalents of palmitate, respectively.

Figure 10

while less intense also appear to reflect the transfer of one or more tyrosine chromophores into a less polar medium (Fig. 11). In contrast to the results with decanoate, however, nearly identical difference spectra are obtained upon addition of palmitate to either HSA or acetyl-HSA (Fig. 11). Thus, acetylation of HSA by NphOAc seems to have no effect on its binding of low mole ratios of palmitate (Fig. 10) consistent with previous results showing that low concentrations of palmitate ion do not affect acetylation (Means and Bender, 1975; see also section D on "Binding of Fatty Acid Anions"). Different changes in absorptivity accompanying the binding of decanoate and palmitate by HSA are revealed in the respective difference spectra both of which resemble that expected upon the transfer of one or more tyrosine side chains into less polar media. The difference between the two as plotted in Figure 12, suggests the perturbation of additional tyrosine residues by decanoate. In contrast to their binding to HSA, the binding of these anions by acetyl-HSA results in slightly more similar absorbing changes as shown by their difference plotted in Figure 12. This difference between HSA and acetyl-HSA as already shown, appears to result exclusively from changes in the effect of decanoate binding after acetylation (see also Fig. 13) as no changes are observed in the case of palmitate after acetylation (Fig. 13.).

Figure 11. Difference spectra of human serum albumin induced by palmitate. The upper half represents the U.V. spectra of 3.12×10^{-5} M HSA with palmitate present in ratios of [palmitate]/[HSA] of (from bottom to top) 0, 0.29, 0.58, 0.81, 1.18, 1.77. The lower half of the figure represents U.V. spectra of 3.38×10^{-5} M acetyl-HSA with palmitate present in ratios of [palmitate]/[HSA] of, (from bottom to top) 0, 0.27, 0.54, 0.75, 1.09, 1.63. All conditions are the same as given in Figure 9.

Figure 11

Figure 12. Differences in U.V. difference spectra of human serum albumin upon binding decanoate and palmitate. The upper half of the figure represents differences in molar absorptancy of HSA upon binding 0.59 equivalents of decanoate and 0.58 equivalents of palmitate as determined from Figures 9 and 11. The lower half of the figure represents the effect of acetylation of HSA on the same phenomena as determined from Figures 9 and 11 by absorption changes accompanying binding of 0.54 equivalents of decanoate and 0.54 equivalents of palmitate to HSA.

Figure 12

Figure 13. The effect of acetylation of HSA on its fatty acid anion induced U.V. difference spectra. The upper half of the figure represents differences in the molar absorption change of HSA induced by 0.59 equivalents of decanoate and that of acetyl-HSA induced by 0.54 equivalents of palmitate. The lower half of the figure represents differences in the molar absorption change of acetyl-HSA induced by 0.54 equivalents of decanoate and that of HSA induced by 0.58 equivalents of palmitate. The corresponding absorption changes are determined from Figures 9 and 11.

Figure 13

1. Deacylation by phenols.

The reaction of ^{14}C -acetyl HSA (0.53 equivalent of acetyl group per HSA) with nucleophiles slowly liberates its radioactive acetyl group. The reaction involving the nucleophilic displacement of the acetyl group from the protein can be monitored using a simple dialysis cell as described in "Methods" by determining the increase in the radioactivity with time in samples collected from the side of the cell initially containing only HSA and nucleophiles but no radioactivity. The reactions appear to follow first order kinetics, but because they are very slow, are only followed for the initial 4 to 30 % of completion, depending on the nucleophiles. Apparent zero order rate constants were calculated from no more than the first 10 % of the reaction.

Different nucleophiles differ in their ability to liberate ^{14}C -acetyl group from ^{14}C -acetyl-HSA (Fig.14). With fixed HSA concentration and concentrations of the nucleophile varied, reaction rates vary such that dissociation constants, K_M , and unimolecular rate constants, k_y , can be determined from double reciprocal plots, wherein the inverse of the apparent first order rate constants are plotted against the inverse of the concentrations of the nucleophiles.

$$\frac{1}{k} = \frac{1}{k_y} + \frac{K_M}{k_y [\text{Nucleophile}]}$$

These double reciprocal plots for various phenols are shown in Figures 15 through 18. Dissociation constants and k_y values obtained are listed in Table 3.

Figure 14. Deacylation of acetyl-HSA by nucleophiles as a function of time. At pH 7.5 in triethanolamine/HCl buffer, ionic strength 0.02 M and $30.5 \pm 0.3^\circ\text{C}$, with 4.32×10^{-5} M ^{14}C -acetyl-HSA and 0.8 mM p-chlorophenol (■), p-methoxyphenol (▲), phenol (●), p-nitrophenol (□); 1 mM NH_2OH (△); and H_2O (○). The amount of ^{14}C -acetyl group in 25 μl of samples collected using the dialysis method (see "Methods") is plotted as a function of time.

Figure 15. Double reciprocal plot of the deacylation reaction of ^{14}C -acetyl-HSA by p-methoxyphenol. Individual data points were determined by the dialysis technique with p-methoxyphenol in excess at pH 7.5 in triethanolamine/HCl buffer, ionic strength of 0.02 M and 30.5° from initial rates in the presence of p-methoxyphenol less than in its absence. The ratio $[\text{acetyl}]/[\text{HSA}]$ is 0.53. K_M and k_p values thus determined are 2.75×10^{-4} M and $5.67 \times 10^{-2} \text{ hr}^{-1}$.

Figure 15

Figure 16. Double reciprocal plot for the deacylation of ^{14}C -acetyl-HSA by phenol. With phenol in excess and conditions are same as given in Figure 15. K_M and k_p values obtained are $7.8 \times 10^{-4} \text{ M}$ and $3.66 \times 10^{-1} \text{ hr}^{-1}$.

Figure 16

Figure 17. Double reciprocal plot for the deacylation of ^{14}C -acetyl-HSA by p-chlorophenol. With p-chlorophenol in excess and conditions are the same as given in Figure 15. K_M and k_p values thus determined are 1.30×10^{-4} M and $6.15 \times 10^{-2} \text{ hr}^{-1}$.

Figure 18. Double reciprocal plot for the deacylation of ^{14}C -acetyl-HSA by p-nitrophenol. With p-nitrophenol in excess and conditions are the same as given in Figure 15. K_M and k_p values thus obtained are 1.85×10^{-4} M and 1.61×10^{-2} hr $^{-1}$.

Figure 18

TABLE 3

DEACYLATION OF ^{14}C -ACETYL-HSA BY PHENOLS.^a

ArO^-	pK_a	$K_m \times 10^4$ (M)	$k_p \times 10^2$ (hr ⁻¹)	$k_p \times 10^4$ (min ⁻¹)	$[\text{ArO}^-]/[\text{ArOH}]$	$(k_p/K_m)_{\text{ArO}^-}$ (min ⁻¹ . M ⁻¹)	$\log(k_p/K_m)_{\text{ArO}^-}$
p-Cl-	9.38	1.30	6.15	10.25	1.32×10^{-2}	606.4	2.78
p-NO ₂ -	7.14	1.85	1.61	2.69	2.29	2.09	0.32
p-CH ₃ O-	10.20	2.75 4.58 ^b 4.68 ^c	5.67 4.83 ^b 3.90 ^c	9.45 8.05 ^b 6.50 ^c	2.0×10^{-3}	1720.9 881.76 ^b 696.4 ^c	3.24 2.95 ^b 2.84 ^c
H	9.95	7.8	3.66	6.10	3.55×10^{-3}	221.91	2.35

a. Experimental conditions are the same as in Figure 3 except as noted.

b. Conditions are the same as in Figure 3 except the ratio $[\text{acetyl}]/[\text{HSA}]$ of ^{14}C -acetyl-HSA is 0.73.

c. The reaction is followed by toluene extraction of the reaction mixture incubated at 29.8°; the ratio $[\text{acetyl}]/[\text{HSA}]$ of ^{14}C -acetyl-HSA is 0.72.

The nucleophilicity of the phenols listed in Table 3 appears⁶¹ to be a function of their para substituents. As shown in Fig 20 the k_p/k_M values of the reactions appear to be a linear function of the substituent constants, σ^- .

Small fatty acid anions were found to inhibit the acetylation of HSA by NphOAc (Means and Bender, 1975). Similarly, the deacetylation of acetyl-HSA is also inhibited by small fatty acid anions. In the presence of decanoate, the rate of p-chlorophenol reaction with ^{14}C -acetyl-HSA is thus decreased (Fig 21) and the extent of inhibition increases with increasing amounts of decanoate anion added. These results suggest the same apolar binding site is involved in both the acetylation and deacetylation of HSA.

The reaction of phenols with acetyl-HSA apparently involves rapid reversible binding of these nucleophiles to the reactive site. Therefore, the reactivity is largely dependent on the intact structure of the reactive site. As shown in Fig 22 at pH 7.8 the presence of 6.46M urea has brought about 80% decrease in the initial rate of reaction.

2. Deacylation by hydroxylamine.

Hydroxylamine, a stronger nucleophile than would be expected for an amine of the same basicity (Jencks and Carriuolo, 1960), has been used to deacylate various esters. In 40 mM hydroxylamine at pH 6, the half-life of phenylacetate is less than 20 minutes (Smyth, 1967), and in 20mM hydroxylamine at pH 7.5, deacetylation of two active site tyrosine residues of carboxypeptidase A is complete in less than 2 hours (Riordon and Vallee, 1963). With acetyl-HSA in 50 mM hydroxylamine at pH 7.8, however, incubation for 6 hours only results in about 50% deacetylation (Fig 23). In comparison with the phenols, hydroxylamine reacts very slowly

Figure 20. Substituent effects in the reactions of para-substituted phenols with acetyl-HSA. Substituent constants for the phenols are 1.24 (σ^-), NO_2 ; 0.23 (σ_p), Cl ; 0, H ; and -0.27 (σ_p), OCH_3 (from Ritchie and Sager, 1964). Individual data points are as shown in Table 3.

Figure 20

Figure 21. Inhibition of deacylation by fatty acid anions. The deacylation of 9.2×10^{-5} M ^{14}C -acetyl-HSA at pH 7.5 in triethanolamine buffer, ionic strength 0.02 M and $30.5 \pm 0.3^\circ$ in the absence of added nucleophiles (O), with 8.04×10^{-4} M p-chlorophenol, $\square$; and with the same concentrations of p-chlorophenol and 9.2×10^{-5} M sodium decanoate, Δ ; 1.8×10^{-4} M sodium decanoate, $\bullet$; 4.6×10^{-4} M sodium decanoate, $\circ$; as determined by the dialysis technique.

Figure 21

Figure 22. The effect of urea on the deacylation of acetyl-HSA by p-methoxyphenol. Deacylation of 2.1×10^{-5} M ^{14}C -acetyl-HSA by 0.05 M p-methoxyphenol, ■; p-methoxyphenol plus 6.46 M urea, ▲; and by H_2O , ●. At pH 7.8 in 0.02 M triethanolamine/HCl, total ionic strength 0.07 M adjusted with KCl at $30.5 \pm 0.3^\circ$ determined by the dialysis techniques.

Figure 22

Figure 23. The effect of urea on the deacylation of acetyl-HSA by NH_2OH . At pH 7.8 deacylation of ^{14}C -acetyl-HSA by H_2O (●), 0.05 M NH_2OH (▲), and hydroxylamine plus 6.46 M urea, (■). All in 0.02 M triethanolamine/ buffer, total ionic strength 0.07 M adjusted with KCl at $30.5 \pm 0.3^\circ$ determined by the dialysis technique.

Figure 23

with ^{14}C -acetyl-HSA at pH 7.5 (see Fig. 14) despite its lower pK and similar nucleophilicity as compared to the phenols. Deacylation proceeds in direct proportion to concentrations of hydroxylamine up to 50 mM consistent with a second order rate constant of $0.0224 \text{ min.}^{-1} \text{ M}^{-1}$ (Fig. 24), or about 30 times slower than the hydroxylaminolysis of phenylacetate under similar conditions (Jencks and Carriuolo, 1960).

In the presence of 6.46 M urea, at pH 7.8, the rate of deacylation by 50 mM hydroxylamine is increased by four-fold as compared to that in its absence (see Fig. 23). This increase in rate in the presence of urea implies the existence of a specific conformation in the protein that retards hydroxylaminolysis.

3. Deacylation by hydroxide ion.

As shown in Fig. 14 and Fig. 21 through 23 even in the absence of added nucleophiles, acetyl-HSA undergoes slow deacylation. Apparently this reflects the hydroxide ion, water and buffer promoted reactions. Buffer promoted deacylation is negligible, however, as at pH 7.5 and 0.02 M ionic strength increasing the concentration of triethanolamine buffer from 0.002 M to 0.02 M has no effect. Using phosphate buffer instead of triethanolamine also does not affect the observed reaction rate.

At constant ionic strength and with no added nucleophiles, the rate of deacylation increases rapidly with increasing hydroxide ion concentrations. As shown in Fig. 25, first order rate constants obtained from pH 6.2 to 9.3 can be described by the equation,

$$\log (k_2 \times 10^5) \text{ min}^{-1} = 0.62 \text{ pH} - 3.01.$$

Figure 24. Deacylation of acetyl-HSA by NH_2OH . With NH_2OH in excess, pseudo-first order rate constants determined by the dialysis technique is a linear function, with a slope of $2.25 \times 10^{-5} \text{ min}^{-1} \text{ mM}^{-1}$, of NH_2OH concentration. All in 0.02 M triethanolamine/HCl buffer, total ionic strength 0.07 M adjusted with KCl at $30.5 \pm 0.3^\circ$.

Figure 24

Figure 25. The deacylation of acetyl-HSA at different pH values in triethanolamine/HCl buffer ionic strength 0.02 M and $30.5 \pm 0.3^\circ$. Each data point corresponds to a single determination obtained using either the dialysis technique ($\bullet$), or spectrophotometrically (lower line, slope = 0.62). Upper line represents data obtained in the presence of 5.36×10^{-5} M p-methoxyphenol monitored spectrophotometrically ($\blacktriangle$).

Figure 25

4. Spectrophotometric measurement of deacylation of acetyl-HSA by p-methoxyphenol.

The slow process of deacylation of acetyl-HSA can be followed by measuring the absorbance increase at 278 nm. The process follows pseudo-first-order kinetics with or without the presence of a large excess of phenols. The logarithm of the pseudo-first-order rate constants of the reaction at different pH values, are shown in Figure 25. Pseudo-first-order rate constants for hydrolysis of acetyl-HSA at pH 8.27 and 9.4 determined spectrophotometrically are in agreement with that by equilibrium dialysis (Fig. 25).

5. Identification of the reaction product by toluene extraction.

Radioactivity obtained by toluene extraction of a reaction mixture containing ^{14}C -acetyl-HSA and excess p-methoxyphenol increases as the incubation time is increased. Kinetic study of the reaction, presumably the deacylation of ^{14}C -acetyl-HSA by p-methoxyphenol at pH 7.5, gives a K_M of $4.63 \times 10^{-4} \text{ M}$, and k of $7.8 \times 10^{-2} \text{ hr}^{-1}$ (see Fig. 26 and Table 3) both compatible with data obtained by the dialysis method. Furthermore, the toluene extract of the reaction mixture when applied to a thin layer chromatograph plate indicates the radioactive species is ^{14}C -p-Methoxyphenyl acetate (Mph $[\text{}^{14}\text{C}]$ OAc). The radioactivity migrated with cold MphOAc on the plate under two developing systems (Hexane: Ethyl-acetate = 85:15, $R_f = 0.67$; Benzene : Hexane = 1:4 $R_f = 0.72$) on silica gel G coated plastic sheets (Eastman Kodak Product No. 13179).

To verify that the radioactivity found in the toluene extract is indeed the product of the reaction of p-methoxyphenol and ^{14}C -acetyl-HSA, which in turn is formed by reacting HSA with Mph $[\text{}^{14}\text{C}]$ OAc, the following experiments were conducted. As shown in Table 4, the toluene extractable

TABLE 4

FRACTIONS OF THE RADIOACTIVITY
EXTRACTED BY TOLUENE FROM THE
REACTION MIXTURES^a

Sample ^b	cpm		
	1 hr	2 hr	3 hr
1. Nph ¹⁴ C OAc + pCH ₃ OPhOH	0.92	0.88 (0.84) ^c	0.42
2. HSA + Nph[¹⁴ C]OAc	0.02	0.03 (0.01) ^c	0.01
3. HSA + Nph[¹⁴ C]OAc + pCH ₃ OPhOH	0.21	0.26 (0.37) ^c	0.11

a. In triethanolamine buffer pH 7.5, 0.02 M ionic strength and 25°. For detailed experimental procedures, see "Methods".

b. Constituents added according to the order indicated in the Table; while [HSA] = 1.02×10^{-4} M, Nph[¹⁴C]OAc = 6.12×10^{-5} M and [pCH₃OPhOH (p-methoxyphenol)] = 1.26×10^{-3} M.

c. Duplicates of samples 1, 2 and 3.

Figure 26. Double-reciprocal plot for the deacylation of acetyl-HSA by p-methoxyphenol (ArOH). Each data point is determined by the toluene extraction technique. With ArOH in excess at pH 7.5, triethanolamine/HCl ionic strength 0.02 M and 29.8°. The kinetic parameters thus obtained are shown in Table 3.

Figure 26

radioactivity, which comes from phenylacetates, is indicative of the²⁹ formation of Mph[^{14}C]OAc during the course of reactions. The reaction mixture which does not contain HSA to be acetylated by Nph[^{14}C]OAc has the highest toluene extractable radioactivity at all times. Its toluene extractable radioactivity decreases with time, a result of OH^- hydrolysis of Nph[^{14}C]OAc, and formation of p-nitrophenol, non-extractable ^{14}C -acetate ion. The lowest radioactivity was found in the reaction mixture containing HSA and Nph[^{14}C]OAc, in which the ^{14}C -acetyl group is almost totally non-extractable as the result of formation of ^{14}C -acetyl-HSA. Addition of pCH₃OPhOH (p-methoxyphenol) to the mixture of Nph[^{14}C]OAc and HSA, increases the toluene extractable radioactivity indicating the formation of Mph[^{14}C]OAc. Similar to that of Nph[^{14}C]OAc, Mph[^{14}C]OAc also is subjected to OH^- hydrolysis and results in some loss of toluene extractable radioactivity after incubation for three hours. A reaction mixture of $2.61 \times 10^{-4}\text{M}$ HSA, $1.56 \times 10^{-4}\text{M}$ Nph[^{14}C]OAc and $3.09 \times 10^{-3}\text{M}$ p-methoxyphenol, or about three times as concentrated as Sample 3 in Table 4, was incubated for one hour followed by extraction with toluene, and after evaporation, was applied to a silica gel G coated plastic sheets (Eastman Kodak product 13179) with a small amount of unlabeled MphOAc. As illustrated in Fig 27, the sample and the unlabeled MphOAc migrate as one spot (in Hexane: Benzene = 4:1) with the same R_f (0.72) value as the unlabeled Mph OAc ($R_f=0.73$) and contains 61% of the total added radioactivity, while 4% of the radioactivity stays at the origin giving a 65% recovery of radioactivity from the plate. This discrepancy may possibly reflect the quenching effect of the silica gel.

Figure 27. Identification of p-methoxyphenyl acetate as the product of p-methoxyphenol reaction with acetyl-HSA by thin layer chromatography. Radioactivity in the toluene extract of the reaction mixture and unlabeled p-methoxyphenyl acetate migrate as one spot with an Rf value of 0.72 (upper left) close to a value of 0.73 for the authentic p-methoxyphenyl acetate. A 64% recovery of radioactivity is obtained from the material scraped from the indicated region of the plate. Conditions are given in the Method.

Figure 27

Figure 28. Inhibition of the reaction between NphOAc and HSA by Nonanoate. The reactivity of 6.61×10^{-5} M HSA with NphOAc decreases linearly in the presence of increasing amount of nonanoate at pH 7.5 in triethanolamine/HCl 0.020 M ionic strength and 25° [$k_0 = (3.34 \pm 0.04) \times 10^{-2} \text{ sec}^{-1}$].

The rapid reaction of HSA with one equivalent of p-nitrophenyl acetate has been shown to be inhibited by small fatty acid anions (Means and Bender, 1975). Competitive binding of these anions to the binding site of p-nitrophenyl acetate appears to account for the inhibition. In the present study, the binding site concentration is thus determined by titrating this site with strong inhibitors, fatty acid anions. Furthermore, a homologous series of fatty acid anions are employed to study the contributions of hydrophobic and electrostatic interactions to the binding of small fatty acid anions to the p-nitrophenyl esters reactive site.

1. Titration of HSA with small fatty acid anions.

The reactivity of HSA with p-nitrophenyl acetate is strongly inhibited by small fatty acid anions. As shown in Figure 28, observed first-order rate constants decrease in direct proportion to added nonanoate. Under the conditions used (i.e. HSA in at least eleven-fold excess over p-nitrophenyl acetate) the reactions remain pseudo-first order even in the presence of excess nonanoate although reactivity decreased by about 95%. The 5% reactivity remaining in the presence of as large excess of nonanoate appears to reflect reactions of p-nitrophenyl acetate with solvent and other less reactive groups of HSA (Means and Bender, 1975). The minimum amount of nonanoate, or other strong inhibitor required, as determined by extrapolation of the initial k/k_0 to reach the limiting value (see Figs. 28, 29), is highly reproducible, and appears to reflect the concentration of binding sites.

Binding site concentrations determined by this method are consistently lower than the HSA concentration determined spectrophotometrically.

Figure 29. Titration of the NphOAc reactive site on HSA. Relative reactivity, k/k_0 in the presence of octanoate ($\blacktriangle$), nonanoate ($\blacksquare$), and decanoate ($\bullet$) at concentrations much higher than the K_d 's is a linear function of the fatty acid anion concentration. Extrapolation of the initial decrease in reaction to 5% reactivity indicates the binding site concentration of 4.5×10^{-5} M. Conditions are as given in Figure 28.

Figure 29

Thus a binding site concentration of 4.5×10^{-5} M is obtained for a ⁸²HSA solution with spectrophotometrically determined concentration of 6.61×10^{-5} M (Figs. 28, 29). As shown in Table 5, difference of 25 to 3% have been observed for other samples of HSA as determined under varied conditions, all with its lipids removed (Chen, 1967). As plotted according to equation 6 (see "Methods") inhibition of the reaction with 1.74×10^{-5} M and 6.61×10^{-5} M HSA by heptanoate indicates binding site concentrations of 1.2×10^{-5} M and 4.5×10^{-5} M and calculated slopes of 4.8×10^{-5} and 5.1×10^{-5} M, respectively corresponding to the dissociation constants (Fig. 30). The same data are shown in Figure 31 in the form of simple binding isotherms assuming stoichiometry of 0.68 binding site per HSA and dissociation constants of 5.1×10^{-5} M. For an individual HSA sample, even as determined under different conditions, i.e. different HSA concentrations, reactive site titers varied by no more than 3% (i.e. see Table 5).

2. Dissociation constants.

Inhibition of the reaction between p-nitrophenyl acetate and HSA by fatty acid anions appears to reflect their competitive interactions in the reaction site. Thus at HSA concentrations comparable to their respective dissociation constants, inhibition by fatty acid anions appear to describe simple binding isotherms consistent with the formation of unreactive 1 : 1 ligand : albumin complexes. As shown in Figure 32, the inhibition of 6.61×10^{-5} M HSA by the homologous fatty acid anions, butyrate through decanoate, follows a series of smooth curves consistent with dissociation constants from 3.2×10^{-4} M to 1.0×10^{-7} M and a binding site concentration of approximately 4.5×10^{-5} M. The same data for valerate, hexanoate, and heptanoate replotted according to

Figure 30. Inhibition of the reaction between NphOAc and HSA by Heptanoate. Data obtained at 1.74×10^{-5} M (lower line) and 6.61×10^{-5} M HSA (upper line) plotted according to equation 6 see "Methods"; Dissociation constants corresponding to the slopes of the two lines are 4.8×10^{-5} M and 5.1×10^{-5} M, respectively. Binding site concentrations determined from the intercepts are 1.2×10^{-5} M and 4.5×10^{-5} M. Conditions are as given in Figure 28.

Figure 31. Inhibition of the reaction between NphOAc and HSA by Heptanoate. The data given in Figure 30 is plotted and the theoretical binding isotherms is calculated using K_d of 5.1×10^{-5} M and binding site concentrations ($[HSA']$) of 1.2×10^{-5} M and 4.5×10^{-5} M for the upper and lower lines, respectively, and a 5% residual reactivity.

Figure 31

Table 5

Titration of HSA with Decanoate^a

<u>Source</u>	<u>Concentration x 10⁵ (Molar)</u>		<u>Titer (%)^d</u>
	<u>Spectrophotometric^b</u>	<u>Titration^c</u>	
Miles, human mercaptalbumin lot 7	6.90	4.35	63.0
Miles, human serum albumin lot 32	2.57	1.95	75.9
	5.52	4.09	74.1
	11.1	8.41	75.8
	13.4	9.82	73.5
Sigma, Fraction V lot 24C-1632-8	2.98	2.02	67.8
	5.22	3.51	67.2
	7.45	4.97	66.7
	14.9	10.3	69.1
	3.37	2.35	69.7
	6.61	4.53	68.5
Sigma, Fraction V (essentially fatty acid free) lot 16C7280 ^e	3.74	2.72	72.7
	5.81	4.36	75.0
	8.05	6.09	75.7

- a. In triethanolamine/HCl pH 7.5, 0.02 M ionic strength and 25°, after treatment to remove bound lipids (Chen, 1967) except as noted.
- b. Using $\epsilon_{278}^{1\%} = 5.31$ (Kirschenbaum, 1968, Cohn *et al.* 1947), and molecular weight of 66,400.
- c. By inhibition of the reaction between HSA and NphOAc as determined in Figures 28, 29.
- d. Concentration determined by titration as a percentage of that determined spectrophotometrically.
- e. As obtained from Sigma; not treated further to remove lipids.

Figure 32. Inhibition of the reaction between NphOAc and HSA by Fatty Acid Anions. Relative reactivity, k/k_0 , in the presence of various fatty acid anions versus the mole ratio of fatty acid anion to binding site; butyrate ($\blacksquare$); valerate ($\blacktriangledown$); hexanoate, ($\bullet$); heptanoate (Δ); octanoate ($\blacktriangle$); nonanoate, ($\square$); and decanoate, (O). Conditions as described in Figure 28. Isotherms calculated according to equations 1 through 5 for dissociation constants as given in Table 6, and 5% residual reactivity.

Figure 32

equation 6 (see "Methods") give three lines of different slope converg⁹⁵ing at the ordinate and consistent with dissociation constants of 1.0×10^{-4} M, 2.6×10^{-5} M, and 5.1×10^{-5} M, respectively and a binding site concentration of 4.53×10^{-5} M (Fig. 33).

Dissociation constants and free energies of association for the anions butyrate through decanoate are given in Table 6. Affinities increase in nearly direct proportion to the number of methylene groups. As shown in Figure 34, the regular increase in affinity of approximately 0.8 kcal per methylene group and an extrapolated free energy presumably reflecting largely ionic interactions of the carboxylate group with the protein of 2.3 kcal at zero methylene groups is obtained according to the following relationship:

$$-\Delta G^{\circ} \text{ (kcal)} = 0.804 (n) + 2.3$$

where n is the number of constituent methylene groups. Larger fatty acid anions like undecanoate and dodecanoate, although both fairly strong inhibitors of the reaction with p-nitrophenyl acetate, are much less potent than decanoate and do not conform to the same relationship (see Table 6).

3. The effect of ionic strength.

The discrepancy between HSA concentration determined spectrophotometrically and the binding site concentration obtained from titration by strong inhibitors of the reaction of p-nitrophenyl acetate and HSA does not appear to be affected by changing the ionic strength. As shown in Table 7, with 4.86×10^{-5} M HSA, the reactivities remaining, after correction for the respective background reactivities, with same amount of decanoate added do not vary significantly with the ionic strength varied between 0.011 M and 0.092 M, all suggesting less than one binding

Table 6

Dissociation Constants and Free Energy of Association for the
Binding of Ligands to Human Serum Albumin.^a

	K_d	(n)	$-\Delta G^0(\text{kcal})$	$\Delta\Delta G^0(\text{kcal})$
Butyrate	3.2×10^{-4}	3	4.77	----
Valerate	1.0×10^{-4}	4	5.45	0.68
Hexanoate	2.6×10^{-5}	5	6.25	0.80
Heptanoate	5.1×10^{-6}	6	7.21	0.96
Octanoate	1.6×10^{-6}	7	7.90	0.69
Nonanoate	4.0×10^{-7}	8	8.72	0.82
Decanoate	1.0×10^{-7}	9	9.54	0.82
Undecanoate ^b	$(\sim 5.8 \times 10^{-6})$	10	----	----
Dodecanoate ^b	$(\sim 1.0 \times 10^{-5})$	11	----	----

a. Conditions used are the same as in Table 5.

b. Apparent values; appear to reflect the influence of interactions at other sites.

TABLE 7
EFFECT OF IONIC STRENGTH ON THE DECANOATE
INHIBITION OF THE REACTION OF
p-NITROPHENYL ACETATE WITH HSA.^a

$[\text{Dec}]_0/[\text{HSA}]_0$	$((k - k_b)/(k_0 - k_b))^b$		
	$\mu = 0.011 \text{ M}$	$\mu = 0.047 \text{ M}$	$\mu = 0.09 \text{ M}$
0.12	0.71	0.83	0.78
0.18	0.63	0.79	0.72
0.30	0.58	0.65	0.61
0.48	0.33	0.39	0.42
0.72	0.13	0.18	0.20

- a. $[\text{HSA}]_0 = 4.86 \times 10^{-5} \text{ M}$; buffers maintained at constant ratio of $[\text{triethanolamine} \cdot \text{HCl}]/[\text{triethanolamine}] = 2$ and pH 7.5; other conditions same as in Figure 28.
- b. Background activities (k_b) are observed as 3.3%, 7.8% and 15.3% of that in the absence of decanoate at ionic strengths of 0.011 M, 0.047 M, and 0.09 M.

Figure 33. Inhibition of the reaction between NphOAc and HSA by Valerate, Hexanoate, and Heptanoate Ions. The data given in Figure 32 for these anions is plotted according to equation 6. Dissociation constants as determined from the slopes are 9.97×10^{-5} M, 2.6×10^{-5} M and 5.1×10^{-6} M respectively. The binding site concentration determined from the intercept is 4.5×10^{-5} M.

Figure 33

Figure 34. Variation in Free Energy of Association for Homologous Fatty Acid Anions as Inhibitors of the Reaction between NphOAc and HSA. Data from Table 6 for the anions butyrate through decanoate are plotted versus their number of methylene groups.

site per HSA molecule. On the other hand, the dissociation constants of fatty acid anions are sensitive to the ionic strength. For example, the dissociation constant of caproate increases 3 folds as the ionic strength is increased from 0.012 M to 0.082 M.

E. Binding of Nitrophenol

p-Nitrophenol, one of the products of the reaction of p-nitrophenyl acetate with HSA, is an inhibitor of the reaction. Assuming the inhibition results from the reversible binding of p-nitrophenolate ion at the reactive site, the dissociation constant of 8.2×10^{-6} M is calculated from the binding isotherms (see "Methods") consistent with values obtained by equilibrium dialysis; e.g. K_d of 6×10^{-6} M for HSA at 23° pH 8.3 triethanolamine/HCl and ionic strength of 0.02 M (Means unpublished) and 5.12×10^{-5} M and 1.69×10^{-5} M at pH 7.6 and 8.2 for nondefatted BSA at 0.22M ionic strength (Teresi and Luck, 1948).

Free p-nitrophenolate ion absorbs maximally at 400 nm with a molar extinction coefficient of $18,000 \text{ M}^{-1} \text{ cm}^{-1}$. Upon adding incremental amounts of HSA, the spectrum is gradually shifted toward shorter wavelength with a concomitant decrease in the 400 nm absorbance, until a spectrum with a maximum absorbance at 377 nm is obtained ($\epsilon = 14,000 \text{ M}^{-1} \text{ cm}^{-1}$, Fig. 35). An isosbestic point at 380 nm ($\epsilon = 13,800 \text{ M}^{-1} \text{ cm}^{-1}$) indicates the existence of two species of p-nitrophenolate ion, namely the free and serum albumin bound forms. Thus as shown in Figure 36, the fraction of bound p-nitrophenolate reflected by the fractional spectral change, is a function of serum albumin concentrations, from this and applying the equations 3,4,5, (see "Methods"), a dissociation constant of 1×10^{-5} M is calculated. Decanoate binds at the reactive site and strongly inhibits the reaction of NphOAc with HSA (Means and Bender, 1975 see also section D) and also inhibits the reverse

Figure 35. Displacement of the p-nitrophenolate ion spectra by HSA at pH 8.2 and 25°. p-Nitrophenol, 2.00×10^{-5} M in triethanolamine HCl, ionic strength 0.02 M, pH 8.2 was titrated with increments of HSA solution up to the ratios[HSA]/[p-nitrophenol], of (1) 0, (2) 0.42, (3) 0.63, (4) 0.84, (5) 1.05, (6) 1.26, (7) 1.47, (8) 1.68, (9) 1.89.

Absorbance

reaction of p-nitrophenol with acetyl-HSA (see section C on "Deacylation of Acetyl-HSA"). In the presence of 1.00×10^{-5} M decanoate and 2.00×10^{-5} M p-nitrophenol at pH 8.2, the serum albumin induced blue shift of the p-nitrophenolate spectrum is very much retarded. Thus as shown in Figure 36, significant spectral change is observed only when the concentration of HSA added exceeds that of the decanoate present. However, the same isosbestic point and end point spectrum are obtained.

The competitive nature of the binding of p-nitrophenolate, decanoate and another inhibitor of the reaction, L-tryptophan at the p-nitrophenyl acetate binding site of HSA is also indicated by the capability of decanoate and tryptophan to displace the HSA bound p-nitrophenolate ion from its binding site. As shown in Figure 37, the spectrum of serum albumin bound p-nitrophenolate ion is red shifted by tryptophan. Notice an isosbestic point (385 nm and $\epsilon = 13,830 \text{ M}^{-1} \text{ cm}^{-1}$), different from that for p-nitrophenol and HSA bound p-nitrophenol is observed, and even in the presence of a large excess of tryptophan over that of p-nitrophenol the spectrum is still distinguishable from that of the free p-nitrophenol. The fraction of spectral change induced by tryptophan, an index of bound p-nitrophenol being displaced, is a function of L-tryptophan added (Fig. 38). In comparison, decanoate which has a higher affinity to the p-nitrophenyl acetate reactive site than does L-tryptophan by 2-orders of magnitude (see sections D and F for binding of decanoate and L-tryptophan, respectively) induces the same characteristic spectral changes as does L-tryptophan, namely the same isosbestic point and the same end point spectrum.

Figure 36. The fraction of bound p-nitrophenol as a function of HSA concentration in the absence (●) and presence (▲) of decanoate at pH 8.2, 25°. The data of the decanoate free (●) experiments are obtained from Figure 35 and using the fractional change in absorption at 410 nm as an index of the fraction of p-nitrophenol bound. The end point absorbance, 24% of that of free p-nitrophenol is determined experimentally in the presence of, relative to p-nitrophenol, 10 equivalents of HSA. The conditions for experiments in the presence of decanoate are the same as in its absence, except 1.0×10^{-5} M of decanoate is added.

Figure 36

Figure 37. Displacement of HSA bound p-nitrophenol spectra by L-tryptophan. p-Nitrophenol of 5.02×10^{-6} M in triethanolamine/HCl buffer, ionic strength 0.02 M pH 8.45 (dotted spectrum) was added 5.93×10^{-5} M HSA (0), followed by addition of increments of tryptophan to reach the ratio [tryptophan]/[HSA] of (1) 0.27 (2) 0.53 (3) 0.80 (4) 1.01 (5) 1.28 (6) 1.54 (7) 2.07 (8) 2.61.

Figure 37

Figure 38. The fraction of p-nitrophenol displaced by L-tryptophan (●) and decanoate (■) as a function of the concentration of these ligands. The data for the tryptophan displacement experiments are obtained from Figure 4. Conditions for the decanoate displacing experiments are the same as that for tryptophan (Fig. 37) except the following: [p-nitrophenol] total = 5.06×10^{-6} M, [HSA] total = 5.79×10^{-5} M and pH 8.2.

Figure 38

1. Dissociation constant of L-tryptophan.

L-tryptophan is the only common amino acid bound by HSA to a significant extent (Peters, 1975). Equilibrium dialysis has demonstrated that binding occurs primarily at one site with an intrinsic binding constant of $1.3 \times 10^5 \text{ M}^{-1}$ in 0.1 M NaCl and pH 9.2 (McMenamy and Oncley, 1958). In the present study, tryptophan binding is studied by using the reaction of p-nitrophenyl esters with HSA as a probe and thus only the interaction of tryptophan with the p-nitrophenyl ester reactive site is under investigation. Figure 39 shows the reactivity of HSA with p-nitrophenyl acetate at pH 7.5 is strongly inhibited by L-tryptophan. At HSA concentration of $1.32 \times 10^{-5} \text{ M}$, a binding isotherm best described by a dissociation constant of $1.6 \times 10^{-5} \text{ M}$ and a background activity, of 7% of that in the absence of added L-tryptophan indicates that the inhibition results from the formation of an unreactive 1 : 1 HSA : tryptophan complex. Treating the same data according to the equation described by Easson and Stedman (1936), Webb (1963), and Beith (1974) (see equation 6 in "Method"), gives $1.32 \times 10^{-4} \text{ M}$ as intercept at the Y axis corresponding to the binding site concentration, and a slope of $1.65 \times 10^{-5} \text{ M}$ as the dissociation constant. At three different HSA concentrations, $1.12 \times 10^{-4} \text{ M}$, $1.83 \times 10^{-4} \text{ M}$ and $4.27 \times 10^{-4} \text{ M}$, the L-tryptophan dissociation constants of $1.77 \times 10^{-5} \text{ M}$, $2.06 \times 10^{-5} \text{ M}$, and $2.10 \times 10^{-5} \text{ M}$ are calculated respectively from the equation 6 (Fig. 40). At pH 7.78 and an HSA concentration of $1.27 \times 10^{-5} \text{ M}$ a dissociation constant of $9.5 \times 10^{-6} \text{ M}$ is calculated using the same equation, i.e. equation 6 in "Methods", (Fig. 41), whereas using the method of Dixon (1953) as shown in Figure 42, gives a value of $9 \times 10^{-6} \text{ M}$ under the same

Figure 39. Inhibition of the reaction between NphOAc and HSA by L-tryptophan. The reactivity of 1.32×10^{-4} M HSA with NphOAc decreases in the presence of increasing amount of tryptophan at pH 7.5 in triethanolamine/HCl, 0.020 M ionic strength and 25°. ($k_o = (4.76 \pm 0.12) \times 10^{-2} \text{ sec}^{-1}$, $n=3$). Solid line is calculated according to K_d of 1.65×10^{-5} M, HSA concentration ($[HSA]_o$) of 1.32×10^{-4} M and 7% residual activity. Inset: Data treated according to equation 6 (see "Methods"); A dissociation constant corresponding to the slope of the line is 1.65×10^{-5} M. The binding site concentration $[HSA]_o$ determined from the intercept is 1.32×10^{-4} M.

Figure 40. Inhibition of the reaction between NphOAc and HSA by L-tryptophan. Data obtained at HSA concentrations ($[HSA]_0$) of 4.27×10^{-4} M (upper line), 1.83×10^{-4} M (middle line) and 1.12×10^{-4} M (lower line) are plotted according to equation 6 (see "Methods") ($k_0 = (8.19 \pm 0.07) \times 10^{-2} \text{ sec}^{-1}$, $n=3$, (upper line); $(5.85 \pm 0.01) \times 10^{-2} \text{ sec}^{-1}$, $n=2$, (middle line); $(4.50 \pm 0.17) \times 10^{-2} \text{ sec}^{-1}$, $n=2$, (lower line)). Dissociation constants corresponding to the slopes are 2.10×10^{-5} M, 2.06×10^{-5} M, and 1.77×10^{-5} M respectively. Binding site concentrations determined from the intercepts are the same as the HSA concentrations. Conditions are as given in Figure 39.

Figure 40

Figure 41. Inhibition of the reaction between NphOAc and HSA by L-tryptophan. The reactivity of 1.27×10^{-5} M HSA with NphOAc decreases in the presence of increasing amount of tryptophan at pH 7.78 in triethanolamine/HCl, 0.020 M ionic strength and 25° . ($k_o = (2.25 \pm 0.13) \times 10^{-2}$ sec $^{-1}$, $n=2$). Solid line is calculated according to K_d of 9.4×10^{-6} M, HSA concentration ($[HSA]_o$) of 1.32×10^{-4} M and 5% residual activity. Inset: Data treated according to equation 6 (see "Methods"); Dissociation constants corresponding to the slope of the line and the binding site concentration ($[HSA]_o$) determined from the intercept are the same as that obtained from the binding isotherm.

Figure 41

Figure 42. Dixon plots with data in Figure 41 plus that obtained with HSA concentration of 9.83×10^{-6} M, ($k_o = (9.55 \pm 0.21) \times 10^{-3} \text{ sec}^{-1}$, $n=2$). Dissociation constant of L-tryptophan thus obtained is 9.0×10^{-6} M. Conditions are as given in Figure 41.

conditions. At pH 7.15 and a HSA concentration of 1.22×10^{-3} M a dissociation constant of 5×10^{-5} M is calculated from either the isotherms or the equation 6 (Fig. 43).

The homologous p-nitrophenyl esters, p-nitrophenyl acetate through p-nitrophenyl caproate, react rapidly with HSA via a mechanism involving prior rapid reversible binding to the reactive site (see section A on "Acylation of HSA by Homologous Fatty Acid p-Nitrophenyl Esters"). Similar inhibition by L-tryptophan in each case shows all of these reactions occur at the same site. Thus as shown in Figures 44 and 45, inhibition of the reactions of p-nitrophenyl propionate and p-nitrophenyl caproate by L-tryptophan give virtually identical values for the inhibition constant, K_i of 1.76×10^{-5} M and 1.89×10^{-5} M as determined with a Lineweaver-Burk type plot. The competitive nature of L-tryptophan binding with that of p-nitrophenyl propionate and p-nitrophenyl caproate for the same site is apparent.

2. The pH effect.

L-tryptophan binding increases with increasing pH from 7.15 to 7.78. Dissociation constants calculated from the binding isotherms and according to the equation 6 are 5.0×10^{-5} M and 9.46×10^{-6} M respectively, wherein residual activities of 5 % and 9% were observed (Figs 41 and 43). In the plot of K_d vs pH (Fig.46) a slope of approximately unity indicates the ionization of a single group is affecting the binding of L-tryptophan.

3. The effect of ionic strength.

A strong dependence of the ionic strength is observed in the interactions of various ligands (e.g. small fatty acid anions) with the site under study. The affinity of this site decreases with increasing ionic

Figure 43. Inhibition of the reaction between NphOAc and HSA by L-tryptophan. The reactivity of 1.22×10^{-3} M HSA decreases in the presence of increasing amount of tryptophan at pH 7.15 in triethanolamine/HCl, 0.020 M ionic strength at 25°. ($k_o = (5.77 \pm 0.06) \times 10^{-2} \text{ sec}^{-1}$, $n=3$). Solid line is calculated according to K_d of 5.0×10^{-5} M, HSA concentration ($[HSA]_o$) of 1.22×10^{-3} M and 9% residual activity. Inset: Data treated according to equation 6 (see "Methods"); Dissociation constant corresponding to the slope of the line is 5.4×10^{-5} M. Binding site concentration $[HSA]_o$ determined from the intercept is 1.22×10^{-3} M.

Figure 43

Figure 44. Double reciprocal plots for the reaction of p-nitrophenyl propionate with HSA in the presence and absence of L-tryptophan. At pH 7.5 in triethanolamine/HCl, 0.020 M ionic strength and 25°. The L-tryptophan concentrations are 3.46×10^{-4} M (upper), 7.31×10^{-5} M (middle) and 0 M (lower). The corresponding dissociation constants calculated for L-tryptophan are 1.74×10^{-5} M (upper) and 1.76×10^{-5} M (middle), while the dissociation constant for p-nitrophenyl propionate is 5.83×10^{-5} M (lower).

Figure 45. Double reciprocal plots for the reaction of p-nitrophenyl caproate with HSA in the presence and absence of L-tryptophan. At pH 7.5 in triethanolamine/HCl, 0.020 M ionic strength and 25°. The L-tryptophan concentration is 6.27×10^{-5} M (upper) and 0 (lower). The corresponding dissociation constants calculated for L-tryptophan and p-nitrophenyl caproate are 1.89×10^{-5} M and 8.76×10^{-6} M respectively.

Figure 46. Dissociation constants of L-tryptophan with HSA as a function of pH. In triethanolamine/HCl buffer, 0.020 M ionic strength and 25°. Data points are obtained from the results in Figures 39 through 45 at the indicated pH values.

Figure 46

strength, as would be expected for a process with a strong electrostatic involvement (see Discussion). Figure 47 shows a series of binding isotherms with the ionic strength varied from 0.012 M to 0.082 M. The dissociation constants increase as the ionic strength increases and as shown in Figure 48, a slope of 5.63 in the Debye-Huckel plot indicates the involvement of charges of high density.

4. Binding of L-tryptophan analogs.

Two L-tryptophan analogs, both anions were studied for their inhibitory effect on the reaction of p-nitrophenyl acetate with HSA. Figure 49 shows N-acetyl-L-tryptophan inhibits the reaction, the dissociation constant calculated from the binding isotherms and the equation 6 (see "Methods") is 1.6×10^{-5} M. In the case of indolepropionic acid, the loss in reactivity exceeds the equivalents of the inhibitor added to the HSA and is difficult to rationalize (Table 8). The same apparent phenomenon is also observed in fatty acid inhibition of the reaction between HSA and p-nitrophenyl acetate and can not be explained in simple terms.

5. Additive effects of L-tryptophan and decanoate.

Decanoate and L-tryptophan, both inhibitors of the reaction of p-nitrophenyl esters with HSA, are additive in their inhibitory effects. In the presence of 0.49 equivalents of decanoate per HSA, an HSA solution of 2.13×10^{-4} M shows only 43% of its reactivity with p-nitrophenyl acetate, and addition of L-tryptophan causes further inhibition of the reaction in a manner such that there is no breaking point observed in a plot of reactivity vs the ratio of inhibitor to HSA (Fig. 50). Under this condition, a strong inhibitor as decanoate ($K_d = 1 \times 10^{-7}$ M) is bound stoichiometrically to HSA. This result is consistent

Figure 47. Effect of ionic strength on L-tryptophan inhibition of the reaction between NphOAc and HSA. At pH 7.5, HSA concentration of 1.46×10^{-4} M in triethanolamine/HCl buffer and ionic strength of 0.082 M ($\blacktriangle$), 0.043 M ($\bullet$), and 0.012 M ($\blacksquare$), and k_o 's of $(1.90 \pm 0.03) \times 10^{-2} \text{ sec}^{-1}$ (n=2), $(2.95 \pm 0.01) \times 10^{-2} \text{ sec}^{-1}$ (n=2), and $(7.71 \pm 0.03) \times 10^{-2} \text{ sec}^{-1}$ (n=2), respectively. Solid lines are calculated according to the HSA concentration of 1.46×10^{-4} M, K_d 's of 6×10^{-5} M (upper) 13.2×10^{-5} M (middle) and 1.1×10^{-5} M (lower) and the respective residual activity of 16%, 13%, and 6% of that in the absence of L-tryptophan.

Figure 47

Figure 48. Effect of ionic strength on the dissociation constants of L-tryptophan. Data are obtained from Figures 39, 40, 41, 43, 44, 45, and 47 for the corresponding ionic strengths. Conditions are as described.

Figure 48

TABLE 8
EFFECT OF INDOLEPROPIONIC ACID ON THE REACTION
OF HSA WITH p-NITROPHENYL ACETATE. ^a

[IA]/[HSA]	k/k ₀
	(k ₀ = 6.28 ± 0.09 × 10 ⁻² (sec ⁻¹)(n=4))
0.18	0.76
0.27	0.69
0.31	0.62
0.45	0.47
0.58	0.32

- a. [HSA]₀ = 2.00 × 10⁻⁴ M; the observed first order rate constants, k's are the average of two measurements; conditions are same as in Figure 4B.

Figure 49. Inhibition of the reaction between NphOAc and HSA by N-acetyl-L-tryptophan (NA-Trp). The reactivity of 5.03×10^{-5} M HSA with NphOAc decreases in the presence of increasing amount of N-acetyl-L-tryptophan at pH 7.5 in triethanolamine/HCl, 0.020 M ionic strength and 25° , ($k_0 = 2.66 \times 10^{-2} \text{ sec}^{-1}$). Solid line is calculated according to K_d of 1.6×10^{-5} M, HSA concentration $[\text{HSA}]_0$ of 5.03×10^{-5} M and 7% residual activity. Inset: Data treated according to equation 6 (see "Methods"); Dissociation constant corresponding to the slope of the line is 1.60×10^{-5} M, binding site concentration $[\text{HSA}]_0$ determined from the intercept is 5.03×10^{-5} M.

Figure 647

Figure 50. Inhibition of the reaction between NphOAc and HSA by decanoate and L-tryptophan. The reactivity of 2.13×10^{-4} M HSA with NphOAc is inhibited by the addition of 0.49 equivalents of decanoate (relative to $[HSA]_0$; 0) and subsequent addition of L-tryptophan ($k_0 = (6.06 \pm 0.13) \times 10^{-2} \text{ sec}^{-1}$, $n=2$). Conditions are as in Figure 39.

with a common binding site for p-nitrophenyl esters, L-tryptophan and decanoate.

DISCUSSION

The reactions of a series of homologous p-nitrophenyl fatty acid esters from p-nitrophenyl acetate through caproate with HSA, appear to follow Michaelis-Menton mechanism, in which the reactivity is very much enhanced through reversible binding of the esters to the reactive site. Among the kinetic parameters determined in this study, the dissociation constant (K_M) is most easy to interpret. As shown in Table 1, the increase in the interaction, as reflected in the dissociation constant, is a function of the number of methylene group in the fatty acyl chain. An increase of 0.42 k cal / $-\text{CH}_2-$ in free energies of association is obtained for each additional methylene group (Fig.8). This compares with an increment of 0.8 k cal per methylene group observed in the interaction of HSA with a series of homologous fatty acids (see section D on "Binding of Small Fatty Acid Anions"). The result is an indication of either the two series of ligands, namely the p-nitrophenyl esters and fatty acid anions, binding with different orientations at the same site. or binding at two subsites of the one site. The first order rate constant k_p of this series of p-nitrophenyl esters are less regular (Table 1), together with the dissociation constants (K_M) it reflects the specificity (k_p/K_M) of HSA, or as compared to hydrolysis (k_{OH^-}) by the dimensionless parameter ($k_p/K_M \cdot 1/k_{\text{OH}^-}$). The optimum chain length, as reflected by the corrected specificity, appears to be with the propionyl group (Fig. 7). Also shown is that the odd even alternation as shown by

Marshall & Akgun (1971) of elastase and chymotrypsin is not observed.

The reaction of p-nitrophenyl acetate and p-nitrophenyl valerate with HSA proceed at different rates (Table 2), yet the extents of inhibition by equal amounts of decanoate at low decanoate concentrations are identical. At high decanoate concentration, on the other hand, the rate with p-nitrophenyl acetate is about twice as high as that with p-nitrophenyl valerate, which is to be expected as one compares the K_{OH^-} of these two esters. Furthermore, the dissociation constants of L-tryptophan calculated from its inhibition of the p-nitrophenyl esters are independent of the esters (see Figures 39, 44, and 45). These results point to an identical reaction site for these p-nitrophenyl esters, from acetate through caproate, and the conformation of HSA is sustained upon binding of these p-nitrophenyl esters.

Inhibition of the reaction between HSA and NphOAc by small fatty acid anions suggests that the reactive nucleophile may be located in or adjacent to a strong binding site for relatively small apolar anions (Means and Bender, 1975; see also section D in the RESULTS). As shown in Figure 9, interactions with decanoate ion, a strong competitive inhibitor of the reaction with NphOAc (Means and Bender, 1975; see also section D in the RESULTS), alter the ultraviolet absorbance of HSA. The observed changes with increasing amounts of decanoate appear to reflect a red shift in tyrosine absorbance as expected upon its transfer to less polar medium (Herskovitz and Sorensen, 1968) and are essentially the same as observed upon the binding of dodecanoate (Steinhardt et al., 1972). Direct interactions of the reactive nucleophile, presumably a tyrosine residue with the apolar decanoate ion may, in part, account for these changes, but to account for the intensity of the change, other tyrosine residues must also be involved.

Interactions of decanoate ion with acetyl-HSA, as with HSA, are accompanied by characteristic ultraviolet absorbancy changes (Fig. 9:). Again apparently reflecting the transfer of tyrosine residues into less polar environments, the observed changes also appear to indicate extensive binding under the indicated conditions. Although less intense than observed with HSA, the magnitude of the changes and their complexity appear again to reflect the perturbation of more than one tyrosine residue.

The binding of decanoate ion by HSA and acetyl-HSA thus both result in a red shift in tyrosyl absorbance. The two cases are different, however, and as shown in Figure 10, this difference amounts to a red shift of approximately one less tyrosine residue in acetyl-HSA as compared to HSA (Herakovitz and Sorensen, 1968). The binding of decanoate ion thus appears to perturb several tyrosine residues of HSA including one which is no longer present in acetyl-HSA, and thus accounts for the single acetyl group by which they are known to differ. Although located in or adjacent to that binding site, its conversion to O-acetyltyrosine does not appear to greatly influence the binding of deconoate ion.

Although bound to HSA more strongly than decanoate, palmitate ion brings about relatively much smaller changes in the ultraviolet absorbance of HSA (compare Figures 9 and 11.). Similarly, interactions of palmitate ion with acetyl HSA also result in relatively less intense changes in its absorbance (Fig. 11). In both cases the observed changes again appear to reflect a red shift in tyrosine absorbance but, in contrast to the corresponding spectra obtained with decanoate, are almost identical. The binding of palmitate ion thus does not appear to be altered by the conversion of HSA to acetyl-HSA. Similarly the

reaction of NphOAc with HSA is not effect by small amounts of pal¹⁴⁴-
mitate ion (Means and Bender, 1975; see also section D in the
RESULTS). The primary binding site for palmitate ion thus appears to
be separate and distinct from that for the binding of decanoate wherein
the reaction with NphOAc appears to take place. This conclusion is
further supported by the evidence indicating that, at low mole ratios
of fatty acid anions to HSA, additional tyrosine residues are
perturbed by decanoate binding as compared to that with palmitate
(Fig. 12). After acetylation of the reactive tyrosine residue
such difference is abolished upon binding decanoate (Figs. 12, 13). On
the other hand, binding of palmitate after acetylation does not seem to
affect any difference (Fig.13). Comparison of the decanoate and pal-
mitate induced spectral shifts of the acetylated HSA does not indicate
the involvement of additional tyrosine residues which presumably is
acetylated in both the HSA for decanoate and palmitate binding.

One of the approaches in studying the mechanisms of organic reaction is to investigate the change in reactivity with changing structures of the reagents. These studies have provided insights to the electronic structures of the transition states and factors determining reactivity. A quantitative estimation of the sensitivity of the reaction to electron withdrawal and donation groups on the attacking nucleophile may be made by measuring the second order rate constants for the attack of a series of nucleophiles on particular esters. A plot of the logarithms of the rate constants against the pK_a s of nucleophiles can provide the needed information. Its slope is an indication of the charge developed in the transition state. A highly charged transition state would be reflected in a large slope. In the event there is no acid-base catalysis, the slope is also an index of extent of bond formation in the transition state.

Studies of structure and reactivity relationships when applied to enzyme catalyzed reactions are faced with difficulties. These studies tend to measure the effects of changes in the structure of substrate on its interaction with the enzyme rather than the effects on the electronic changes in the transition state. Inductive effects of the substituents are often obscured by effects on binding. The difficulty in examining the effects of substituents on k_y/K_M is that binding energy terms come directly into this, and so the substituent effects on electronic effects and binding are not separable. On the other hand,

choosing k_p as a measurement of the substituents effect is complicated in that all the effects such as the accumulation of intermediates, strain, non-productive binding, and induced fit alter this term. Nevertheless, it was suggested that k_p/K_M is the safer parameter to use since the artifacts that obscure k_p are cancelled. Structure-reactivity relationships have been studied with many enzymes. Studies on the acylation of chymotrypsin by non-specific substrates (e.g. Bender and Nakamura, 1962) and the deacylation of non-specific acylchymotrypsin (e.g. Caplow and Jencks, 1962) have shown that the reactions increased with increasing electron withdrawing ability or lowering the pK_a of the product, indicating that there is an accumulation of negative charge in the transition state. Studies on the hydrolysis of substituted anilides by papain showed the values of k_p and k_p/K_M are increased by electron donating substituents in the aniline ring (Lowe and Yuthavong, 1971). This is consistent with the rate-determining breakdown of a tetrahedral intermediate.

Although human serum albumin is not an enzyme, its reaction with NphOAc apparently follows a simple Michaelis-Menton mechanism; strong binding has been attributed to be the major contributor to the unusually rapid reaction (Means and Bender, 1975). A particular tyrosine with an unusually low pK_a value also appears to partially account for the high reactivity.

Deacylation of acetyl-HSA by phenols, as the microscopic reverse of acylation, also follows Michaelis-Menton mechanism (Fig.15 through Fig.18). A Hammett plot showing the logarithm of the reactivity ($\log k_p/K_M$) decreases in proportion to increasing values for the substituent constants (σ^-) of the phenols (Fig.20) consistent with a highly charged,

presumably tetrahedral intermediate whose breakdown is the rate determining step. Identification of NphOAc as the acetate containing product after deacylation by p-methoxyphenol, together with the detection of a slow first-order-increase of absorbance at 278 nm in the presence of p-methoxyphenol, suggest the deacylation of acetyl-HSA by various phenols follows the mechanism:

An equilibrium constant of 36,000 at pH 7.5, in favor of the acylation reaction, can be calculated from the observed rate constants for the two reactions; NphOAc with HSA (Means and Bender, 1975) and p-nitrophenol with acetyl-HSA (Table 3). By comparison, more electron donating substituents, for example, p-methoxy, accelerate the deacylation reaction and slow down the acylation reaction, and therefore give a more closely comparable partitioning of the putative tetrahedral intermediate and an equilibrium constant closer to unity. However, the electron donating substituents also result in less extensive ionization of the corresponding phenols, and thus partially cancel their effect on the equilibrium.

Small fatty acid anions were found to inhibit the acetylation of HSA by NphOAc (Means and Bender, 1975). Binding of these fatty acid anions to the same site as the p-nitrophenyl esters has been attributed to the observed phenomenon (Means and Bender, 1975; and also see Section C, "Binding of Fatty Acid Anions"). As the same binding site is involved in the deacylation reaction, the reaction is also inhibited by small

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fatty acid anions (Fig 21). Disruption of the conformation of this site by 6.5M urea causes a large decrease in the deacylation rate by p-methoxyphenol (Fig 22).

Hydroxylamine, a nucleophile with higher nucleophilicity than would be expected for a simple amine of the same basicity (Jencks and Carriuolo, 1960) reacts much slower with acetyl-HSA than do the phenols (Fig 14 and Fig 23). Judging from the second-order rate constants in the reaction with phenylacetate, hydroxylamine is approximately as reactive as the phenolate anion. Yet at pH 7.8, the second order rate constant of the reaction of hydroxylamine and acetyl-HSA, $2.25 \times 10^{-2} \text{ min.}^{-1} \text{ M}^{-1}$ (see Fig 24), even after correction for the pK ($\text{pK}_a = 6$) is still several hundred folds less than the phenolate anion ($221.9 \text{ min.}^{-1} \text{ M}^{-1}$, see Table 1). Hydroxylaminolysis of o-acetyltyrosyl residues in other proteins (e.g. Riordan and Vallee, 1963; Smyth, 1967) proceeds at much faster rates than that of acetyl-HSA. The apparently depressed reactivity of hydroxylamine with acetyl-HSA reveals the lack of binding on the reaction site by hydroxylamine, also perhaps electrostatic repulsion and destabilization of the positive charge in the zwitterionic transition state by cationic groups in the proximity of the reactive site, probably the same groups that promote the reactions of phenolate anions and contribute to the electrostatic interaction with small fatty acid anions (see Section C on "Binding of Fatty Acid Anions"). In the presence of urea, the reactivity of hydroxylamine is increased by about four-fold (Fig 23) indicating a decreased influence of groups surrounding the acetyl-tyrosine residue in the native protein conformation.

In the absence of added nucleophiles, acetate is slowly released from acetyl-HSA at a rate more comparable to the saponification of simple

model compound phenyl acetate (Jencks and Carriuolo, 1960; Smyth, 1967).

However, the OH^- hydrolysis of acetyl-HSA is not in direct proportion to the increased hydroxide ion concentration (Fig 25). The linear plot of $\log k_{\text{obs}}$ versus pH between pH 6.2 and 9.4 has a slope of 0.62. Serum albumin is not known to experience a conformational change at this pH. The positively charged groups in the proximity of the reactive site are arginine residues (Means and Bender, unpublished result), and therefore not titratable at this pH range. The reason for this unusual pH dependence is not known. As compared to its reaction with phenolate anions, the reaction of acetyl-HSA with hydroxide ion is not facilitated by strong binding prior to reaction.

Binding of p-nitrophenyl acetate to human serum albumin results in its subsequent rapid reaction with a nucleophilic group (Means and Bender, 1975). A single group reacts and thus appears to involve a single site. The reaction is easy to follow as p-nitrophenol produced has a relatively strong visible absorbance. Competition with other ligands interacting with the same site is easily detected by reduced rates of reaction with p-nitrophenyl acetate. Other ligands bound strongly by HSA under the same conditions do not influence the reaction and are thus presumably bound at other sites (Means and Bender, 1975). As the reaction with p-nitrophenyl acetate appears to be a specific probe to reflect the availability of one particular binding site of HSA, the effects of small fatty acid anions on the reaction of p-nitrophenyl acetate would give insights of this specific site.

At HSA concentrations much higher than the K_d (see the following scheme) inhibition increases in proportion to added ligand (e.g. Fig. 28). Apparent binding site concentrations estimated by a linear extrapolation of the decline in k/k_0 with inhibitor, to the level due to other reactions, however, are consistently about 25 to 35% below spectrophotometrically determined concentrations of total HSA (see Table 5 and Figures 28, 29). These lower values appear to reflect the unavailability or the absence of this site in a fraction of the HSA molecules and are thus consistent with other indications of albumin heterogeneity (e.g., see Franglen, 1974; Peters, 1975). Since the amino acid sequence of HSA is already known and thus also gives an accurate molecular weight the other possibility remaining to account for the discrepancy of about 30% is the inaccurate value for ϵ $\frac{1\%}{278}$ of 5.31 accepted by most of the workers in the field e.g. ϵ $\frac{1\%}{279}$ of 5.30 used by Soltys & Hsia (1977). The

inaccuracy is most possibly caused by the hydrated water present in HSA crystals. After extensively drying the crystalline HSA, values of 5.8-6.0 are obtained, however, does not seem to be able to compensate the full deficiency.

At HSA concentrations comparable to K_d or lower, inhibition is no longer obviously stoichiometric. Plots of k/k_0 versus $[FA]/[HSA]$ nevertheless appear to describe simple binding isotherms (see Fig. 31 and 32) consistent with stoichiometries determined at higher HSA concentrations and reflecting the formation of simple HSA : ligand complexes in competition with the reactive HSA : NphOAc complex as follows:

where K_d is the fatty acid anion : HSA dissociation constant² and K_g is the NphOAc : HSA dissociation constant of approximately 2×10^{-4} M (Means and Bender, 1975; see also section A in the RESULTS).

The dissociation constants for the homologous series of anions, butyrate through decanoate, decrease approximately 4-fold for each additional methylene group (see Table 6). The dissociation constant for decanoate is thus approximately 0.0003 of that for butyrate. The free energy increment per methylene group is relatively constant at about 0.8 kcal, similar to values observed for partitioning other aliphatic compounds between water and apolar solvents (Kiehs et al., 1966; Tanford, 1962). This regular increase in affinity is consistent with a relatively uniform apolar cavity and appears to hold for anions as large as decanoate.

Undecanoate and dodecanoate are also strong inhibitors of the reaction of NphOAc with HSA but not as strong as decanoate and thus depart

from the relationship. Strong interactions with at least one other site, as suggested for the even larger homologs, myristate and palmitate (Means and Bender, 1975), appear to account for the apparently weaker interactions. Weaker inhibitory effects of these compounds are thus also accompanied by apparently greater binding site concentrations as compared to the smaller homologs. The involvement of additional strong binding sites is also consistent with the results of equilibrium partitioning studies wherein affinities increase consistently with chain length (Ashbrook et al., 1972).

The carboxylate groups of small fatty acid anions appear to be important in their binding to HSA. Thus affinities decrease with fewer methylene groups from decanoate through butyrate, but as indicated in Figure 34, extrapolation of the linear change in free energy to zero methylene groups leaves about 2.3 kcal due presumably to interaction of the anionic carboxylate with one or more cationic groups on the protein. Consistent with the probable electrostatic character of these interactions, affinities appear to be strongly dependent on the ionic strength. Similarly, electrostatic interactions have been shown to be important in the binding of L-tryptophan (see Section F in the RESULTS). Binding of other anions by serum albumin (Boyer et al., 1946; Klotz and Walker, 1947; Jonas and Weber, 1971) has been shown to involve electrostatic interactions. The apparent magnitude in the present case is similar to that involved in the binding of monoanionic indole compounds under similar conditions (McMenamy and Seder, 1963; Ray et al., 1966).

Dissociation constants determined by the described method appear to be lower than previously determined. The dissociation constants of hexanoate and octanoate given in Table 6, for example, are approximately

2 and 20 fold lower respectively, than determined by Ashbrook, et al., (1975). Considering the different conditions, however, these differences are relatively small and may largely reflect the considerably lower ionic strength. The dissociation constant determined for decanoate is about 100 fold lower than previously found, however, and no satisfactory explanation is known to account for such a large difference.

The reaction of NphOAc with HSA appears to afford a relatively convenient method to detect and study interactions of certain ligands with HSA. As compared to most other methods it specifically reveals interactions in only one particular site. Interactions at other sites as, for example, in the cases of bilirubin or pyridoxal phosphate, have essentially no effect (Koh and Means, unpublished results). The particular site involved although only one of several anion binding sites, appears to be particularly important in that it is the primary binding site for a wide range of ligands. Thus in addition to NphOAc and small fatty acid anions, it also appears to be the high affinity site for tryptophan and many drugs (see Section F ... in the RESULTS).

Dissociation constants for many strongly bound ligands can be readily determined from their respective binding isotherms (Figs. 31, 32) or as plotted according to equation 6 (see Figs. 30, 33). Some ligands, however, although interacting strongly with the NphOAc binding site and affecting strong inhibition of its reaction, also interact significantly with other HSA binding sites. Thus as already indicated, fatty acid anions larger than decanoate, although good inhibitors of the reaction with NphOAc appear also to interact with at least one other site. With palmitate, for example, these additional interactions are very strong

and the resultant inhibition curves are obviously complex (Means and Bender, 1975). Ligands interacting weakly at other sites, however, pose a special problem as the inhibition curves may resemble simple binding isotherms with only slightly altered stoichiometry. Undecanoate, for example, appears to be such a case and dissociation constants calculated in these cases are presumably incorrectly high.

The described method for determining HSA-ligand dissociation constants is also limited to those cases wherein values of K_d are between approximately 10^{-2} M and 10^{-7} M since for most accurate results, HSA concentrations should be comparable to K_d (Weber, 1965). The higher limit is thus imposed by the maximum solubility of HSA which is approximately 10^{-3} M. At HSA concentrations below 10^{-6} M, on the other hand, correspondingly small amounts of p-nitrophenol are involved which, with the usual spectrophotometric equipment, limits the lower range for such determinations.

The shift of p-nitrophenolate absorption spectrum to lower wavelength and the concomitant decrease in the absorption intensity, which reflects a decrease in the auxochromic effect of the phenolate oxygen and a decrease in polarity of the environment, is consistent with the positively charged hydrophobic binding site for p-nitrophenolate anion on HSA. The observed decrease in pK_a of p-nitrophenol upon binding to HSA (Means, unpublished data) suggests that the electrostatic interaction between the p-nitrophenolate oxygen and the positive group(s) contributes about 2 kcal/mole to the free energy of ionization of p-nitrophenol. The enhanced reactivity of HSA with p-nitrophenyl esters has been attributed to the reversible binding of the esters to a hydrophobic binding site and the stabilization of a negatively charged transition state by the

positively charged group(s) in the vicinity of the reactive nucleophile (see section A in the RESULTS; Means and Bender, 1975). The reaction is inhibited presumably through competitive binding at the same site, by small fatty acid anions (see section D in the RESULTS; Means and Bender, 1975), p-nitrophenolate (see section E in the RESULTS), tryptophan (see section F in the RESULTS), and various drugs (see section G in the RESULTS). Binding studies of a series of homologous small fatty acid anions with HSA at the p-nitrophenyl acetate reactive site have revealed that the electrostatic interaction between the carboxylate ion and the positively charged group(s) on the protein, contributes about 2.3 kcal/mole to the binding force (see section D in the RESULTS).

Decanoate, the strongest known inhibitor, retards the HSA induced p-nitrophenolate spectral change (Fig. 36) and reversely shifts the spectrum of the albumin bound p-nitrophenolate back to one more closely resembling that of the free anion, but actually with a maximum at a slightly longer wavelength (405 nm) and an isosbestic point also longer (385 nm) than that observed in the p-nitrophenol interaction with HSA in the absence of decanoate. While binding at the same primary site as decanoate, L-Tryptophan also displaces the spectrum of the albumin bound p-nitrophenolate to the spectrum with a maximum at 405 nm and an isosbestic point at 385 nm (Fig. 37).

There are many fluorescent probes (e.g. Sudlow et al., 1975) and chromophoric probes (e.g. Stroupe and Westphal, 1978) being used to study specific binding sites on serum albumin. The characteristic changes in properties of electron transition of these usually small molecules upon binding to the proteins, affords a simple and sensitive method to study the constituents of the binding site and its affinities

for various ligands. Nevertheless, the reliability of this method lies on an accurate determination of the end point at each manipulation on the probe and the possibility of separating the effect resulted from the manipulation alone from the secondary effects. For instance, the spectrum of HSA bound p-nitrophenolate anion, after being displaced by a large excess of decanoate or tryptophan, is still distinguishable from that of the free p-nitrophenolate ion (Fig. 37), apparently due to the binding of p-nitrophenolate ion to other sites and the change in solvent property by adding ligands. However, to what extent does each of these factors contribute to affecting the electron transition of the p-nitrophenolate anion, remains difficult to estimate.

Early studies on L-tryptophan binding have indicated there is one primary site with much higher affinity for L-tryptophan and its analogs than other secondary sites (McMenamy and Oncley, 1958). Although the HSA preparations used in the early studies were of high purity, they were not always treated to remove the tightly bound lipids from the protein. Therefore, comparison between the present study with the data published by others should be made with the awareness of this fact. Furthermore, the defatting procedure used in the early studies were that of Goodman (1957), and in the present study HSA is treated according to Chen (1960).

Competitive studies by equilibrium dialysis have shown L-tryptophan, acetyl-L-tryptophan, indolepropionate among other indole compounds bind to the same primary binding site on HSA (McMenamy & Oncley, 1958; McMenamy & Seder, 1963). This result is confirmed by the present study in that all of these three compounds are strong inhibitors of the reaction between p-nitrophenyl esters and HSA (e.g. Figs. 39, 49, and Table 8).

At pH 7.5, ionic strength of 0.02 M and 25°, the L-tryptophan dissociation constants calculated thereby vary from 1.65×10^{-5} to 2.10×10^{-5} M. Comparing these dissociation constants with that obtained at pH 7.15 (Fig. 43) and pH 7.78 (Figs. 41, 42), an increase in the dissociation constant in direct proportion to the H^+ concentration is observed, suggesting that the anionic form of L-tryptophan is the species bound at the primary binding site. McMenamy (1963) calculated the pH profile of the association of L-tryptophan and tryptamine to defatted HSA, by assuming binding occurred only when the amino group was uncharged and on the other hand, for the similar pH profile with nondefatted HSA McMenamy & Seder (1963) assumed binding could take place with the amino group both charged and the noncharged. The result was two sets of thermodynamic parameter values differing from each other significantly. Although McMenamy (1963) attributed the difference to the difference in the binding of indole compounds to the nondefatted and defatted HSA, undoubtedly this is superimposed by the effect of making different assumptions in the two conditions.

N-acetyl-L-tryptophan, at pH 7.5 and ionic strength of 0.02 M, shows about the same inhibitory effect as L-tryptophan (Fig. 49). Without an amino group, N-acetyl-L-tryptophan isn't subjected to the complexity in deciding the charge state of its bound form. Indeed the binding constants for N-acetyl-L-tryptophan to defatted and non-defatted HSA differ, but neither value varies in the pH range 6.5 to 9 where it is at its maximum (McMenamy, 1963; McMenamy & Seder, 1963). Therefore, the dissociation constant calculated from Figure 49, 1.6×10^{-5} M, represents the intrinsic dissociation constant at 0.02 M ionic strength and 25°. This dissociation constant corresponds to a free energy of

association of -6.7 kcal/mole, and after allowing the ionic strength difference (see Fig. 48) this value is in good agreement with -5.8 kcal/mole obtained by McMenemy (1963) in 0.1 M NaCl with defatted HSA.

Indolepropionate behaves very similarly to decanoate in inhibiting the reaction of *p*-nitrophenyl acetate with HSA, in that a greater than stoichiometric inhibition is observed (Table 8). Competitive binding studies (McMenemy & Oncley, 1958; McMenemy and Seder, 1963) had indicated that *L*-tryptophan, *N*-acetyl-tryptophan and indolepropionate are bound at the same primary site. Similar to *N*-acetyl-*L*-tryptophan, the strength of binding of indolepropionate reached a maximum at pH 6.5 and remained constant between pH 6.5 and pH 9.5. When the disorientation entropies of these two compounds, -6 e.u. and -13 e.u. at 25° for indolepropionate and *N*-acetyl-*L*-tryptophan respectively, were taken into account the free energies of association of these two compounds with defatted HSA differed only by 0.6 kcal. These findings are indicative of a similar mode of binding involved in these two compounds. The most likely explanation for the greater than stoichiometric inhibition by indolepropionate is that applied to the decanoate inhibition; the site at which *p*-nitrophenyl esters and other ligands bind to is not completely free in the defatted HSA used. While the phenomenon of less than one binding site per HSA molecule is observed with strong inhibitors such as indolepropionate and decanoate, such does not also appear to be so with weak inhibitors or at HSA concentration below K_d since incomplete association interferes with determinations of exact stoichiometry (see Bieth, 1974).

Strong dependence of the binding of *L*-tryptophan (Fig. 48), and presumably of *N*-acetyl-*L*-tryptophan and indolepropionate, on the ionic

strength, is consistent with that observed in the binding of other ligands, e.g. caproate. Structural studies of the binding site are supportive of the strong ionic strength dependence observed, (see below).

The dissociation constants of L-tryptophan obtained by using various p-nitrophenyl esters as the probes are identical (e.g. Figs. 39, 44, 45) further indicates the reaction site of the p-nitrophenyl esters is identical (see section A in the RESULTS).

The location of the indole binding site has been studied by affinity labels, bromoacetyl-L-tryptophan, dansyl chloride or pyridoxal 5'-phosphate (Gambhir and McMenamy, 1973). Reacting HSA with these labels led to a blockage of the binding of acetyl-L-tryptophan. If labeling was conducted in the presence of indolepropionate, the indole binding site is largely protected. Each of these labels was incorporated mainly into fragment C (Gambhir et al., 1975), which is one of the three cyanogen bromide cleared fragments of HSA and was later assigned as the peptide Cys₁₂₄-Met₂₉₇ (Peters, 1975). The native structure of the cyanogen bromide fragments of HSA is partially preserved as indicated by circular dichroism measurements, showing 70-80% of the α -helix on HSA is retained in the fragments (Sjoholm and Ljungstedt, 1973). Fragments A and C were found to be active in binding L-tryptophan and drugs. Within the fragment C, His 146 and lysine 190 were the two positions labeled and resulted in inhibition of indole binding (Gambhir et al., 1975). It was also found that a tyrosyl OH in fragment A, the C-terminal fragment from the three CNBr digest, was involved in the indole binding site. Furthermore, His 146 is shown to be located in a segment predominated with hydrophobic and basic side chains. From the primary sequence elucidated by Brown et al. (see Peters in the Plasma Proteins, 1975) His 146 itself

is adjacent to two arginine residues. A cluster of three to five positive charged residues present in the binding site was also suggested by the abrupt decrease in the association constants for indole compounds as the pH was increased beyond 9 or 9.5 (McMenamy, 1963). This is supported by the strong dependence of dissociation constant of L-tryptophan on the ionic strength observed in the present study (Fig. 48). These positive charges may be the same as those postulated to be involved in stabilizing the tetrahedral intermediate of the p-nitrophenyl ester reaction with HSA and those involved in the electrostatic interaction with the small fatty acid anion (see section D in the RESULTS). Thus studies of affinity labeling concluded a tryptophan binding site composed of hydrophobic and positively charged residues, and a tyrosine residue folding toward it from a distant region on the HSA, a feature which is consistent with that postulated for "the small apolar anion binding site on HSA" throughout the present study.

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