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THE SIGNIFICANCE OF STEROID STRUCTURE IN INTERACTIONS WITH SERUM ALBUMIN

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To my parents, wife and daughter.

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1. GENERAL REMARKS

Reversible binding by proteins of substances of low molecular weight (ligands) is a phenomenon of considerable fundamental and general significance in Biochemistry. Such a process often leads to alterations in the functional activities of either the ligand or protein or both. These changes serve as important regulatory mechanisms in the living organisms. Many examples can be cited of the processes which involve interaction of a ligand with some protein as the primary step in the overall scheme. One of the most widely encountered is the combination of an enzyme with its substrate molecule. As postulated by Lumry (1959) and Jencks (1966) interaction of an enzyme with its substrate produces strain in the susceptible bonds of the latter which thereby prepares it for undergoing chemical change with lower energy of activation (Rack and Strain theories). X-ray crystallographic studies of the lysozyme molecule have provided convincing support to the validity of this concept (Phillips, 1966). Further, it is now known that the conformation of enzymes is altered and the activity modified by the interaction of a small molecule or ion (called effector) with the catalytic protein (Koshland Jr., 1970). Other biological phenomena in which ligand-protein interactions play vital roles are hormone action, antigen-antibody

reactions, drug action, and transport of physiological substances in the biological fluids as well as their conveyance and partitioning across the cell membranes.

Because of the fundamental importance of ligand-protein interactions in a wide variety of biological processes, considerable effort has been made in recent years to understand the general principles underlying these interactions. Since binding is reversible, the law of mass action can be applied to the final equilibrium. The thermodynamic parameters obtained from such an analysis have provided useful insights into the binding mechanisms. However, as has been pointed out by Klotz (1974), the thermodynamic studies alone are insufficient for the proper understanding of the binding reactions and must be supplemented with spectroscopic investigations. Both kinds of approaches have been applied in the study of small molecule-protein combinations. Further, complete description of the binding equilibrium also requires knowledge of the rates of formation and dissociation of the ligand-protein complexes. However, such reactions are usually too rapid and require application of fast reaction techniques for their measurement. Temperature jump and stopped flow methods have been used for the fluorimeteric determination of kinetic constants of binding in a few instances (e.g. Froese et al, 1962; Stroupe and Westphal, 1975). Stroupe and Westphal have emphasized the significance of values of rate constants of steroid-protein interactions in the mechanism of hormone

action (section 2.c.).

Many of the proteins involved in the biological processes mentioned above have been isolated in sufficiently pure forms and their interactions with ligands of concern have been studied, in some cases in very great detail. These proteins include the various blood proteins and certain enzymes (Steinhardt and Reynolds, 1969), the estradiol-binding proteins of uterus (Williams-Ashman and Reddi, 1971), the biotin-binding protein, avidin (Green, 1963), and galactose-binding protein of E. Coli (McGowan et al, 1974). A conformation change which has been demonstrated in the binding of galactose to the galactose-binding protein (McGowan et al, 1974) may be important in galactose transport in the cell membrane.

It should be pointed out that with the exception of very few proteins such as serum albumins, binding proteins are usually specific for only particular types of ligand so that a very limited amount of general information can be acquired by means of binding studies with any one of these individually. However, serum albumins are especially suitable proteins for the study of such reactions since they possess remarkable ability to bind a seemingly limitless number of molecules or ions of varied chemical nature. These include such physiological substances as fatty acids (Ashbrook et al, 1975), thyroxine (Tabachnik and Giorgio, 1964), tryptophan (McMenamy and Oncley, 1958), bilirubin (Jacobsen, 1969), urea (Farrell et al, 1971), steroids

(Westphal, 1971), calcium (Katz and Klotz, 1953) as well as such non-physiological compounds as drugs (Spector et al, 1973) dyes, detergents and many other organic and inorganic ions (Steinhardt and Reynolds, 1969c). Furthermore, serum albumins are obtainable in a highly pure state at very low price and because of their high conformational flexibility (Foster, 1960) can be subjected to a wide variety of experimental conditions without causing any irreversible alterations. It is for these reasons that serum albumin had been investigated very extensively both in regard to physico-chemical structure and ligand binding properties (Peters, 1975; Steinhardt and Reynolds, 1969).

One of the major aims of studies with serum albumins is to understand the general mechanisms involved in ligand-protein binding and apply the information obtained therefrom in the investigation of the interactions of more specific proteins. However, in spite of the considerable amount of information available on the interaction of various ligands with serum albumin, the most intriguing question which remains still unsettled is whether or not ligand binding involves specific interactions (i.e. structure-dependent reactions) with this protein. It may be noted that since serum albumin is a globular protein, hydrophobic amino acid residues would be buried in the interior of the macromolecule, while polar residues would be exposed on the surface and interact with the water molecules in aqueous solutions. However, as suggested by Steinhardt and Reynolds (1969d) and Tanford

(1973), a few of the apolar residues in a globular protein may also be located on the surface or inside the crevices and can give rise to hydrophobic patches that may constitute binding sites for the nonpolar ligands. The presence of charged residues in these non-polar regions (Green, 1963; Swaney and Klotz, 1970; Jones and Weber, 1971; Gambhir et al, 1975) can explain the binding of ionic ligands. Binding at such sites would be determined by the hydrophobicity and/or charge on the ligand. Such a binding may be either purely non-specific in which case the information derived from ligand binding studies will not be of much value, or it may involve specific combinations of hydrophobic bonds, hydrogen-bonds and/or electrostatic interactions. Most of the data on serum albumin reported in the literature do not provide clear cut answers to the question of specificity in the binding of ligands to this protein (see for example Steinhardt and Reynolds, 1969). Although recent studies involving affinity labeling experiments (Gambhir et al, 1975) and binding measurements on the proteolytic fragments of serum albumin (Reed et al, 1975) and others (Peters, 1975) have led to the identification of separate binding sites for many different kinds of ligands (non-steroid) in this protein, it still does not prove that the interactions involved are specific. This is because binding at some of the regions may be dependent upon the dimensions of the crevices in which they may be located and the size and shape of the interacting molecule.

The understanding of specific interactions requires studies with model compounds as ligands having similar molecular sizes. As pointed out first by Eik-Nes et al (1954), steroids represent a good class of compounds for the study of ligand binding since they contain a common carbon skeleton into which various kinds of functional groups can be introduced in different combinations and their effects on the interaction can be assessed. Thus, a study of the binding of steroids with serum albumins can provide better understanding of the nature of the ligand-binding phenomenon. Although the interactions between steroids and serum albumin have been studied extensively in the past many years, the review on these studies discussed in section 2 reveals that the literature lacks suitable data for the proper understanding of these interactions.

2. REVIEW OF LITERATURE

2.a. Structure and some physico-chemical properties of serum albumin

The chemical structure and physico-chemical behavior of serum albumins have been investigated in considerable detail and a wealth of information has accumulated (Foster, 1960; Peters, 1975). The following is a summary of the various features of bovine serum albumin (BSA) and human serum albumin (HSA) which may be helpful in the understanding of steroid-albumin interactions.

Serum albumin is a globular protein. At physiological pH it exists largely in the monomeric form; dimers and trimers are present in very low concentrations. Each monomer contains about 600 amino acid residues. Serum albumin is a simple protein and contains no non-protein (prosthetic) groups covalently attached to it. However, crystalline preparations may contain 2 - 2.5 moles of fatty acid per mole protein in the case of HSA and relatively smaller amounts (up to one mole) in the case of BSA (Chen, 1967). These fatty acid molecules are bound to the protein by means of non-covalent linkages, but are not removed by repeated crystallizations. However, serum albumins can be freed of most of the lipids by the application of defatting procedures devised by Goodman (1957) and Chen (1967). The

solutions of serum albumin may be sometimes yellow due to the presence of traces of hematin arising from blood hemolysis. Hematin is bound very tightly to the protein and is difficult to remove.

The amino acid sequences of both bovine and human serum albumins have been worked out completely by the combined efforts of a number of workers in various laboratories (Peters, 1975). These analyses (fig. 2.a.1.) reveal the presence of repeated sequences in both proteins. The Cys-Cys pairs are found to occur seven times in the total sequence. The other Cys residues are located at the same distances on either side of each of these pairs. On the basis of these sequence data Brown (Brown, 1975; Behrens et al, 1975) has proposed a model (see fig. 2.a.1.) of serum albumin (BSA or HSA) according to which it contains nine double loop domains which arise due to the formation of disulfide bonds between the cysteine residues. The amino acid sequences in some of the loops are very similar. Repeated sequences are probably a consequence of gene duplication. These sequences may contain identical binding sites within a given class for the same ligand (Spector, 1975).

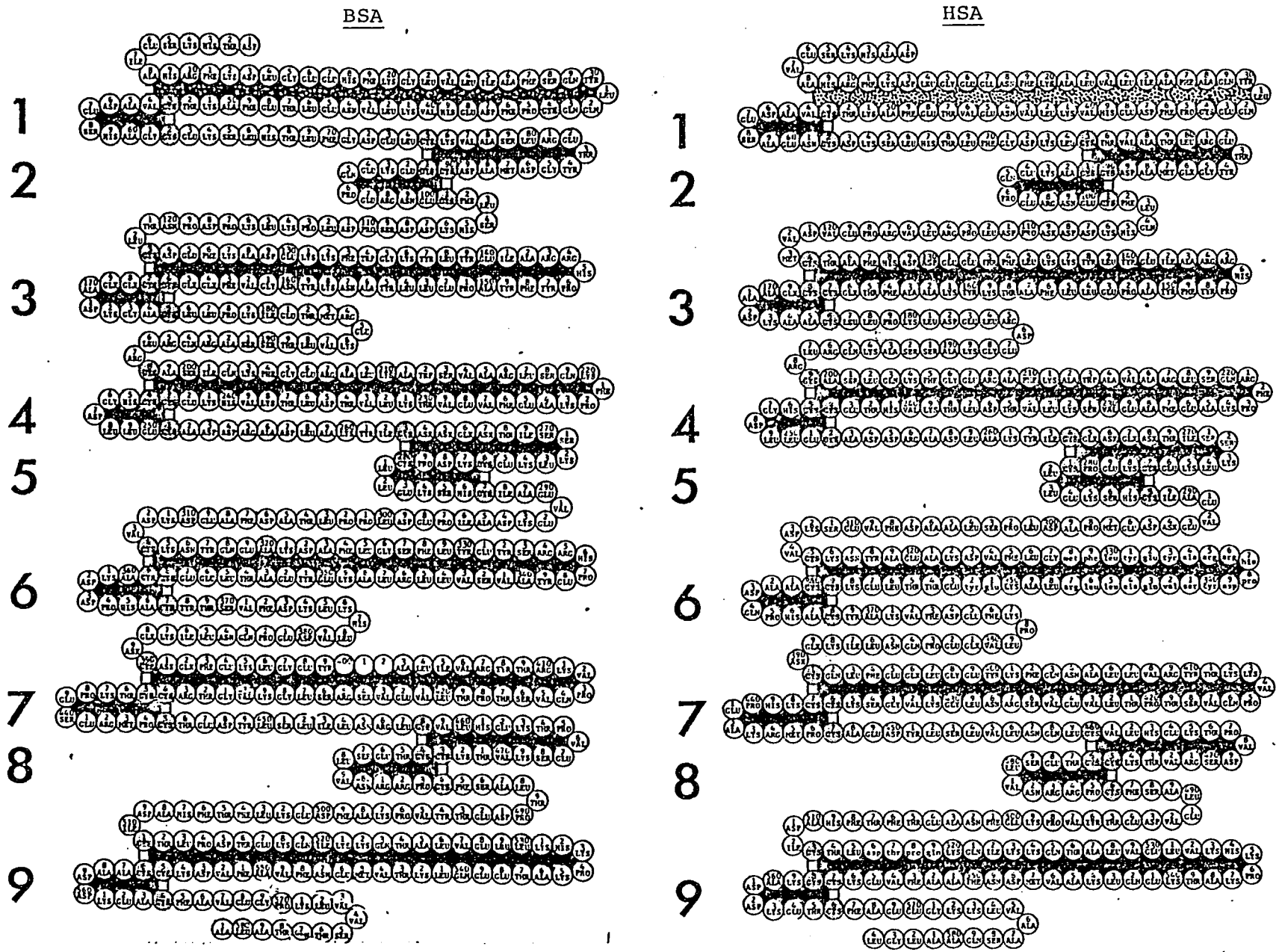
The secondary structure of serum albumin has been studied by means of deuterium-hydrogen enchange (Benson et al, 1963), circular dichroism and optical rotatory dispersion measurements (Reed et al, 1975). Studies of Reed et al have shown that bovine albumin contains 68% α-helix and about 18% pleated-sheat structure. It has been suggested that the

FIGURE 2.a.1.

PRIMARY STRUCTURE MODELS OF BSA (BROWN, 1975)

AND HSA (BEHRENS ET AL, 1975)

Reproduced (without permission) from Peters (1975).


BSA
HSA
1
2
3
4
5
6
7
8
9

disulfide bridges may impose limits on, as well as help to stabilize, the secondary structure of albumin (Marcus and Karush, 1957; Reed et al, 1975).

The three-dimensional structure of serum albumin has not yet been worked out by means of x-ray crystallography. Such studies have begun only recently as a result of the availability of monoclinic crystals of HSA (McClure and Craven, 1974). In the native protein the double loop domains may coalesce to form larger globular regions. Thus, from their fluorescence polarization studies of complexes of BSA with 1-anilino - 8 - napthalene - sulfonate, Anderson and Weber (1969) have proposed that the polypeptide chain contains three globular domains in a linear arrangement with transverse clefts. However, on the basis of their studies of the enzymatic cleavage of BSA and reversible association of peptide fragments, Pederson and Foster (1969) have suggested another model that contains four such domains.

The ultraviolet spectra of serum albumins are similar to those of other simple proteins. The exinction coefficient ($\lambda 279$) of BSA is greater than that of HSA. This is because BSA contains two tryptophan residues which are located in loops 3 and 4, whereas HSA contains only one that is present in loop 4. Further, the fluorescence intensity of BSA is more than twice that of HSA. This suggests that the tryptophan residue in loop 3 is present in a strained environment (Peters, 1975). Most of the tyrosine residues are contained in loops 3 and 6; the tryptophan-

containing loop 4 contains no tyrosine.

Solvent perturbation studies of Herskovits and Laskowski (1962), indicate that two-thirds of the tyrosyl residues in BSA are inaccessible to the solvent of the medium. The two tryptophans in the BSA are exposed to the extent of 50 - 70% (1 - 1.5 residues) in the native protein.

2.b. Nature of binding of some non-steroid ligands to serum albumin

Since serum albumin is known to bind a wide variety of non-steroid ligands (section 1.) the interactions involved in the binding of these may bear some relation to the binding of steroids to this protein. It is therefore necessary to review briefly what is known about the interaction of some of these ligands with serum albumin.

According to the studies of Goodman (1958) plasma albumin contains three classes of binding sites for the anionic ligands. The two primary sites have much higher binding affinity than the remaining secondary and tertiary sites. Goodman proposed that while the primary sites are occupied only by fatty acids, the secondary and tertiary sites are shared by many types of organic ligands. Positive entropy changes have been noted in the binding of fatty acids to serum albumin (Foster, 1960) which suggests that the interactions involved are largely hydrophobic. Morrisett et al (1975) studied the interaction of palmitic acid with BSA by means of fluorescence quenching and electron paramagnetic resonance spectroscopy using spin-labeled analogues.

Their data indicated that the binding involves hydrophobic interactions along the entire length of the fatty acid tail. The methyl esters of fatty acid were bound with slightly higher energy than the free anions which indicated that the carboxyl group may be involved in hydrogen bonding or electrostatic interactions.

The studies of thyroxine binding to HSA have shown interesting relationships between chemical structure and binding activity. Tabachnik (1964) reported two sets of sites for the binding of thyroxine to HSA. Tabachnik and Giorgio (1964) studied the binding of thyroxine and a number of its derivatives (by means of their ability to compete with thyroxine) to HSA. Their results showed that neither charged carboxyl nor charged amino group is required for binding. The binding activity was completely abolished when four iodine atoms were replaced by hydrogen atoms. However, only slight activity was left when they were replaced by $-CH_3$ groups. Replacement of iodines by bromine atoms caused a small decrease in binding affinity for the protein. The esterification of carboxyl group and removal of amino group increased binding affinity significantly.

Out of all the naturally occurring amino acids only tryptophan is bound to plasma albumin with strong affinity (McMenamy and Oncley, 1958). The binding involves a single site and is specific for the L-form. Cunningham et al (1975) showed that fatty acid binding at primary sites inhibits tryptophan binding only slightly. However, marked inhibition

is observed when fatty acids are bound to the secondary sites.

Binding affinity of bilirubin for serum albumin is very large. The binding is so strong that the measurement of binding affinity presents considerable difficulty. Binding takes place at a single site (Jacobsen, 1969) and is competitively inhibited by fatty acid binding to the secondary anion sites (Starinsky and Shafrir, 1970).

Rudman et al (1971) studied the effect of palmitic acid on the binding of a large number of drugs to BSA and HSA. Their results showed that the drugs were not displaced from the albumin binding sites until fatty acid levels exceeded 3.5 moles/mole protein. It was suggested that since plasma fatty acid levels under normal states rarely exceed 1.5, drug binding would not be affected by them.

Recently attempts have been made to locate the positions of binding sites for various ligands in the amino acid sequences of human and bovine serum albumins. Gambhir et al (1975) showed by means of affinity labeling that indole binding may involve two positions in the peptic fragment C of human serum albumin. It may be noted that fragment C corresponds to residues 124 - 237 of the amino acid sequence and contains the entire loop 3 and a large portion of the loop 4. Results of Gambhir et al showed that the affinity labels blocked indole binding at His-146 and Lys-189. These authors made the following deductions from the amino acid sequence which they determined for the

fragment C: Since His-146 and Lys-189 are far removed in the amino acid sequence, the polypeptide chain must be folded to bring these residues close together at the binding site in the native protein. The amino acid sequences in the vicinity of these two residues are especially favorable for the interaction of anionic ligands. The residues 133 - 166 contain two proline residues and a large number of hydrophobic and basic amino acids. The presence of proline residues suggests absence of helical structure in this region which can permit rearrangement of hydrophobic and basic groups for the accommodation of the hydrophobic portion of the anionic ligand. The sequence near Lys-189, however, contains very few non-polar residues and numerous hydrophilic amino acids. There are relatively few acidic and basic amino acids present in equal numbers. This region constitutes one-third of the binding site and appears more suitable for the interaction of charged group of the ligand. It was further pointed out that Lys-194 is also involved in the binding of salicylic acid (Hawkins et al, 1969; Pinckard et al, 1974) and acetrizoate (Farr et al, 1961) and that a number of dyes, drugs and other compounds such as bilirubin inhibit acetrizoate binding (Pinckard et al, 1974), Gambhir et al postulated that the residues 124 - 214 is the general region for the major binding sites of all ligands.

In this connection it is pertinent to note that Swaney and Klotz (1970) have suggested that a hexapeptide sequence in the neighbourhood of Trp-213 in HSA forms part of

the binding sites of many types of organic anions as well as the (neutral) steroids. The binding of the neutral steroids in this area is supported by the spectroscopic observations of Ryan (1968) and Ryan and Gibbs (1970a), who have noted that the tryptophan spectrum in the HSA is perturbed as a result of interaction with the steroid.

Reed et al (1975) employed a different approach for the identification of ligand binding sites in the bovine serum albumin. This involved the determination of binding activities of the fragments of BSA obtained following limited hydrolysis with trypsin or pepsin. Although it may be questioned that the action of the enzyme might lead to fragments which have conformations different from those in the native protein, these authors showed by means of CD measurements that the native conformation is largely retained in the individual fragments. The results suggested that bilirubin binding involves residues 186 - 238 which form loop 4 and a part of the segment between loops 3 and 4. The results further indicated that the strong palmitate binding sites are located in the carboxyl two-third end of the molecule.

2.c. Biological significance of steroid binding by serum proteins

Some substances such as fatty acids and bilirubin are very water insoluble. However, these are found to occur in the blood at concentrations much higher than their solubilities in water (Peters, 1975). Binding of these

metabolites to serum proteins is responsible for high levels in the blood. Protein binding therefore plays a significant role in the solubilization and hence transport of these substances in the blood. However, although steroids are non-polar molecules, their aqueous solubilities are known to be markedly greater than their normal physiological or even pathological levels in the blood. Serum protein binding therefore does not seem to perform any useful function in the process of steroid-transport in the blood (Sandberg et al, 1966). However, evidence available from several studies indicates that steroid-protein interaction in the plasma is fundamentally significant in the regulation of physiological action and metabolism of steroids in the organism. These studies have shown that the bound steroid is both biologically and metabolically inert and that the unbound fraction is the active form of the steroid in the blood.

Thus Mills et al (1960) examined the influence of estrogen administration on cortisol metabolism and plasma levels of free and bound cortisol in the human subjects. As a result of this treatment the concentration of total corticosteroid in the blood increased (hypercorticism). The percentage of free cortisol decreased but its actual concentration remained at the control level. These changes were attributed to the estrogen induced increase in the concentration of a cortisol binding protein in the plasma (see section 2.d.). Since depression of eosinophils, a condition associated with hypercorticism could not be

detected in the treated subjects it was suggested that the unbound cortisol is the biologically active form of the steroid. Further, the half-life of disappearance of plasma cortisol was considerably increased after estrogen treatment suggesting that the bound cortisol is metabolically inert.

With the development of techniques for the isolation and purification of serum proteins it became possible to define the role of steroid binding to the individual serum proteins separately. Thus Westphal and Forbes (1963) injected protein-free solutions of progesterone or solutions containing progesterone and AAG (α_1-acid glycoprotein), a progesterone binding protein of plasma (section 2.d.), into ligated uterine horns of adult mice and determined the progestational activity of the steroid by means of a bioassay procedure. The use of this system avoided the possibility of any effect of administered protein on the metabolic clearance of cortisol. A positive response was seen when progesterone was injected alone and no response was observed when either AAG or mixture of AAG and progesterone was injected. Matsui and Plager (1966) studied the inhibitory effect of cortisol on the in vitro oxidation of glucose by mouse ear slices and found that the effect was markedly diminished when either BSA or CBG (cortisol binding globulin, section 2.d.) was added to the incubation medium.

Recently Stroupe and Westphal (1975) determined the rate constants for the interaction of steroids with progesterone binding globulin (PBG) of guinea pig serum

(section 2.d.) by means of stopped flow fluorescence measurements. Both association and dissociation rate constants were found to be higher than those reported in the literature for any other steroid-protein binding system. The authors suggested that rapid uptake of the steroid hormone by steroid binding proteins such as PBG may be necessary to protect the organism against toxic effects of the release of steroids in large quantities in the blood and further that faster dissociation may be required for immediate supply to the target cells. The slow dissociation of the hormone from the receptor complex may be required for protection against metabolic transformation during the transfer of the complex form from the cytoplasm to the nucleus.

2.d. Experimental demonstration of binding of steroids to serum proteins

The binding of steroids to serum proteins was first recognized by Brunelli (1934). He found that the estrogenic hormones of the plasma could not be completely extracted with ether and that a significant fraction remained in the precipitated proteins. By means of equilibrium dialysis it was further demonstrated that these hormones were bound reversibly by the plasma proteins. The significance of these observations of Brunelli was not realized until some years later when the binding of these and other steroids to serum proteins was studied more systematically.

As it became known that serum albumin possessed the ability to bind various substances of different chemical natures, steroid binding to this protein was investigated first by a number of workers (Bischoff and Pilhorn, 1948; Eik-Nes et al, 1954; Sandberg et al, 1957; Daughaday, 1958a,b; Slaunwhite et al, 1963). These studies involved measurements of increased solubility of steroids in protein solutions compared to their solubility in protein-free aqueous solutions or the determination of bound steroid by the classical method of dialysis equilibrium. They demonstrated clearly that steroids such as testosterone, progesterone, dehydroisoandrosterone, estrogens and others are bound to serum albumin with considerable avidity and in addition provided some useful information about the fundamental nature of these interactions. The binding of the more polar steroids (such as cortisol) was found to be much weaker compared to the binding of the less polar ones (polarity rule). This led to the belief that plasma might contain other proteins capable of binding cortisol and other corticosteroids in the blood.

Experiments conducted by Daughaday (1956, 1958a) by means of equilibrium dialysis and paper electrophoresis seemed to show that albumin was chiefly responsible for the binding of cortisol in the plasma. It should be pointed out that these studies employed large quantities of cortisol which would prevent the detection of any low-capacity binding system in the plasma. Daughaday (1958b) then studied the

effect of steroid concentration on the binding of ^{14}C-labeled cortisol, corticosterone, testosterone, progesterone and estrone to the human plasma by equilibrium dialysis and his results showed the presence of at least two binding systems for each of these steroids except estrone. Daughaday (1958c) showed by means of continuous flow equilibrium paper electrophoresis that an α-globulin fraction in the human plasma had high affinity for cortisol and named it "Corticosteroid Binding Globulin" (CBG).

Sandberg et al (1965) presented evidence of the high affinity of this protein also for progesterone and called it by the more appropriate name "transcortin." Transcortin has been isolated from human plasma by the combined application of a number of techniques such as chromatography on DEAE-cellulose or hydroxy apatite C columns, gel filtration, and disc electrophoresis on polyacrylamide gel columns (Seal and Doe, 1962a,b; Sandberg et al, 1966). Its amino acid composition and molecular weight have been determined (Seal and Doe, 1962a; 1966).

Although the investigations of Daughaday (1958b) indicated the presence in plasma of non-albumin proteins capable of binding steroids other than cortisol, the value of his results was not fully appreciated at that time. Systematic studies of their demonstration in the plasma started only some years after. Thus Mercier et al (1966) reported a specific testosterone binding protein in the plasma which was shown to be a β-globulin by means of

electrophoresis. The studies of Guériguian and Pearlman (1968) involving both electrophoresis and chromatographic experiments showed that testosterone binding globulin and CBG of plasma are not identical. Rosenbaum et al (1966) demonstrated an estradiol binding β-globulin in the human plasma. The work of Steeno et al (1968) suggested that the activities of estradiol binding globulin and testosterone binding globulin are associated with the same β-globulin fraction and applied the term steroid-binding β-globulin to this fraction.

Diamond et al (1969) reported that a large increase in progesterone binding activity seen in the plasma of pregnant guinea pig did not correlate with simultaneous rise in binding activity observed for cortisol. Studies of Burton et al (1970) showed that the high progesterone binding activity in the guinea pig serum is due to the presence of another progesterone binding protein which was later designated by Westphal (1971b) as PBG. Chromatography of serum on hydroxyapatite and Sephadex G-200 (Burton et al) revealed that PBG is different from CBG.

It may be mentioned that, with the exception of serum albumin, all of the steroid binding serum proteins mentioned above have been shown to be glycoproteins. Another serum glycoprotein called α_1-acid glycoprotein (AAG or orosomucoid) was isolated by Weimer et al (1950) even before its steroid binding ability was demonstrated. Westphal and coworkers found that AAG binds progesterone (Westphal et al, 1961) and a number of other steroids (Westphal, 1964) with

affinities intermediate between those of serum albumin and CBG. AAG is present in plasma at much higher concentrations compared to other high affinity serum proteins and therefore can be readily isolated in large quantities. Further, AAG is very soluble in water and is exceptionally stable to heat. These factors have enabled detailed investigation of its chemical structure and physico-chemical properties in a number of laboratories. AAG appears to be a good model protein for the study of the relation between steroid structure and protein binding behavior. The possibility of the binding of other ligands to this protein has not been examined as yet. Such studies may further add to the suitability of AAG as a steroid-binding model.

2.e. Relation between steroid structure and protein binding

Many studies have been carried out to determine how the various structural features of steroids affect their affinity for proteins. These studies have provided useful information about the nature of steroid binding sites and the types of interactions involved. This section describes the results obtained with serum albumins and in addition also deals with some data on other serum proteins and some enzymes.

2.e.1. Serum albumin

In general steroids are bound to serum albumin with relatively lower affinity and specificity as compared to their interaction with other steroid binding plasma proteins mentioned in the previous section. However, albumin is the

most abundant protein of the blood plasma. Steroid binding to albumin would not be of much physiological value, except possibly in some case (e.g., 5α-androstan, 3α, 17β-diol), if its concentration in the plasma were not so high. The other steroid binding proteins of plasma have high affinity but low capacity. When steroid binding sites on these proteins are saturated with steroid, binding to the albumin becomes physiologically significant. Thus, albumin serves as an overflow reservoir in the binding of steroids in the blood. However, since albumin is a general ligand binder and more readily available (section 1.) it has since long served as the choice protein for the investigation of steroid-protein interaction as a general phenomenon. For this reason more data are available in the literature for the interaction of steroids with serum albumin than for any other serum protein.

However, in spite of numerous data available on steroid-albumin interaction there have been very few systematic studies of the relationship between chemical structure of steroids and their binding to the plasma albumin. Some detailed studies that have been made are not based on the analysis of actual thermodynamic constants of binding but rather these involve comparison of the binding abilities of various steroids determined by measurements of the binding induced alterations in the ultraviolet absorbance or fluorescence of the protein or steroid. The thermodynamic data which are available in the literature were acquired with different albumin preparations under varying conditions

of temperature, pH and buffer. Recently Westphal (1970) has compiled the binding data for a number of steroids and HSA derived from a number of sources up to 1968. The results reported for some steroids show marked variations in the values of binding constants for a given steroid, most probably because of the differences in the experimental conditions under which they were acquired. Such data are obviously not suited for comparison of the binding of different steroids. However, in spite of large variations in the data for individual steroids these results show a consistent decrease in the values of association constants with increasing number of polar groups in the steroid (polarity rule). The less polar progesterone shows higher value than the more polar testosterone. Affinity values for estrogens are however contrary to the definition of polarity rule.

Eik-Nes et al (1954) studied the binding of a number of different kinds of steroids to BSA. Their data did obey the polarity rule in the same fashion as noted above in the case of steroid binding to HSA. However these authors determined binding at only a single steroid concentration for each steroid and expressed their values as $^{r}/_{[S]}$, where r is the moles of bound steroid per mole protein and [S] is the free steroid concentration, which are not always true measures of the binding equilibrium.

Here it is necessary to point out that bovine and human serum albumins show some clear cut differences in their

steroid binding properties and caution should be exercised in extrapolating information derived with HSA to BSA and vice versa. Thus a number of workers have independently shown that binding affinities of various steroids to HSA are higher than their affinities to BSA (Daughaday, 1956; Sandberg et al, 1957). Further Ryan (1973) in his study of the effect of steroids on the tryptic hydrolysis of albumin (section 2.m.) has also noted marked differences in the behavior of the two proteins. Ryan found that whereas BSA hydrolysis was stimulated, the hydrolysis of HSA was inhibited by binding of C_{21} steroids. Interestingly, Kniewald et al (1975) determined the enthalpy values for the binding of a number of steroids to bovine and human serum albumins by means of microcalorimetery. These represent the first ΔH values measured for steroid binding by a direct method. The authors showed that the values determined for HSA followed the polarity rule but the values for BSA did not. It is possible that the polarity rule observed for steroid binding to BSA by Eik-Nes et al (1954) may be a coincidence. A more systematic study of the binding parameters by complete thermodynamic analysis would be required to confirm the operation of the polarity rule in the case of BSA. However, it must be mentioned that in their studies of the fluorescence quenching, Attallah and Lata (1968) have noted the adherence to the polarity rule in the interaction of steroids with BSA.

Westphal and Ashley (1958, 1959, 1962) measured the decrease in absorbance of Δ^4 - 3 - ketosteroids at 249 mμ

resulting from their interaction with protein. The $\Delta\varepsilon$ values obtained were used to compare the binding of various steroids with HSA and β-lactoglobulin (BLG). Since a close agreement was claimed between the binding parameters derived from spectrophotometric and equilibrium dialysis results (Westphal et al, 1958), the $\Delta\varepsilon$ values which were determined at the same protein and steroid concentrations for different steroids were assumed to represent good relative measures of the binding strengths. However, later Ryan and Gibbs (1970a) showed that such an assumption may not always be valid since they found that while the relationship between $-\Delta\varepsilon$ and $\bar{\nu}$ (the ratio of the bound steroid concentration to the total protein concentration) was linear for the binding of testosterone to HSA it showed considerable departure from linearity in the interaction of cortisol and HSA.

Results of Westphal and Ashley showed clearly that the $\Delta\varepsilon$ values for HSA - steroid interaction decreased uniformly with increasing number of polar groups in the steroid. The affinity decreasing effect of a hydroxyl group was found to be greater than that of an oxo group. The introduction of a non-polar alkyl group increased the strength of interaction with HSA. Further, the presence of a double bond in conjugation to the 3-keto group destabilized the HSA-steroid complex. It was concluded that the binding of steroids involves hydrophobic interactions with the protein. The electron-repelling (alkyl) groups which increase the hydrophobicity of the steroid lead to

strengthening of the complex whereas reverse holds true for the electron-attracting groups. Comparison of the $-\Delta\varepsilon$ values for the epimeric steroids revealed that an α-hydroxyl group decreased the strength of interaction more than a β-hydroxyl group. Further, transfer of a methyl group from α- to the β- position resulted in lowering of the binding strength. These findings led the authors to propose that the interaction involves the rear (α-) surface of the steroid molecule.

Attallah and Lata (1968) and Romeu et al (1975) measured the changes in the native fluorescence of BSA produced by steroid binding to the protein. In contrast to the spectrophotometeric method of Westphal and Ashley which required the presence of a Δ^4-3-keto function in the steroid, fluorescence quenching measurements allowed the study of interaction of not only the α, β- unsaturated-3-ketones, but also of various other steroid derivatives in the androstane, progestane and estrane series. In addition to verifying the above mentioned conclusions of Westphal and Ashley these studies revealed the involvement of other structural relationships in the interaction of steroids with serum albumin. From the data they obtained with a number of androgenic steroids Attallah and Lata (1968) suggested that although the interaction involved is largely hydrophobic, some functional groups in the A-ring and at C-17 have a determining influence on binding. Thus, although polar groups are generally known to hinder binding, introduction of

an oxo group at C-17 was found to stimulate the interaction with BSA. Further, while the addition of Δ^1-double bond in the 17-keto-steroids resulted in weakening of the complex, similar changes in the 17β-hydroxy-steroids caused enhancement of interaction.

The fluorescence quenching results of Romeu et al (1975) are in general accord with the polarity rule. However, some data indicate specific influences of steroid structure on the interaction. Thus, the pregnane derivatives exhibited increased binding with the introduction of oxo and hydroxyl groups at C-20 and this was interpreted by the authors as possibly being due to hydrogen bonding of these groups to proton acceptor residues in the albumin. Comparison of other steroids showed that presence of an aromatic function in the ring A increased interaction quite markedly. This led the authors to propose that the binding of estrogens may involve specific interaction of the Π electrons in the ring A with the aromatic residues of the protein. The data also indicated that planarity of the A/B ring system is not important in determining the strength of binding since 5α- and 5β-pregnane-3, 20-diones had almost identical values. However, it must be pointed out that both Attallah and Lata and Romeu et al made an unverified assumption that the fluorescence quenching at a single wavelength employed is linearly related to the binding levels. As pointed out by Steinhardt and Reynolds (1969b) such an assumption may not be actually valid. Thus the findings of these authors

be considered with some caution.

2.e.2. Other serum proteins

CBG of human plasma contains one binding site per molecule which has very high affinity for cortisol (Muldoon and Westphal, 1967). The entropy change accompanying this binding is -17 cal/mole/degree. It was suggested that this high value is indicative of a very strong interaction of the steroid and protein. The binding of steroids to CBG differs in this respect from their binding to plasma albumin which is associated with positive entropy changes. Further, many steroids are known to displace cortisol from CBG-Cortisol Complex and are apparently bound at the same common site (Sandberg et al, 1966). The binding affinities of progesterone and cortisol for human CBG are in agreement with the polarity rule (Westphal, 1967). However, the values in the case of rabbit CBG do not obey this rule. It appears that binding sites in the CBG's of the two species may be fundamentally different.

Like CBG the number of primary steroid sites on AAG is also one and the binding affinity of various steroids to this protein also shows systematic decrease with increasing steroid polarity (Kerkay and Westphal, 1968, 1969). The work of Vermeulen and Verdonck (1968) suggests that the presence of a 17β-hydroxyl group is essential for the binding of steroids to steroid-binding-globulin. Steeno et al (1968) have shown that testosterone and estradiol are bound at common sites on this protein. This is in clear contrast to the specific

binding of estradiol by receptor protein of the uterus (Rochefort and Baulieu, 1969; Westphal, 1971c).

2.e.3. Enzymes

Some similarities as well as some differences are also encountered in the interaction of steroids with plasma proteins and their interaction with some enzymes. Marcus and Talalay (1955) studied the kinetics and specificity of β-hydroxy-steroid dehydrogenase for a number of steroid substrates. Analysis of the data led these authors to propose that the interaction with the enzyme takes place at the rear plane of the steroid molecule. Further, the stability of the complex decreased with increasing number of hydroxyl groups in the steroid molecule (Talalay, 1957). This suggested that the steroid-enzyme complex involves largely hydrophobic linkages.

Studies of the interaction of steroids with glucose-6-phosphate dehydrogenase have shown that an oxo group at C-17 or C-20 is necessary for binding to the enzyme (Rainieri and Levi, 1970). The interaction of the steroid was hindered by the introduction at C-17 of groups larger than an acetyl group. From this these authors concluded that the binding sites must be located in a crevice having the opening of the size of a C_{21} steroid.

Using their spectroscopic method Westphal and Ashley showed that binding affinity to lysozyme, trypsin and chymotrypsin increases with number of hydroxyl groups in the steroid. This relationship named by the authors as

"inverse polarity rule" was suggested as an indication of specific interaction between steroids and the enzyme. Later, however, Ryan (1968) could not demonstrate such a correlation for the same steroids and enzymes as employed by Westphal and Ashley. Difference spectral measurements showed no indication of steroid band perturbations in the 249 mu region. Ryan suggested that $(\Delta O.D)249$ is not a valid measure of the interaction of Δ^4-3-ketosteroid with these enzymes.

2.f. Competition in the binding of steroids to serum albumin

Attempts have been made to determine the structural specificity of binding sites on serum albumin i.e. whether they are the same or different for different steroids. Sandberg et al (1957) studied the binding of ^{14}C-labeled testosterone, progesterone, corticosterone and estrone to HSA in the absence and presence of a saturating concentration of a second unlabeled steroid by equilibrium dialysis. No significant change in the binding was observed in these experiments. Similarly in another investigation (Slaunwhite et al, 1963) these authors found that the binding of testosterone and progesterone to HSA was not altered by the presence of estradiol and estriol. It should be mentioned that none of the steroids mentioned above (except corticosterone) is very water soluble and this may have prevented the attainment of sufficiently high binding levels at which competition among the steroids may be experimentally

demonstrable. Thus Slaunwhite et al found that the addition of carrier estrone and estradiol did not significantly influence the binding of their corresponding ^{14}C - analogues. However, the binding of the highly polar cortisol was reduced by about 65% in the presence of corticosterone and vice versa.

The possibility of competition in the binding of progesterone and deoxycorticosterone to HSA was examined by Westphal and Harding (1971b) by means of equilibrium dialysis. In their competition experiments Slaunwhite et al made binding measurements only at a single steroid concentration. However, since such measurements allow comparisons of only binding affinities (expressed as $\frac{\bar{\nu}}{[S]}$, where $\bar{\nu}$ = level of bound steroid and [S] = free steroid concentration) and provide no estimates of the number of binding sites for the steroid they are not sufficient for the analysis of competitive binding. For a more quantitative assessment of competition in binding Westphal and Harding measured complete binding curves for each of the steroids, progesterone and deoxycorticosterone in the absence and presence of the other. The data were analyzed in terms of competitive binding equations and suggested that progesterone and corticosterone compete for the same sites on HSA.

2.g. Binding of steroid-sulfates to serum albumins

Many studies have revealed that serum albumin is chiefly responsible for the binding of steroid-sulfates in

the plasma. Thus after the injection of labeled steroids to human subjects Sandberg et al (1957) and Antoniades et al (1957) studied the distribution of radioactivity among different plasma protein fractions separated by Cohen's low temperature-ethanol procedure. A considerable portion of the injected radioactivity was found in the albumin fraction. However the application of the alcohol procedure may have affected the binding of these steroids to the other plasma proteins. In order to avoid this difficulty, Oertel et al (1962, 1965) studied the distribution of steroid sulfates among plasma proteins by means of electrophoresis and gel-filtration. After oral administration of dehydroisoandrosterone-7α-^{3}H-sulfate and separation of proteins it was found that the steroid associated mainly with albumin and almost no binding occurred to the other plasma proteins.

Plager (1965) studied the binding of androsterone sulfate, etiocholanolone sulfate and dehydroisoandrosterone sulfate to HSA, human serum and plasma by means of equilibrium dialysis and ultrafiltration. The results indicated that albumin is primarily involved in the binding of all three steroid sulfates in the plasma. Analysis of the data by means of Scatchard plots showed two sets of sites for each steroid sulfate. The binding values for both sets of sites were almost identical for androsterone sulfate and etiocholanolone sulfate. The first set of sites in the case of dehydroisoandrosterone sulfate had lower affinity than the affinity of other two steroid sulfates for the same set.

However, the affinity values for the second set of sites were the same for all three steroid sulfates. From these and competition data Plager concluded that androsterone sulfate and etiocholanolone sulfate are bound at common sites on the HSA molecule. Purdy et al (1961) similarly reported two sets of sites for the binding of estrone sulfate to HSA. The binding affinities were similar to those of androgen sulfates. Displacement studies showed that binding sites for estrone sulfate and androgen sulfate on HSA are the same. It may be pointed out that Puche and Nes (1962) have suggested that binding of steroid sulfates to serum albumin may involve electrostatic interactions with the arginyl groups.

2.h. Relationship between steroid solubility and binding to serum albumin

In section 2.e.1. it was noted that the binding of steroids to serum albumin follows the polarity rule. This rule suggests that the steroid binding sites on albumin are non-polar in nature so that as the number of polar groups in the steroid increases the affinity for water is increased whereas affinity for binding sites is decreased. Scholton et al (1968) have demonstrated a linear relationship between the partition coefficient of a steroid between isooctane and water and its binding affinity for HSA. These findings appear to indicate that binding may involve simple partitioning of the steroid between the aqueous phase and the non-polar regions of the protein. However, if this were true, one would expect the binding process to follow a non-stoichiometric

(partition law) type of behavior. But results of several studies have shown that binding of steroids to albumin can be unequivocably described by the law of mass action, giving rise in most cases to a finite number of sites (table 2.h.1.). Thus there must be definite sites on albumin for the binding of each steroid. As the polarity rule is obeyed, it appears that the steroid binding at these sites must be largely hydrophobic. Since the strength of hydrophobic interactions will be strongly dependent on (i.e. inversely related to) steroid solubility in water, it appears that the latter would play a dominant role in steroid binding.

In most studies of steroid-albumin interaction the steroid concentrations are generally assumed to be equal to the activities. However, Eik-Nes et al (1954) argued that since steroids have very low solubility in water, molar concentration may not be an appropriate choice of standard state for these substances. They preferred to choose steroid solubility as the standard of unit activity to determine the binding energies for the interaction of a number of steroids with BSA. In this operation the crystalline form of the steroid becomes the standard state and the free energy contributions arising due to transfer of the steroid from the aqueous phase to the non-polar binding sites are eliminated. The calculated values of binding energy (designated by the authors as ΔG_1^o values) therefore represent free energies due to non-hydrophobic interactions. Eik-Nes et al found that $-\Delta G_1^o$ values for the steroid-BSA interaction increased with the number of polar groups in the

TABLE 2.h.1.

BINDING PARAMETERS FOR SOME STEROID-PROTEIN INTERACTIONS

Steroid	Protein	n	nk	Reference
TESTOSTERONE	BSA	10.0	$1.9 \times 10^{4} M^{-1}$	Schellman et al, (1954)
TESTOSTERONE	HSA	14.0	$3.7 \times 10^{4} M^{-1}$	Ryan and Gibbs, (1970b)
PROGESTERONE	HSA	2.2	$8.9 \times 10^{4} M^{-1}$	Westphal (1966)
CORTISOL	HSA		$0.34 \times 10^{4} M^{-1}$	Ryan and Gibbs, (1970b)
PROGESTERONE	AAG	0.98	$8.9 \times 10^{5} M^{-1}$	Kerkay and Westphal, (1969)
CORTISOL	CBG	1.0	$5.0 \times 10^{8} M^{-1}$	Seal and Doe, (1966)

n: Number of steroid binding sites.

nk: Binding Affinity.

steroid, and therefore these must be attributed to polar interactions between the steroid and the BSA. In the case of neutral steroids such interactions will be most likely hydrogen-bonding (section 2.1.). The results of Eik-Nes et al showed that the magnitudes of $-\Delta G_1^o$ values were only a fraction of the total binding energies (ΔG_2^o values) determined for the same steroids on the basis of one molar concentration as the concentration of unit activity. Since the later values represent measures of the total binding energies, it seems that the polarity rule observed in the binding of steroids to albumin is due to large contribution of the solubility effect to the total binding energy.

2.i. Relation of steroid structure to aqueous solubility

If steroid solubility exerts a large influence on binding of steroids to albumin, it is important to know what structural features of the steroid determine the magnitude of this property. However, although much is known about the molecular structure and various physical and chemical characteristics of the steroids in the crystalline form, very little information is available on the physical state of these substances in aqueous solutions and on the relation of their structure to solubility in water. Some studies that have been made on this subject will be considered in this section.

The solubilization of testosterone, progesterone and α- estradiol was first studied by Bischoff and coworkers

(Bischoff and Pilhorn, 1948; Bischoff and Stauffer, 1954). These authors showed that when solid testosterone was equilibrated by shaking with water at room temperature, a supersaturated solution was obtained in five hours. On shaking further the steroid concentration in the solution decreased to a lower equilibrium value (steroid solubility). Equilibrium in the case of progesterone required a much longer time compared to that in the case of testosterone. In the case of testosterone at 25^{o} the same solubility value was obtained whether the equilibration was approached from supersaturation or from undersaturation. However, different solubility values were obtained with progesterone under these conditions. The authors suggested that progesterone exists in solution in two different polymorphous forms, one having a higher solubility than the other and that the progesterone solubility depends upon the relative amounts of the two crystalline forms at equilibrium. The pH had no significant effect on the solubilities of the three steroids studied.

Other data reported in the literature on the solubilities of steroids in buffered and un-buffered aqueous solutions indicate that the solubility increases with the number of polar groups in the steroid molecule (Eik-Nes et al, 1954; Slaunwhite et al, 1963). However, low solubilities of estradiol and estrone are not in keeping with the general experience that the aromatic compounds are less hydrophobic than the corresponding non-aromatic substances (Tanford,

1973a). This has been explained as being due to the high lattice energies of the estrogenic steroids (Eik-Nes et al, 1954). Further, although the hydroxy compounds are known to be more polar than their keto-analogues, the solubility of Δ^4-androsten-3, 17-dione has been reported to be significantly higher than that of testosterone (Slaunwhite et al, 1963). By means of model building studies Warner (1965) has pointed out that specific interactions can take place between the steroid and the complementary tetrahedral water lattice which may play a vital role in the biological action and metabolism of steroid hormones.

2.j. Factors affecting steroid binding to serum albumin

As pointed out in a previous section (section 2.e.1.) the values of binding parameters for steroid-albumin interaction may be dependent upon the conditions under which the determinations of such values are made as well as on the source and quality of the albumin preparations employed. For this reason several workers have examined the influence of various factors on steroid binding to albumin. A detailed knowledge of these factors is fundamental to the investigation of any facet of steroid-albumin interactions. Moreover, studies of the factors influencing steroid binding to albumin have provided some fundamental information about the nature of the interactions involved.

2.j.1. Source and purity of albumin

It was mentioned in section 2.e.1. that human albumin in general binds steroids with greater affinity than bovine serum albumin. The reason for this difference is not known with certainty. Schellman et al (1954) studied the binding of testosterone to various preparations of BSA obtained with different degrees of crystallization. These preparations showed varying levels of purity on electrophoresis. The binding data revealed that the preparation of highest electrophoretic purity had the largest binding affinity for the steroid. Westphal (1966) found that HSA defatted by treatment with $CHCl_3$:CH_3OH::3:1 at 4^o had a binding affinity for progesterone which was more than twice that of the untreated HSA. The number of binding sites, however, remained unchanged after this treatment. This led the authors to believe that the lipids removed by solvent might compete for progesterone binding sites on the protein. The nature of these lipids was, however, not examined. It may be recalled (section 2.a.) that crystalline HSA usually contains 2 - 2.5 moles of fatty acids per mole protein. Westphal and Harding (1971a) therefore studied the effect of addition of lauric acid (5 moles per mole protein) on progesterone binding to defatted and untreated HSA. Defatting increased the nk value for progesterone four fold and n value from 2 to 3. Addition of fatty acid to the defatted HSA caused lowering of the nk value to about half and a decrease in the n value back to the n value for the original

untreated protein. Since the n value increased after defatting and decreased after refatting, the mechanism of this effect does not appear to be competitive.

In this context it should be recalled that fatty acids are also known to reduce binding affinity of albumin for many other ligands such as tryptophan, bilirubin, thyroxine and drugs (section 2.b.). Spector et al (1973) have proposed a model to explain the fatty acid effect on the binding of certain drugs to serum albumin. According to this scheme the two primary sites on albumin can bind only fatty acid and not the drug whereas the secondary sites can bind both. Occupancy of primary sites by fatty acid causes conformation change in the protein which transforms the secondary anion sites. Competition then takes place between fatty acid and drug molecules at the modified (secondary) sites.

2.j.2. Effect of temperature

The affinity of association of steroids with albumin decreases with rising temperature (Oyakawa and Levedahl, 1958; Slaunwhite et al, 1963; Ryan, 1973; Romeu et al, 1976). Slaunwhite et al found that, in the case of HSA, the effect is more marked for cortisol than for testosterone and progesterone. Ryan (1973), however, showed that the effect of temperature on the binding-induced inhibition of tryptic hydrolysis of HSA is essentially the same for testosterone, progesterone and dehydroisoandrosterone. In the case of BSA the stimulatory effect of progesterone showed the

greatest temperature sensitivity while the inhibitory effect of testosterone was comparitively less influenced.

Westphal (1971a) has calculated the entropy changes accompanying the formation of testosterone-BSA and progesterone-HSA complexes. These data were calculated from the values of binding affinites published in the literature for these complexes at different temperatures. The entropy changes were found to be highly positive values. Many explanations were offered to account for these entropy changes. Klotz (1950) interpreted positive entropy changes in the binding of methyl orange to serum albumin as due to the displacement of the hydration (immobilized) water molecules from the protein and the ligand. Similarly, in the case of steroid-albumin interaction, to account for the highly positive entropy values it is considered that the structured water molecules are removed from both steroid and protein as a result of interaction (Westphal, 1971a).

An additional explanation was provided by Schellman et al (1954) to explain the higher magnitude of entropy changes associated with testosterone binding to BSA. Their hypothesis was based on the Karush's concept of conformational adaptability (Karush, 1950). Schellman et al suggested that steroid binding may lead to breaking or bridging of the ammonium-phenolic links in the protein. This may decrease the degree of order in the polypeptide chain.

2.j.3. Effect of steroid concentration

Brunkhorst and Hess (1965) measured the binding of cortisol to BSA and HSA over a very wide range of steroid concentration. They found that binding affinity in the case of both proteins decreased with increasing cortisol concentration. Analyses of the data by means of reciprocal plots showed two sets of binding sites for cortisol on BSA as well as HSA. This was attributed to microheterogenity of the serum albumin (Foster, 1960).

2.j.4. Effect of albumin concentration

Most of the binding data for steroid-albumin interaction available in the literature have been obtained by varying steroid concentration at constant concentrations of the protein. However, different workers have employed different concentrations of the protein for the measurement of the binding values. Because of the variations in the reported data many workers have studied the effect of protein concentration on the steroid-albumin binding parameters. (Brunkhorst and Hess, 1965; Attallah and Lata, 1968; Kerkay and Westphal, 1969; Ryan and Hanna, 1971). Brunkhorst and Hess found that Scatchard plots of the data for BSA and HSA obtained at constant cortisol concentration and varying protein concentration were linear parallel curves. It was therefore suggested that microheterogenity of albumin is not

responsible for the observed effect of protein concentration on binding. The authors proposed that this effect can be attributed to the presence of a competitive inhibitor in the protein.

Later, Kerkay and Westphal (1969) performed binding measurements in which the concentrations of both cortisol and HSA (delipidated) were varied simultaneously but the ratio of steroid to protein was held constant over the entire range of protein concentration. A similar effect of protein concentration on cortisol binding was still observed. This indicated that the effect is not due to the presence of any competitive inhibitor in the protein. Further, progesterone binding was not altered with similar changes in protein concentration under the same conditions. No explanation is so far available to explain the effect of protein concentration on cortisol binding to HSA.

2.j.5. Effect of pH and buffer

The affinity constant of the steroid-albumin interaction is markedly affected by alterations in pH. Many studies have shown that the binding of steroid increases with increasing pH (Bischoff and Pilhorn, 1948; Levedahl and Perlmutter, 1956; Sandberg et al, 1957; Romeu et al, 1976). Levedahl and Perlmutter noted a steady increase in binding affinity between testosterone and BSA over the pH range 2.3 - 11. Increase of pH above 11 resulted in a sharp decrease in the affinity constant, which was attributed to denaturation of the protein. Romeu et al (1976) found that

the effect of pH on the binding affinity to BSA is somewhat dependent on the structure of the steroid. Cortisol showed the highest sensitivity compared to the other steroids. These authors observed a gradual increase in the binding energy up to pH 9.5 and a rather sudden lowering above this pH. Since the tyrosyl residues in proteins have a pKa of 9.5, the data was interpreted as indicating the involvement of unionized tyrosyl groups in steroid binding.

Little is known as to the influence of ionic strength on the interaction of steroids with serum albumins. Sandberg et al (1957) observed a significant increase in binding affinity between estrone and HSA when the ionic strength of the buffer was doubled. However, in the equilibrium dialysis study of progesterone-HSA binding, Ganguly and Westphal (1968) observed that the binding affinity increased by more than 100% when NaCl concentration was increased from 0 to 4M. For a similar increase in NaCl concentration the increment in binding affinity in the case of cortisol-HSA interaction was comparitively less significant (Westphal, 1971a).

2.k. Amino acid residues involved in steroid binding to serum albumin

The operation of the polarity rule in the binding of steroids to serum albumin (section 2.e.1.) indicates that the hydrophobic amino acid residues may be present at the

steroid binding sites. By means of difference spectroscopic studies of steroid-albumin interaction, Ryan (1968) has provided strong evidence to support this view. Although the participation of non-polar residues in steroid binding is fairly apparent, direct involvement of specific amino acid groups in the interaction is not yet completely established. Several studies indicate that the tyrosine residues may participate in the interaction. This is suggested by: (a) decrease in binding affinity of albumin after deprotonation and nitration (Romeu et al, 1976) and ketenization (Oyakowa and Levedahl, 1958); (b) binding induced perturbation of tyrosyl spectrum (section 2.1.) and quenching of tyrosyl fluorescence (Attallah and Lata, 1968; Romeu et al, 1976); (c) evidence of complex formation between steroid and L-tyrosine (Abelson et al, 1960) and (d) preferential binding of steroid by a tyrosine-rich peptide fragment of albumin (Pearlman and Fong, 1972).

Many literature reports suggest that tryptophan groups also take part in the steroid-albumin interaction. Thus Romeu et al (1976) have noted decrease in the binding affinity of albumin for steroids after modification of tryptophan residues with 2-nitrophenyl-sulfonyl chloride. Difference spectroscopic studies (section 2.1.) suggest that the tryptophan spectrum is perturbed in the interaction of steroids with albumin. Further it has been observed that steroid induces quenching of L-tryptophan and albumin tryptophyl fluorescence (Attallah and Lata, 1968; Romeu et

al, 1976). Studies of Abelson et al (1960) indicate that interaction takes place between steroid and L-tryptophan. Romeu et al (1976) found that modification of seventeen arginyl residues with glyoxal results in decrease in binding affinity by about 26% which suggests that these residues may also play a role in steroid binding. However, these authors found that modification of -SH groups by iodoacetamide caused no alteration in binding affinity. Thus -SH groups do not seem to perform any important function in the steroid binding to albumin.

It should be pointed out that the modification procedures used in the above mentioned studies are usually not completely specific. Further, the various treatments employed in these procedures may alter the native conformation of the protein which may be responsible for the observed decreases in the binding values. Further, it is also possible that the aromatic perturbations and fluorescence quenching effects seen in the steroid-albumin interactions may arise due to binding induced conformation changes (section 2.m.) in the protein affecting the environment of aromatic residues.

2.1. The use of difference spectroscopy to examine the chromophoric environment of bound steroid

The absorption of light by proteins in the near ultraviolet region is mainly due to tyrosine and tryptophan residues. Small perturbations in the protein spectra are generally observed in the interaction of proteins with small

molecules. These changes can be readily recognized by means of difference spectroscopy, a technique first introduced by Herskovits and Laskowski (1960). Perturbation of both tyrosyl and tryptophyl spectra have been reported in the interaction of proteins. It is generally considered that these perturbations arise either because of direct interaction between the ligand and the aromatic residues or due to change in the environment of these residues brought about by ligand induced alterations in the conformation of the protein.

Spectral perturbation studies performed with aromatic amino acids or their model compounds and with certain tyrosine and tryptophan rich proteins have greatly facilitated the interpretation of difference spectra for the ligand-protein interactions (Bigelow and Geschwind, 1960; Herskovits and Sorensen, 1968; Ananthanaryan and Bigelow, 1969a,b). In these studies red-shifts in the spectra, giving rise to difference extrema in the 280 - 295 mμ region, were observed with a variety of perturbants including some non-polar solvents. Herskovits and Sorensen (1968) showed that the actual positions of these extrema (minima) in proteins are determined by the relative numbers of tyrosine and tryptophan residues perturbed. In general as the tryptophan involvement increases the difference troughs appear at longer wavelength and when tyrosine involvement dominates the minima appear at shorter wavelengths. The magnitudes of these minima represent extent of perturbation of aromatic residues or the strength of

interaction with the perturbant (e.g. the ligand). In tryptophan-rich proteins two minima are generally seen at 281 - 284 mμ and 291 - 294 mμ; however in the case of tyrosine-rich proteins the usual positions of minima are 278 - 281 mμ and 286 - 288 mμ.

Ananthanaryan and Bigelow (1969a,b) have shown by means of spectral perturbation studies on tryptophan model compounds as well as some tryptophan containing proteins that a difference peak at 300 mμ arises due to perturbation of a tryptophan minor band as a result of alterations in the charge environment of this chromophore. These authors found that a blue shift accompanies an increase in the positive charge or decrease in the negative charge in the vicinity of tryptophan.

Zakrzewski and Goch (1968) showed by means of difference spectroscopy that the binding of fatty acid to HSA produces marked red shifts in the spectra of tyrosyl residues with no indication of any perturbation of tryptophyl spectrum. From their difference spectroscopic and other data these authors postulated that tyrosyl red shifts may be brought about by a direct interaction of the fatty acid molecule with the environment of the tyrosyl residues.

Ryan (1968) and Ryan and Gibbs (1970a) applied the method of difference spectroscopy to the investigation of the nature of interaction between steroids and serum albumins. They found that the interaction of steroids such

as dehydroisoandrosterone, lacking a Δ^4-3-keto system, results in a red shift in the aromatic spectra giving rise to difference minima at 282 and 288 - 289 mμ, which the authors attributed to perturbation of tyrosine residues. However, in the interaction of Δ^4-3-ketosteroids with serum albumins two spectral shifts were noted, a blue shift of the steroid band giving rise to a difference peak at 259 mμ and a red shift of the protein spectrum resulting in the appearance of two minima at 286 and 292.5 mμ. The authors proposed that the steroid binding sites in serum albumin are largely hydrophobic in nature and that the blue shift of the steroid band results from transfer of the steroid from the aqueous medium into a hydrophobic environment. Since the solvent perturbation difference spectrum of the steroid in Tris/ethanol pair also showed a difference peak at 259 mμ, it was further suggested that the environment of steroid at the binding site may be as apolar as ethanol. The location of the difference minima at 286 and 292.5 mμ in the interaction of Δ^4-3-ketosteroids with albumin were interpreted as suggesting an involvement of tryptophan and tyrosine residues in the perturbation in a ratio of not less than 0.5 - 1:1 based on the data of Herskovits and Sorensen (1968). A plot of the height of the difference peak at 259 mμ against level of bound steroid ($\bar{\nu}$) for testosterone was found to be linear over the measured binding range. From this plot a value of the molar perturbation of bound steroid (see section 5.b.) was determined. Since this value was

found to be lower than the value of the molar perturbation of the steroid in the Tris/ethanol pair, Ryan and Gibbs concluded that interactions other than hydrophobic may be responsible for lowering this value. It was proposed that the interaction of Δ^4-3-ketosteroids may involve hydrogen bonding of the steroid carbonyl to the aromatic residues of the protein. In this context it may be recalled that the fluorescence quenching studies in section 2.k. have also indicated the involvement of tryptophan in the binding of steroids to BSA.

2.m. Studies of steroid-induced conformation changes in the albumin

Since the conformation changes in the ligands and/or proteins arising in their mutual interaction are often important in the execution and control of their biological functions (section 1.), it is necessary to understand the nature of these changes and the types of mechanisms by which they may be actually produced. However, although the binding induced changes in the ligand have not yet been examined experimentally, those produced in the protein have been investigated by a number of workers. Three types of general mechanisms have been proposed to explain the unfolding in the proteins brought about by binding of the ligands (Steinhardt and Reynolds, 1969a). These are:

(1) Unfolding can occur as a result of repulsion between the electrostatic charges on the bound ligand and on the protein.

(2) Binding of a non-polar ligand may involve replacement of the hydrophobic interactions holding different parts of the polypeptide chain by ligand-segment interactions. This may reduce the stability of protein conformation and thereby lead to unfolding of the polypeptide chain.

(3) A third type of mechanism was proposed by Reynolds et al (1967) according to which the protein molecules in solution exist in two conformational states (native and unfolded) which are in equilibrium. Each conformation contains a set of identical binding sites for the ligand. However, the numbers of binding sites and/or their association constants in the two forms may be different. The ligand arrests (or "stabilizes") the conformation to which it binds and leads to shift of the equilibrium towards that conformation. The position of the equilibrium at any level of bound ligand ($\bar{\nu}$) is thereby determined by the relative numbers of binding sites and their association constants in the two forms as well as the equilibrium constant for the conformational forms. Both stabilization and unfolding can be explained by means of this model. Reynolds et al proposed the following conditions for the two types of transitions:

Stabilization:

$$K \gg J, \quad n \leqslant m$$

$$K \geqslant J, \quad n > m$$

Unfolding:

$$m > n, \quad K \geqslant J$$

$$m \leqslant n, \quad K \ll J$$

where n and m are the numbers of ligand binding sites and J and K their respective association constants for the native and unfolded forms of the protein.

Several types of methods have been employed for the qualitative and quantitative evaluation of conformation changes in the proteins. These include determination of changes in the various properties of the protein such as viscosity, light adsorption, ligand binding behavior susceptibility to enzymatic hydrolysis and others. Some of these approaches have been applied in the investigations of steroid induced conformation changes in albumin.

Thus by means of optical rotation measurements Alfsen (1963) showed for the first time that testosterone binding to albumin results in stabilization (an increase in the helical content) of the protein structure. Although the fluorescence quenching studies of Attallah and Lata (1968) do suggest similar changes in the albumin structure (stabilization) induced by binding of various steroids, CD studies of Tucker and Lata (1975) have not shown any significant effect of testosterone binding on the conformation of BSA.

Ryan (1973) studied the influence of steroids on tryptic hydrolysis of BSA and HSA and suggested that the observed changes in the rates can serve as a useful guide in the investigation of steroid induced conformation changes in the protein. Ryan observed both stimulatory and inhibitory effects depending on the structure of steroid and the nature of protein. In the case of HSA all steroids produced inhibition of tryptic hydrolysis. However in the case of BSA all the neutral and charged derivatives of estrane and androstane caused inhibition while all the pregnane and cholestane derivatives produced stimulation of hydrolysis. Preliminary results showed that the steroids exert no influence on the enzyme itself and the steroid effects result mainly from changes in the protein. Since gel filtration chromatography in the presence of steroid showed no change in the content of dimer or higher polymeric forms of albumin the authors proposed that the inhibitory effects could be due to conformation changes in the protein (stabilization) brought about by steroid binding, although the possibility of steroid induced dimerization in the transition state complex was not excluded. Measurement of steroid binding to HSA under the conditions of tryptic hydrolysis (in the absence of trypsin) and correlation of the binding data to the tryptic hydrolysis results suggested that a maximum of four steroid binding sites on the average may be involved in the inhibition by testosterone and dehydroisoandrosterone. Further, marked qualitative and quantitative differences

in the influence of various steroids on the hydrolysis of the two serum albumins suggested that the nature and degree of conformation changes involved may be dependent not only on their different binding affinities for a given steroid but also on the occupancy of different binding sites.

3. PLAN OF WORK

3.a. Study of specific interactions in the binding of steroids to BSA

Although several workers have carried out detailed studies of the relationship between the structure of steroids and the strength of steroid-albumin interactions using spectrophotometeric and fluorescence quenching techniques, the thermodynamic validity of results obtained by these procedures cannot be fully trusted (section 2.e.1.). Actual thermodynamic values for steroid-albumin interactions have been reported in the literature for some steroids by various workers (Westphal, 1971a; section 2.e.1.) but relatively few comparisons can be made with such data. In some cases the binding parameters reported by different workers for the same steroids show marked variations because of difference in the conditions employed for their measurement and the source and purity of albumin preparations used (section 2.j.) although some variations may also be due to differences in the methods of measurement. Therefore definite conclusions cannot be drawn by comparison of different steroids from these data. These factors have prompted us to undertake a systematic study of steroid-albumin binding on a single preparation of the BSA (lot 30). Seventeen steroids of related chemical structures were carefully selected and their binding to the BSA was determined under the same set of

experimental conditions and protein concentration using only one technique, equilibrium dialysis. This method, although the oldest among all others so far available, is considered to yield valid results for the thermodynamic analysis of ligand-protein binding equilibria.

In order to examine the phenomenon as purely a steroid-protein interaction, the non-protein contaminants were removed from the BSA prior to the actual binding measurements by means of deionization on a column of mixed-bed resin. The deionization procedure is believed to remove most of the inorganic ions (Dintzis, 1952) as well as residual fatty acids (Ryan and Chopra, 1976).

It is evident from literature cited in the review that binding to the serum albumin of highly polar (more soluble) steroids such as steroid sulfates (section 2.g.) and cortisol (section 2.j.3.) involves more than one set of binding sites. However, heterogenity has not yet been reported in the binding of the less polar steroids. In an attempt to examine the possibility of heterogenity we tried to cover a maximum binding range in the case of the less polar steroids by adding these to the protein solutions in small aliquots of ethanol.

Since the review in section 2.h. revealed that steroid solubility plays a significant role in the binding of steroids to serum albumin, we determined these values for all the non-sulfated steroids in Tris buffer at room temperature and applied the corrections for these values to the binding data in the manner suggested by Eik-Nes et al

(1954). It was pointed out in section 2.i. that the structural features of steroids which determine their solubility in aqueous solutions are not known in detail. Therefore solubility values for the various steroids were compared in order to see any structural relationships involved.

The possibility of competition between steroids has not been examined systematically (section 2.f.). Except for the results of Westphal and Harding (1971a) on competition between binding of progesterone and deoxycorticosterone to HSA such studies for other steroids have not been made by means of complete binding analysis. In the present study we determined the binding of ^{3}H-labeled steroids, 5α-dihydrotestosterone, progesterone, estradiol-17β, dehydroisoandrosterone and androsterone to the BSA in the absence and presence of a fixed concentration of testosterone-4-^{14}C. Since in our study we found that fatty acid markedly alters specificity in the binding of steroids to BSA (section 5.d.), we also examined the possibility of competition between progesterone-1,2-^{3}H and testosterone-4-^{14}C, androsterone-1,2-^{3}H and testosterone-4-^{14}C, and androsterone-1,2-^{3}H and dehydroisoandrosterone-4-^{14}C in the presence of 5 moles lauric acid per mole of BSA.

3.b. Changes in the environments of chromophoric groups in the steroid-BSA interaction as examined by difference spectroscopy

Studies of Ryan (1968) and Ryan and Gibbs (1970a) indicate that difference spectroscopy can be fruitfully applied to the investigations of steroid-albumin interactions and, in particular, for the determination of chromophoric environment

of the bound steroid at the binding sites on the protein (section 2.1.). Their results have also indicated that the type of aromatic residues present at the binding sites and the extent of their involvement in the interaction may be dependent upon the structure of the interacting steroid. Therefore in addition to the determination of effects of steroid structure on the binding parameters for the interaction of various steroids with BSA we have also studied the interaction of each of the steroids by means of difference spectroscopy performed under the same set of experimental conditions as employed in the binding measurements by equilibrium dialysis. In the case of some steroids (Δ^4-3 keto-) an attempt was made to correlate the difference spectral results with equilibrium dialysis data in the manner described by Ryan and Gibbs (1970a).

3.c. The effect of steroid-albumin interaction on the aromatic amino acid exposure

Although the involvement of aromatic amino acid groups in the binding of steroids to albumin is suggested by several studies, definitive evidence of a direct interaction between these residues and steroid is still lacking (section 2.k.). Ryan and Gibbs (1970a) suggested that aromatic red-shifts seen in the interaction of steroids with serum albumins may arise due to hydrogen bonding of the steroid with the aromatic groups of the protein (section 2.1.). However, an alternative possibility could not be excluded that these shifts may also result from decrease in exposure of these residues brought about by steroid-induced conformation changes in the protein. In order to test the second possibility we measured the

solvent perturbation difference spectra of BSA in the absence and presence of the steroid. Since this technique measures the magnitude of exposure of aromatic residues (see section 4.b.12.), it allowed the assessment of changes in exposure resulting from steroid binding to the protein.

3.d. Effect of fatty acid on steroid-albumin interaction

Studies in section 2.b. indicate that competition takes place between binding of various non-steroid ligands and fatty acid to serum albumin. Studies of Westphal and Harding (1971a) indicate that fatty acid markedly inhibits binding of progesterone to HSA but their results show that the inhibition is rather non-competitive (section 2.j.1.). In order to clarify the nature of the discrepancy between the two types of ligands (steroid and non-steroid) we re-examined the effect of fatty acid on steroid-albumin interaction with BSA and HSA using two different techniques, viz--equilibrium dialysis and difference spectroscopy.

3.e. Effects of steroids on tryptic hydrolysis of BSA

Although controversy exists as to the influence of testosterone binding on the conformation of BSA (Alfsen, 1963; Tucker and Lata, 1975), studies of Ryan (1973) suggest that effects of steroids on tryptic hydrolysis of serum albumins (BSA and HSA) can be attributed to conformation changes in the protein (section 2.m.). The results obtained by Ryan in the case of HSA have yielded useful information about the nature of binding process and the identity of steroid-binding

sites on the protein. Preliminary data obtained in parallel in the case of BSA led Ryan to propose that steroid binding sites on this protein may be different in nature from those on the HSA. In the present study, effects of some steroids were studied on the tryptic hydrolysis of BSA and the results obtained analyzed on the basis of Ryan's assumption that alterations in the rates are brought about solely by steroid-indiced changes in the albumin.

Thus the main objectives of this study were the following:

(i) To examine the influence of steroid structure on the steroid-BSA binding parameters (obtained by means of equilibrium dialysis) and determine the structural specificity of the binding sites in competition experiments with different steroid pairs.

(ii) To study the spectral perturbations arising in the steroid-BSA interaction and to see how they might correlate with the structure of the bound steroid.

(iii) To study the effect of fatty acids on steroid-BSA interaction using both equilibrium dialysis and difference spectroscopy.

(iv) To investigate the nature of steroid induced conformation changes in the BSA by studying the effects of some steroids on the triptic hydrolysis of the protein.

4. EXPERIMENTAL

4.a. Materials

Non-radioactive steroids were purchased from Steraloids Inc., Mann Research Labs. and Sigma Chemical Company. The purity of these steroids was checked by melting point determinations and by thin layer chromatography (TLC) in two different solvent systems, benzene: acetone: :I:I and choloroform: acetone:: 95:5 for the neutral steroids and ethylmethyl ketone : ethanol : water :: 3:3:I and I-butanol : acetic acid : water :: 3:I:I for steroid sulfates. Absence of other spots on the TLC plate after spraying with concentrated sulfuric acid and charring in the oven was used as the final criterion for steroid purity. Testosterone and progesterone were recrystallized from aqueous ethanol.

Radioactive (^{14}C- or ^{3}H-labeled) steroids were obtained from New England Nuclear Corporation and Amersham/ Searle Corporation as solutions in benzene or ethanol. After diluting the neutral steroids with benzene: distilled ethanol ::9:I and steroid sulfates with ethanol they were stored at 4^{o}. Their radiochemical purity was re-examined and in case of doubt they were purified using the above mentioned solvent systems. The radiochemical purity of all steroids used in our experiments was not less than 95%.

Crystallized bovine and human serum albumins were supplied by Miles Lab. Inc. These were stored in the refrigerator. They were dissolved in deionized water prior to use.

Cellulose dialysis tubings were obtained from Union Carbide, Arthur H. Thomas Company and Fisher Scientific Company. These were cleaned as follows. The tubing was soaked successively for one day in hot distilled water, for one day in 0.1N HNO_3 and for three days in 0.01N HNO_3. This was followed by exhaustive rinsing with distilled and deionized water. In order to check if the membrane was sufficiently clean after washing, a 10 inch piece was soaked in 10 ml of Tris buffer and another piece of the same size in 10 ml of deionized water. After leaving them overnight at room temperature the optical density at 240 mμ of Tris buffer (against fresh Tris buffer solution as blank) and electrical conductivity of deionized water were measured. These values respectively were found to be less than 0.03 and 0.1. If found necessary, the washing procedure was repeated again. The cleaned dialysis tubing was cut into pieces of appropriate size and each tied at one end. They were dried with a stream of nitrogen gas and stored at room temperature until used.

Pairs of tandem cells originally designed by Herskovits and Laskowski (1962) were obtained from Hellma (Canada) Ltd. For cleaning they were soaked in a mixture of nitric acid : sulfuric acid ::1:3 in a beaker and sonicated with ultrasonic cleaner (EMC Corp.). The acid was

removed with a Pasteur pipet and the cells rinsed thoroughly with distilled and deionized water. The cells were further rinsed and filled with distilled ethanol and a base line was recorded in a Cary 15 spectrophotometer in the 240 - 350 mμ range. The cells were considered clean if the alcohol - alcohol base line was closely identical with the air - air base line which was also run at the same time. The alcohol was removed and the cells were dried with a stream of nitrogen gas.

Charcoal (Norit A) was obtained from J. T. Baker Company. It was washed with deionized water in a porcelain funnel containing a filter paper and dried under vacuum at room temperature. Fatty acids came from J. T. Baker Company and Sigma Chemical Company. They were found to be more than 99% pure by gas liquid chromatography.

Deuterium oxide (D_2O), Trizma base and N-bromo-succinimide (NBS) were supplied by Sigma Chemical Company. NBS was recrystallized with deionized water before use.

Hydroxysteroid dehydrogenase and nicotinamide adenine dinucleotide (NAD^+) were obtained from both Worthington Biochemical Corporation and Sigma Chemical Company. Five mg of the enzyme was dissolved in 5 ml of deionized water. A 0.006 M solution of NAD^+ was prepared by dissolving 41 mg of crystalline solid in 10 ml of deionized water. The pH of the solution was adjusted to 7.0 with solid sodium bicarbonate.

Hydrazine hydrate (99 - 100% pure) was obtained from B.D.H. Chemical Company. For preparing a 1.5 M solution, 7.5 ml was dissolved in approximately 25 ml of deionized water in a 100 ml volumetric flask. The flask was chilled in ice and 4.5 ml of 1N H_2SO_4 was added. The volume was adjusted up to the mark with deionized water.

Methylene blue chloride was supplied by National Aniline Division (USA). Methylene blue reagent was made by dissolving 250 mg methylene blue chloride, 50 gm of sodium sulfate and 10 ml of 98% sulfuric acid in one liter of deionized water.

2,5-Diphenyloxazole (PPO) was obtained from Kent Labs., Vancouver, Canada and 1,4-Bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) was supplied by Allan Labs. (Wash.). Scintillation liquid was made by dissolving 4.4 gm of PPO and 74 mg of POPOP in 500 ml toluene and 240 ml of ethanol.

Deionized water was prepared by passing glass distilled water through a column of Puritan ION-X-CHANGER (Model II) obtained from Illinois Water Treatment Company. Deionized water was used for making all aqueous solutions and washing all glassware.

Bio-Rad Laboratories supplied the mixed-bed (AG^R501-X8, 20 - 50 mesh size) and cation exchange (AG^R50W-X4) resins. Nile blue A was received from Allied Chemical Co. Trypsin was obtained from Worthington Biochemicals. Streptomycin sulfate was supplied by Mann Research Labs. Other reagents used were reagent or analytical

grade and were supplied by Fisher Scientific Co. All organic solvents used in this study were distilled prior to use.

4.b. Methods

4.b.1. Deionization of protein

A deionization column was prepared by packing a seven inch layer of mixed-bed resin over a one inch layer of cation exchange resin in the acid form. The column was washed with 125 ml of deionized water. A 10% solution of the protein (BSA or HSA) was layered on the top of the column and allowed to drain slowly (7 - 8 drops per minute) into a beaker. The protein left in the column was eluted with deionized water.

4.b.2. Defatting of protein

The method of Chen (1967) which is the mildest procedure available was employed. A 10% solution of the protein was mixed with an amount of charcoal equal to half the protein content by weight, brought to pH 3.0 and shaken gently in an ice bath with a magnetic stirrer for one hour. Charcoal was removed by repeated centrifugation at 2^{o}. The pH was adjusted to 7.0 and the defatted protein dialyzed against deionized water for 48 hours.

4.b.3. Refatting of protein

Refatting was done by means of two different procedures. In the method of Spector and Hoak (1969) sufficient fatty acid to give 0.1 mm/gm celite was dissolved in a

suitable volume of hexane and poured gently over a thin layer of celite in a large beaker. The hexane was evaporated under nitrogen at less than 50°. The dried particles were thoroughly mixed in a stoppered flask containing some glass beads. To 25 ml of protein solution at pH 7.4 was added 5 gm of fatty acid-coated celite and the mixture was stirred gently for 30 minutes at room temperature. The celite was removed by centrifugation and the solution filtered through a Millipore filter (1.5 μm pore size). The pH of the solution was adjusted to 7.4 with 1N NaOH. In the alcohol procedure a concentrated solution of fatty acid was made in ethanol and 0.1 ml added slowly to 12 ml of a 192 μM solution of protein in Tris buffer at pH 8.0 by means of a microsyringe with constant and gentle mixing with a stirrer. Immediately following addition of fatty acid, the protein solution was diluted with either a saturated solution of steroid in Tris buffer or Tris buffer itself.

4.b.4. Determination of fatty acid content

This was done by a modification of Dole's method described by Chen (1967). To 1 ml sample containing 35 - 70 mg of protein was added 5.0 ml of extraction mixture (isopropanol : isooctane : 1N H_2SO_4 : :40: 10:1) in a 15 ml stoppered glass tube. After vigorous shaking for 30 seconds the tube was allowed to stand for 15 minutes. The isooctane (upper) layer was removed and washed successively with 2.0 ml of 0.1N H_2SO_4 and 5.0 ml of 0.01N H_2SO_4. The

isooctane phase containing fatty acid was titrated with 0.02N NaOH to a light pink end point using 0.002% Nile blue A (in 90% ethanol) as an indicator. During titration nitrogen gas (CO_2-free) was bubbled through the solution to expel carbon dioxide and allow free mixing of the indicator with the isooctane phase. Appropriate blanks and standards were treated in the same manner as the protein samples. This procedure was used for the determination of the fatty acid contents of the defatted, deionized and refatted protein preparations used in the present study. It was found that within the limits of accuracy of this method defatted protein contained essentially no fatty acid and the deionized preparation contained 0.3 mole/mole protein. Polet and Steinhardt (1968), however, report that deionized albumin preparations may retain up to one mole of fatty acid per mole protein.

4.b.5. Extinction coefficient of protein

A one ml sample containing 50 - 80 mg of protein was dried in a weighing bottle to constant weight at 110°. Using the same pipet another aliquot was drawn into a 50 ml volumetric flask and the volume adjusted up to the mark using Tris buffer or deionized water. The optical density of the diluted protein was determined on a Cary 15 spectrophotometer. The extinction value was expressed as $E^{1\%}_{279}$. Since we found that the extinction values in the literature for serum albumins vary, we determined these values for all albumin preparations used in this study and employed them

for calculating protein concentrations in various experiments. These data are collected in table 4.b.1. An examination of these results appears to throw some light on the possible cause of the variability of literature data. As can be seen, the extinction coefficient of HSA decreased after deionization and defatting. The value for BSA was, however, unaffected by either of these treatments. Further, the value for HSA showed no change after refatting. It appears that some UV-adsorbing chromophore such as protein-bound tryptophan or bilirubin (see section 2.b.) is released from HSA by the first two treatments.

4.b.6. Storage of protein

Protein solutions obtained after the above mentioned treatments were diluted to suitable concentrations with deionized water and stored in glass containers in aliquots of 3 ml or more at -20°. The samples were thawed immediately prior to use.

4.b.7. Determination of steroid concentration

Steroid concentrations in the various experiments were determined by one or a combination of more than one of the following procedures.

4.b.7.1. Measurement of UV absorbance

Testosterone, progesterone and cortisol were determined by optical density measurements at their absorption maxima (λ_{max}) and steroid concentration calculated using published values of extinction coefficients. All

TABLE 4.b.1.

VALUES OF THE EXTINCTION COEFFICIENT ($E_{279}^{1\%}$) OF VARIOUS ALBUMIN PREPARATIONS IN TRIS BUFFER pH 8.0, $\mu = 0.1$

Serum Albumin	Lot No.	Treatment	$E_{279}^{1\%}$
Bovine	22	Defatted	6.68 , 6.68
		Defatted + 3 moles Palmitic/mole Protein	6.67
	30	Untreated	6.91
		Deionized	6.91
Human	31	Untreated	6.28
		Defatted	5.59 , 5.66
			5.47[(a)] , 5.64[(a)]
		Defatted + 3 moles Palmitic/mole Protein	5.52
	32	Untreated	6.29
		Deionized	5.56

[(a)] Values determined in deionized water.

spectral values used were those of Ryan (1968) and Ryan and Gibbs (1970a).

4.b.7.2. Enzymatic Assay

The method of Talalay (1960) was used for this assay. To a glass cuvet were added 0.1 ml of a methanolic solution of the steroid, 2.2 ml of pyrophosphate buffer, pH 9.5, 0.5 ml of hydrazine (1.5M) and 0.1 ml of 0.006M NAD^+. The blank cuvet contained all reagents except that 0.1 ml of methanol was added instead of the methanolic steroid solution. The cells were placed in appropriate compartments in the Cary 15 spectrophotometer. The recorder pen was set at the base line and 0.1 ml of hydroxysteroid dehydrogenase added to both cells. After exactly 15 seconds the instrument was turned on again and the reaction was traced as increase in $(O.D.)_{340}$ versus time at a constant chart speed. The final O.D. was measured from the base line. The initial straight line portion of the curve was extrapolated back to zero time. This allowed correction for any possible difference in O.D. between the sample and blank cells at zero time. Steroid concentration was calculated using 6.22×10^3 as the extinction coefficient of NADH formed in the reaction.

Some difficulties were encountered when the steroid assays were performed in Tris buffer (pH 8.0) instead of pyrophosphate buffer. We found that a slow but continuous reaction was taking place in the blank. This reaction appeared to be faster in the sample mixture since when the

assay was carried out with the blank cell in the reference compartment the reaction did not reach completion but continued as a slow increase in O.D. for long periods of time. Although it requires further study, it appears from our observations that the blank reaction may be due to the action of some alcohol dehydrogenase contamination in the hydroxy-steroid dehydrogenase used in this assay and further that such a reaction may be stimulated by the presence of the steroid. We further found that the blank reaction could be completely prevented by addition to the incubation mixture of BSA or HSA to a final concentration of 32 μM. For this reason assays in Tris buffer were performed in the presence of deionized BSA.

For the determination of very low concentrations of steroid, as in the equilibrium dialysis, measurements of the fluorescence intensity rather than optical density were made in the Aminco-Bowman spectrofluorimeter after completion of the enzymatic reaction. The activating and fluorescence wavelengths were 340 mμ and 460 mμ respectively. A linear relationship was observed between the relative fluorescence intensity and steroid concentration in the 0 - 10 μM range (fig. 4.b.1.). In order to calculate the actual steroid concentrations (free and bound) testosterone standards were run along with the equilibrium dialysis samples. An agreement was found between the binding curves determined by radioactive and enzymatic (fluorescence) analyses of the steroid. This is illustrated in fig. 4.b.2.

FIGURE 4.b.1.

STANDARD CURVE FOR TESTOSTERONE OBTAINED BY HYDROXY-STEROID DEHYDROGENASE (FLUORESCENCE) ASSAY IN TRIS BUFFER, pH 8.0 AT 32 µM PROTEIN CONCENTRATION

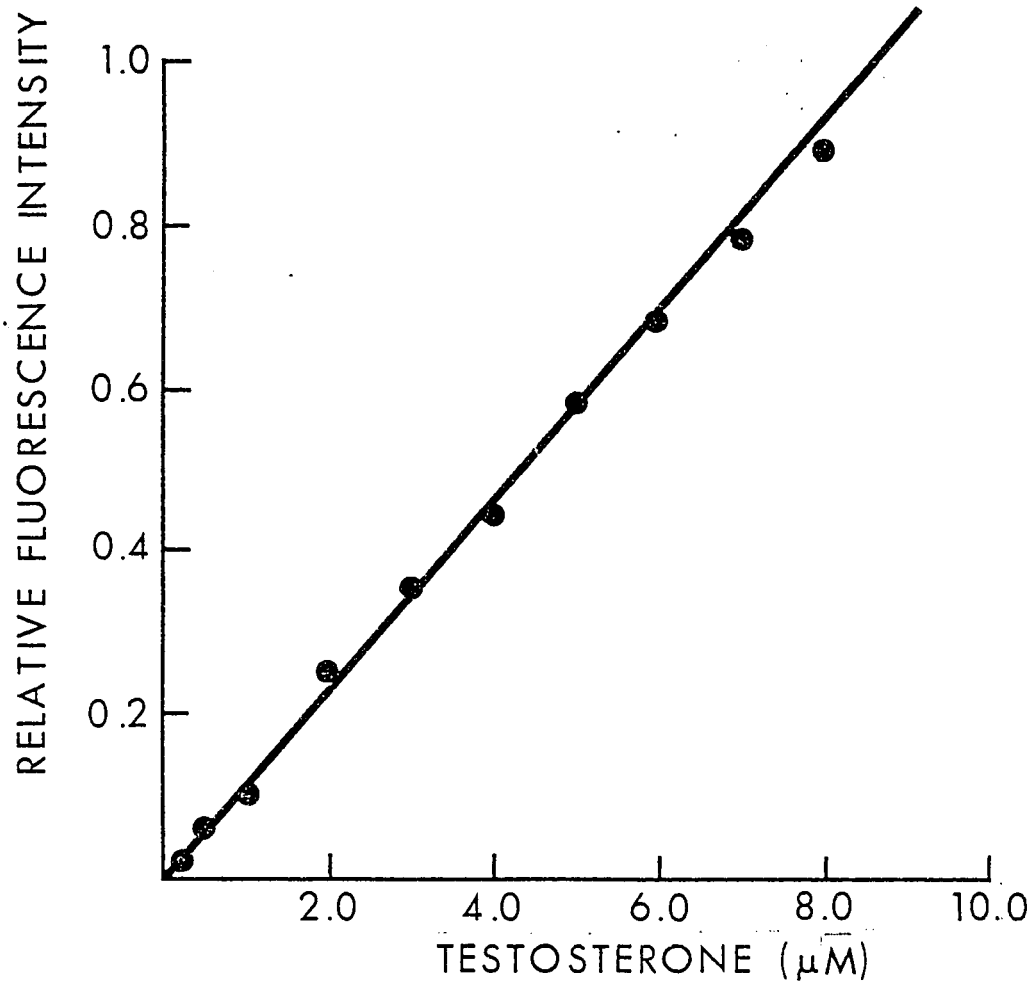
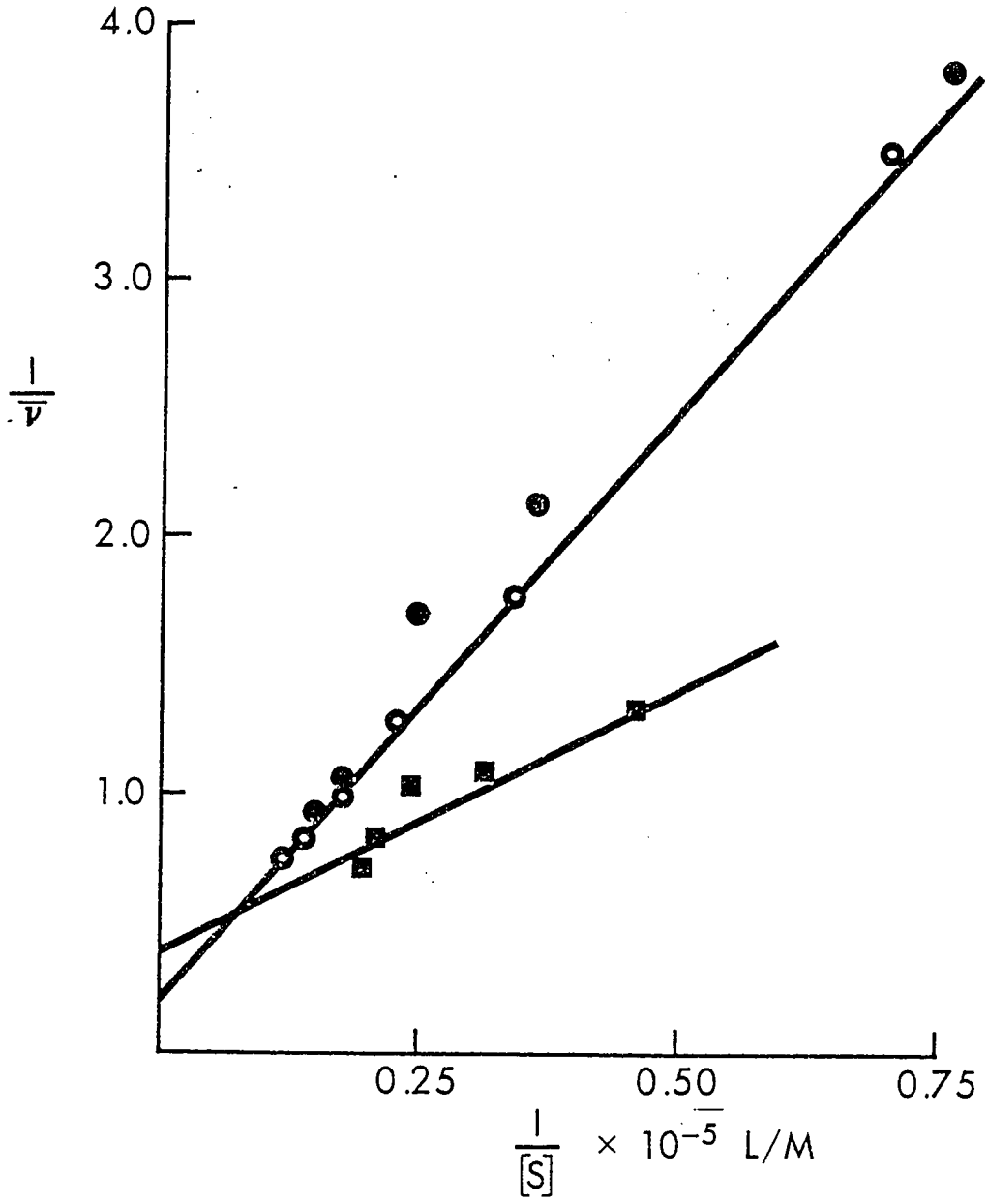

RELATIVE FLUORESCENCE INTENSITY
1.0
0.8
0.6
0.4
0.2
2.0
4.0
6.0
8.0
10.0
TESTOSTERONE (μM)

FIGURE 4.b.2.

VALIDITY OF ENZYMATIC (FLUORESCENCE) ASSAY OF STEROIDS IN THE DETERMINATION OF STEROID-ALBUMIN BINDING PARAMETERS BY EQUILIBRIUM DIALYSIS

Data presented in the form of reciprocal plots. ⊗, testosterone by fluorimetric (enzymatic) assay; ○, testosterone by radioactive determination; ▣, 5β-dihydrotestosterone by enzymatic assay. Binding of the steroids was determined with deionized BSA (lot 30) in Tris buffer, pH 8.0, I = 0.1. Enzymatic assays were performed under the conditions as described in fig. 4.b.1. Other procedure details are described in the text.


4.0
3.0
2.0
1.0
$\frac{1}{v}$
0.25
0.50
0.75
$\frac{1}{[S]}$ × 10^{-5} L/M

4.b.7.3. Colorimeteric determination of steroid-sulfates

Since androsterone sulfate was not available in the radioactive form its concentration was determined in the equilibrium dialysis experiment by means of the assay method described by Pasquilini (1967). A 1.0 ml sample of the steroid sulfate was mixed with 1.0 ml of methylene blue reagent and 7.0 ml of sodium sulfate (50 gm of sodium sulfate and 10 ml of 90% sulfuric acid dissolved in one liter of deionized water). The steroid sulfate-methylene blue complex was extracted in 5.0 ml of distilled chloroform and the optical density of the organic phase was measured at 650 mμ. A standard curve prepared for testosterone sulfate using this method is shown in fig. 4.b.3.

4.b.7.4. Concentration by weight

In some experiments solutions of steroids were prepared by dissolving a weighed amount of steroid in distilled ethanol. The steroid concentration in these solutions was either calculated on a weight basis or verified by optical density measurement or enzymatic assays.

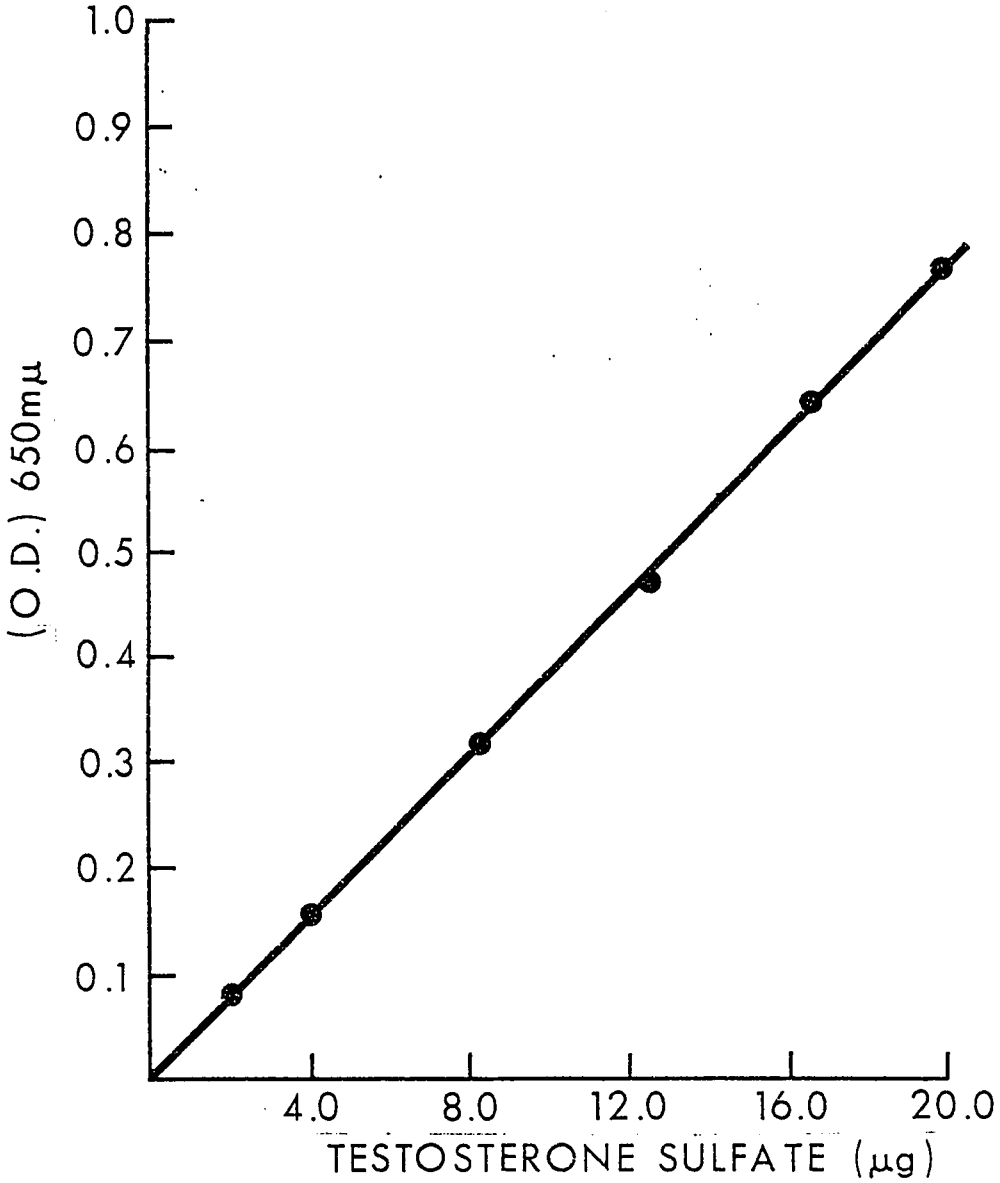
4.b.7.5. Radioactive analysis

Radioactive (^{14}C- or ^{3}H-) samples (0.5 ml) were added to glass vials containing 15 ml scintillation liquid and counted in a Mark II Nuclear Chicago Scintillation Counter to a standard error of 1% and at an efficiency of 75% in the case of ^{14}C- and 30% in the case of ^{3}H-steroids. The data were obtained in a punched tape format and the

FIGURE 4.b.3.

STANDARD CURVE FOR TESTOSTERONE SULFATE OBTAINED BY THE METHOD OF METHYLENE BLUE COMPLEX FORMATION

A stock solution of testosterone sulfate was diluted to different concentrations with Tris buffer. Each of the diluted solutions also contained streptomycin sulfate to a final concentration of 10 µg/ml. Other procedure details are the same as described in the text.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
(O.D.) 650mμ
4.0
8.0
12.0
16.0
20.0
TESTOSTERONE SULFATE (μg)

calculations were done using a Hewlett-Packard Model 9280A Calculator with programs written for quench correction and for calculations of steroid solubility and free and bound steroid in the single and double isotope equilibrium dialyses.

4.b.8. Steroid solubility

A thin layer of the steroid sufficient to provide an excess was deposited in an Erlenmeyer flask by evaporating a concentrated solution of the steroid in ethanol or methanol under nitrogen. An appropriate volume of Tris buffer was added and the flask rotated gently on a rotating machine (Kraft Corp.) for 72 hours. The solution was centrifuged or filtered through a medium porosity sintered glass funnel. Steroid concentration was determined by means of one of the methods described above.

4.b.9. Determination of bound steroid by equilibrium dialysis

This was performed according to the procedure described by Ryan and Gibbs (1970a). In the case of less soluble steroids, higher binding levels were reached (section 3.a.) by addition of a concentrated solution of steroid (radioactive) in ethanol to the protein. The precipitated steroid was removed by centrifugation. A 4-5 ml aliquot of protein solution (32 µM or 91 µM) or protein containing steroid was added to cleaned dialysis bags (20"/32"). The bag was tied at the other end after adding two glass beads to ensure proper mixing and was placed in a wide mouth (25 ml) Erlenmeyer

flask containing a solution (12 - 15 ml) of steroid of known specific (radioactive) activity or concentration. Steroid concentration was varied from one flask to another; the protein concentration was the same in all bags. Six sets of duplicate flasks (one set for each steroid concentration) were run in each experiment. Streptomycin-sulfate was added to each flask to prevent bacterial growth; the concentration of the antibiotic in the final dialysis system was 10 µg/ml. In order to duplicate conditions of the pH - stat some binding experiments were made at 91 µM protein concentration in 0.1M NaCl containing 5% Tris buffer. Equilibrium was achieved by placing the flasks in a shaking water bath at 25^{o} for 48 hours. At the end of equilibration, triplicate samples were removed from the external and internal phases for the determination of steroid concentration by the above methods. In order to check the attainment of equilibration, duplicate flasks ("control") containing the supporting medium (Tris buffer or NaCl) instead of protein in the bags were run simultaneously in each experiment. Steroid analysis in the "control" flasks revealed that equilibration was complete in the 48 hour time period employed. The bound steroid was calculated as the difference between internal (total) and external (free) steroid concentrations.

4.b.10. Study of competition

These experiments were performed by measuring binding to BSA of one steroid (^{3}H-labeled) by equilibrium dialysis in the absence and presence of a second steroid (^{14}C-labeled). Thus, 12 ml of a saturated solution of

^{14}C-steroid was placed outside the bag. The protein solution saturated with tritiated steroid was prepared as described in the last section and diluted with protein solution of the same concentration. The steroid concentration was thus varied and 5 ml of the steroid-protein mixture was added to the bags. The ethanol concentration in the final dialysis system was kept low (0.09%). Samples from outside and inside bags were counted in triplicate using a double isotope counting mode and calculations done as already described (section 4.b.7.5.).

4.b.11. Difference spectroscopy

These studies were carried out as described by Ryan (1968) on a double beam Cary 15 spectrophotometer. All difference spectra were obtained at a fixed protein concentration of 32 μM. To one ml of a 192 μM protein solution was added 5.0 ml of either Tris buffer or a solution of steroid in Tris buffer. To a one ml aliquot of Tris buffer was added 5 ml of steroid solution. All additions were made using the same pipets repeatedly, the order of additions being such that buffer was pipeted out first and protein or steroid solution was transferred after rinsing the pipet into a large volume of that solution. This facilitated the avoidance of errors which might arise in the use of different pipets of the same nominal capacity for different solutions.

Two types of double chamber cells were used for recording the difference spectra. The cylindrical cells

had a path length of 1 cm per compartment and were used for measuring difference spectra at low steroid concentrations. In order to measure difference spectra at high steroid concentrations and at the same time keep the total absorbance below the recommended upper limit of 2.0 (Herskovits and Laskowski, 1962) a second pair of rectangular tandem cells was used which had a shorter path length of 0.438 cm per chamber. In the case of all difference spectra, protein solutions were placed in the front (facing radiation beam) compartments and the non-protein solutions in the rear compartments. The sample cell contained steroid and protein in separate chambers and the reference cell contained steroid-protein mixture in one and buffer in the other compartment. Difference spectra were recorded in the 240 - 350 mμ range at a dynode setting of 2.0 on 0.1 and 1.0 absorbance scales. For each steroid, the records were obtained at more than one steroid concentration. Before measuring each difference spectrum, an air - air base line (with no cells) was linearized using the spectrophotometer "multipots." Difference spectra were expressed as ΔO.D. versus wave length.

Solvent difference spectra for steroids in the alcohol/Tris pairs were measured in the manner described by Ryan and Gibbs (1970a). A concentrated solution of radioactive steroid in ethanol was diluted with Tris buffer and with ethanol using the same pipet and volumetric flask and the concentrations of steroid in the diluted solutions

verified by radioactive measurements. Records were made by placing ethanolic solution of steroid in the reference (tandem) cell and Tris buffer solution of steroid in the sample cell. Compensating blanks (ethanol and Tris) were put in the appropriate cell compartments.

4.b.12. Solvent perturbation difference spectroscopy

This technique was developed by Herskovits and Laskowski (1962) for the determination of the degree of exposure of chromophoric groups in proteins. It is based on the principle that alterations in the solvent environment of such exposed groups results in perturbation of their absorption spectra. The groups buried in the interior folds of the protein do not come into free contact with the solvent and therefore their spectra are not affected. The changes produced by the solvent are measured in the form of a difference spectrum. Many types of perturbants which have been employed do not significantly alter protein conformation when added to aqueous solutions at low concentrations (10 - 20%). These include sucrose, glycerol, ethylene glycol and D_2O (90%). Using the method of solvent perturbation Sogami and Ogura (1973) were able to demonstrate a two-step opening of the crevices to large perturbants in BSA with changes in pH in the acid region. This work illustrates the potential of this technique in the recognition of conformation changes in the proteins.

Three kinds of spectral perturbants namely 90% D_2O, 20% ethylene glycol and 20% glycerol were employed in the

present study to observe changes in the exposure of aromatic chromophores produced by steroid binding to the BSA. To 5.0 ml of Tris buffer, pH 8.0 prepared in deuterium oxide was added 0.5 ml of either 352 μM deionized BSA or the same protein solution containing steroid. The final protein concentration in each of these solutions was 32 μM and D_2O concentration 90% (V/V). Similar solutions were made using Tris buffer in ordinary (deionized) water. Again, as described in the previous section, single pipets were used for the volumetric transfers. Difference spectra were obtained by placing protein solution containing perturbant (D_2O) in the reference cell and those lacking perturbant in the sample cell. The solutions for the other two perturbants were prepared as follows. To a 10 ml volumetric flask was added 5.0 ml of 40% perturbant solution and 1.0 ml of 320 μM deionized BSA and the volume was adjusted with Tris buffer. The flask was rinsed several times with deionized water and dried under a stream of nitrogen. Other solutions were prepared using this same flask after washing and drying it each time. Steroid solutions were made in Tris buffer and added in 1.0 ml or larger aliquots. Records were made using cylindrical cells with protein and perturbant in separate chambers in the reference beam and a mixture of the two and buffer in the sample beam.

4.b.13. Effect of steroid on the rate of tryptic hydrolysis of albumin

The trypsin catalyzed hydrolysis of BSA was followed in the absence and presence of various concentrations of each steroid by means of a pH - stat assembly described by Ryan (1973). All measurements were made at 25^{o} and a fixed protein concentration of 91 µM. The pH during the reaction was maintained constant at 8.5. The reaction was followed as the amount of 0.02N NaOH consumed versus time on a Sergeant multirange chart recorder. An 0.5 ml aliquot of 728 µM deionized BSA was added to 2.0 ml of a 0.1 M NaCl solution in a titration vessel surrounded by a thermostated water jacket. The pH was adjusted to 8.5 by adding 0.02N NaOH. 50 µl of either distilled ethanol or a solution of steroid in ethanol were added and the pH adjusted again. The final volume was made up to 3.6 ml with 0.1M NaCl. The reaction was initiated by the addition of 1.6 mg of trypsin dissolved in 0.4 ml of 10^{-3}N HCl. The volume of NaOH required to neutralize HCl was determined by extrapolation of the initial linear portion of the curve to zero time. The apparent enzyme activity was expressed in terms of µls of alkali consumed in the first ten minutes of the reaction. Experience with this assay procedure (Ryan 1973) showed that different values of activities are obtained for the same protein and enzyme concentrations at different times (day-to-day). Therefore for the calculation of stimulatory or inhibitory effects (as %) assays were always run along with a duplicate (matching) control containing no steroid.

4.b.14. N - bromosuccinimide (NBS) oxidation of tryptophyl residues

This treatment was performed by the method described by Spande and Witkop (1967). For the determination of percentage exposure of tryptophan in the native BSA, titration was performed with NBS as follows: A 2.5 ml aliquot of 32 µM BSA solution in 0.05M acetate buffer, pH 4.0 was placed in a UV cuvet. The blank cell contained 2.5 ml of buffer. A small magnetic bar was placed in each cell. A 10 mM solution of NBS was prepared by dissolving freshly crystallized reagent in deionized water. The NBS solution was added in 10 µl aliquots to each cell, the solution mixed on magnetic stirrer and decrease in optical density of the protein solution recorded at 280 mµ in a Cary 15 spectrophotometer. The titration was continued until no further decrease in O.D. was observed. The total decrease in optical density was determined and corrected for the dilution due to reagent volume. The titration was also performed in 8M urea to determine the number of tryptophyl groups in the unfolded protein. The number, N, of tryptophan residues/mole protein was calculated using the expression

$$N = \frac{(\Delta O.D.)_{280} \times 1.31 \times M.W.}{C \times 5500}$$

where $(\Delta O.D.)_{280}$ = corrected optical density decrease at 280 mµ; 1.31 is the factor which corrects for the optical density of oxidation product, C = protein concentration in mg/ml,

5500 = molar extinction coefficient of tryptophan at 280 mμ.

For the study of the effect of NBS oxidation on the binding of steroid to BSA the treatments (5 moles/mole protein or 10 moles/mole protein) were performed as follows. The protein was dissolved in 0.05M acetate buffer, pH 4.0. A suitable volume of a concentrated solution of NBS was added drop-wise with constant shaking on a magnetic stirrer. Deionized water was added to the control protein solution. The protein solution containing NBS was left at room temperature for 30 minutes for the oxidation of tryptophyl residues to take place. Both control and NBS-treated proteins were dialyzed against deionized water for two days in the cold room. After dialysis the optical densities were determined at 280 mμ. The number of exposed tryptophyl groups oxidized was calculated from the optical density difference at 280 mμ.

5. RESULTS AND DISCUSSION

5.a. Relationship between structure, dipole moment, aqueous solubility and steroid-BSA binding behavior for various steroids

5.a.1. Results

Table 5.a.1. includes values of the dipole moments, Tris buffer solubilities and binding parameters for the interaction of a number of steroids with 32 μM deionized BSA (lot 30). The binding data for all steroids were determined by equilibrium dialysis (section 4.b.9.) performed under the same set of experimental conditions, i.e. in Tris buffer, pH 8.0, I = 0.1 at 25°. The solubility values were determined by shaking solid steroid gently in Tris buffer, pH 8.0, I = 0.1 at room temperature (section 4.b.8.). The values of dipole moments were derived from the literature sources (McLellan, 1963; Neudert and Ropke, 1965). For the calculation of binding parameters, nk and n, the equilibrium dialysis data were graphed in the form of double reciprocal plots (e.g. fig. 4.b.2.). These plots (not all shown) were found to be almost linear for all the steroids studied. The values of n and nk were obtained respectively from the slopes ($\frac{1}{nk}$) and the y-axis intercepts ($\frac{1}{n}$) of these curves.

In order to cover the maximum binding range (see section 3.a.) some steroids were added to the protein as ethanolic solutions; the concentration of ethanol in the

TABLE 5.a.1.

RELATIONSHIP BETWEEN DIPOLE MOMENTS, TRIS BUFFER SOLUBILITIES AND STEROID-BSA BINDING PARAMETERS[a] FOR VARIOUS STEROIDS

No.	Name	Dipole Moment[b] $\times 10^{-17}$ e.s.u.	Solubility in Tris (μM)	$nk \times 10^{-4}$ (M^{-1})	n	$\bar{\nu}$ max[c]
(A)	C_{19} STEROIDS					
I	Δ^4-ANDROSTEN-3, 17-DIONE	3.35	246.3	0.88	13	1.33
II	5β-DIHYDROTESTOSTERONE	2.96	105.6	4.52	3.0	1.4
III	TESTOSTERONE	4.35	91.6	2.13	8.0	1.93
IV	DEHYDROISOANDROSTERONE	2.54	89.2	5.27	10.8	1.4
V	EPIANDROSTERONE	2.9	56.5	4.09	1.2	0.82
VI	EPITESTOSTERONE	5.15	51.0	1.58	5.0	
VII	ANDROSTERONE	3.73	41.6	3.74		0.073
VIII	6-DEHYDROTESTOSTERONE		30.7	2.62	20	0.33
IX	5α-DIHYDROTESTOSTERONE	2.92	20.5	4.43	5.5	1.24
X	5α-ANDROSTAN-3α, 17β-DIOL	2.28	10.3	19.32	5.0	1.93
XI	ANDROSTERONE SULFATE	-	-	11.0	7.0	
XII	DEHYDROISOANDROSTERONE SULFATE	-	-	11.8	12.0	5.08
(B)	C_{21} STEROIDS					
XIII	CORTISOL	-	835	0.15	17.5	1.21
XIV	PROGESTERONE	2.72	31	3.83	7.0	
(C)	C_{18} STEROIDS					
XV	ESTRADIOL-17β	2.33	8.8	7.85		2.67
XVI	ESTRONE	3.07	9.1	2.97	4.0	
XVII	ESTRONE SULFATE			20.0	8.0	

TABLE 5.a.1.

(Continued)

(a) nk and n. Binding was measured by means of equilibrium dialysis with 32 μM deionized BSA (lot 30) in Tris buffer, pH 8.0, I = 0.1 at 25° and values of nk and n determined from reciprocal plots (e.g. see fig. 4.b.1.) of the binding data (see text for details).

(b) Values taken from the literature (McLellan, 1963; Kuksis, 1967).

(c) Maximum level of bound steroid.

final equilibrium dialysis system in these experiments was 0.138%. It may be noted that such low concentrations of ethanol would not have any significant influence on the steroid-BSA binding parameters in comparison to the effect of higher concentrations of ethanol (e.g. 1.25%) observed by Ryan et al (1977) on testosterone binding to the HSA. The values of maximum binding levels ($\bar{\nu}$ max) reached for these steroids in the equilibrium dialysis are also listed in table 5.a.1. It may be mentioned that although the $\bar{\nu}$ max values given are the maximum obtainable values in Tris buffer, comparison of these with the corresponding n values indicates that they still represent values in the low binding range. It should be noted that, although in the (low) binding range measured the reciprocal plots for the steroids were linear, the n values obtained are still subject to the usual uncertainties of extrapolation (see Schellman et al, 1954). For this reason only the nk values were considered and no attempt was made to derive the k values from them.

The steroids in table 5.a.1. are arranged in the descending order of their solubilities in Tris buffer in three different sections as C_{19}, C_{21}, and C_{18} steroids. All of the C_{19} steroids contain two oxygen atoms, but the steroids differ with regard to whether they are in their oxidized (oxo) or reduced (hydroxyl) forms. Some steroids also differ with respect to presence or absence of double bonds as well as their locations in the molecule.

5.a.1.1. Structure and solubility

As can be seen, the solubility values show a marked dependence on the structure of steroid. Thus there is about a 27 fold difference between the solubilities of cortisol (XIII) and progesterone (XIV). This difference can be attributed to a greater number of polar groups in cortisol than in progesterone. However, although all C_{19} steroids contain the same number of oxygen containing groups, large variation is seen in the solubility values of these, the most soluble Δ^4-androsten-3, 17-dione (I) having about 25 times the solubility of the least soluble 5α-androstan-3α, 17β-diol (X). It is further seen in the case of the two C_{18} steroids estradiol-17β (XV) and estrone (XVI) that although they differ in the type of polar group at C-17, their solubilities are very close.

It is evident in the case of C_{19} steroids that the structural features of the molecule other than the number of polar groups are important in determining the solubility in water. It is therefore of interest to compare the values of solubility and dipole moments.
Thus the 3-ketosteroid, 5α-dihydrotestosterone (IX) has twice the solubility of the 3-hydroxy compound, 5α-androstan-3α, 17β-diol (X). The dipole moment of the former steroid is also higher than that of the latter, by about 28%. However, the solubility of the 17-ketosteroid, androsterone (VII) is about 4 times the solubility of the 17-hydroxysteroid, 5α-androstan-3α, 17β-diol (X). The increase in dipole moment

accompanying oxidation is also correspondingly higher, i.e., about 63%. Further while the 17-ketosteroid, Δ^4-androsten-3, 17-dione (I) has a solubility which is 2.7 times that of the 17-hydroxy compound, testosterone (III), the dipole moments of these two steroids show a contradictory trend, the value of the reduced compound being about 30% higher than that of the oxidized steroid. These results indicate that no direct correlation exists between the aqueous solubility of these steroids and their dipole moments. This is perhaps so because the water molecules interact with the individual group dipoles and not with the resultant vector of these dipoles in the steroid molecule. A similar situation holds in the case of disubstituted benzenes (Hildebrand and Scott, 1964). While the ortho, meta and para isomers have widely differing dipole moments their solubilities (in chloroform) were found to be similar. It was suggested by these authors that since the solvent molecules are small they interact almost identically with the two chlorogroups in the three different isomers.

The introduction of double bonds shows a very large influence on the solubility of C_{19} steroids. Thus dehydroisoandrosterone (IV) has a significantly higher solubility compared to epiandrosterone (V), although the dipole moment of the former steroid is lower than that of the latter by about 12%. Introduction of Δ^{4-5} double bond into 5α-dihydrotestosterone (IX) giving rise to testosterone (III) increases solubility by a factor of 4.5 and the dipole

moment is also increased markedly. However, although the introduction of Δ^{4-5} double bond in 5β-dihydrotestosterone (II) decreases solubility only slightly, the dipole moment is increased quite appreciably (by about 47%). It may be noted that the observed increases in solubility on introduction of the double bond are in keeping with known effects of unsaturation (Tanford, 1973a). However, it can be further seen that the introduction of Δ^{6-7} double bond in testosterone (III), giving rise to 6-dehydrotestosterone (VIII), causes marked reduction in the solubility value. It therefore seems that the effect on solubility is particularly dependent on the position of double bond and further that its effect on dipole moment is again not consistently related to the solubility effect.

Examination of the values in table 5.a.1. reveals that although the two C-5 isomers, 5α-dihydrotestosterone (IX) and 5β-dihydrotestosterone (II) show a five fold difference in solubility their dipole moments are almost identical. The difference in solubilities of the two isomers is in keeping with the observation that in paper and gas chromatographic systems the 5α-compound favors the non-polar phase (Kuksis, 1967). The solubility of the 3β(OH) isomer, epiandrosterone (V) is higher than the corresponding 3α(OH) isomer, androsterone (VII) by about 36%, although the dipole moment of the former is lower by about 22% than the latter compound. The 17β(OH) steroid, testosterone (III) has about twice the solubility of the 17α(OH) isomer, epitestosterone

(VI), and again values of dipole moments are contradictory. The very low solubilities of estrone (XVI) and estradiol-17β (XV) in relation to epiandrosterone (V) and 5α-androstan-3α, 17β-diol (X) respectively are not consistent with the general belief that aromatic compounds are less hydrophobic than the corresponding aliphatic analogues (Tanford, 1973a). These observations seem to further point out that structural factors in addition to those responsible for dipole moments of steroids are important in determining their solubility in water.

It may be mentioned that most of the literature data on solubility of steroids has been obtained either at 4 - 5^{o} or 37^{o} (Eik-Nes et al, 1954; Bischoff and Stauffer, 1954; Sandberg et al, 1957; Slaunwhite et al, 1963), and are not suitable for comparison with our values at room temperature. However, our values for Δ^{4}-androsten-3, 17-dione, testosterone, androsterone and dehydroisoandrosterone are related in the same manner as the solubility values obtained for these steroids by Eik-Nes et al (1954) at 37^{o}.

5.a.1.2. Structure and binding

Comparison of the nk values of steroids in table 5.a.1. suggests that steroid-BSA interaction is significantly increased after reduction of keto groups at C-3 (steroids IX versus X) and C-17 (steroids VII versus X and I versus III or VI) positions and following the introduction of Δ^{5-6} and Δ^{6-7} double bonds into the steroid (steroids V versus IV and III versus VIII). It may be noted that

fluorescence quenching studies have also indicated enhancement of steroid-BSA interaction with reduction of C-17 keto group (Attallah and Lata, 1968) and introduction of Δ^{5-6} and Δ^{6-7} double bonds (Romeu et al, 1975). However, our data indicate that nk value is markedly decreased with insertion of a Δ^{4-5} double bond(steroids II or IX versus III) and the same has also been found by Attallah and Lata (1968). It is further evident from table 5.a.1. that inversion of a hydroxyl group from β- to the α- configuration at either C-3 (steroids V versus VII) or C-17 (steroids III versus VI) lowers binding affinity. This is in keeping with the spectrophotometeric observations of Westphal and Ashley (1959) that in the case of steroid-HSA interaction the affinity decreasing effect of an α-hydroxyl group is greater than a β-hydroxyl group and supports the general belief (section 2.e.1.) that the interaction involves hydrophobic bonding at the rear (α-) plane of the steroid. Our nk values for the 5α-dihydrotestosterone (IX) and 5β-dihydrotestosterone (II) are almost identical which seems to suggest that conformation of the steroid ring has no influence on these values. Romeu et al (1975) have similarly reported identical values for the interaction of 5α- and 5β-pregnane-3, 20-diones with BSA (section 2.e.1.).

The data in table 5.a.1. suggest that introduction of a sulfate group into the steroid molecule has a very strong influence on the binding affinity to BSA. Thus the binding affinities of dehydroisoandrosterone sulfate (XII),

androsterone sulfate (XI) and estrone sulfate (XVII) are respectively 2.2, 2.9 and 6.7 times the binding affinities of their corresponding non-sulfated analogues (IV, VII and XVI). It is evident that the affinity increasing effect of sulfate group is more marked on the binding affinity of estrone than on the binding affinities of androsterone (VII) and dehydroisoandrosterone (IV). However, this effect is less marked on the binding of dehydroisoandrosterone compared to androsterone. Our data indicate nearly the same nk values for androsterone sulfate (XI) and dehydroisoandrosterone sulfate (XI) and in this respect our results agree with those of Puche and Nes (1962) who also found almost the same values for dehydroisoandrosterone sulfate and pregnenolonesulfate which are structurally very different.

It may be noted that the relative order of our nk values androsterone, dehydroisoandrosterone, Δ^4-androsten-3, 17-dione, testosterone and cortisol agrees with the one-point data (r/[S]) of Eik-Nes et al (1954) determined by the solubility method. The value for estradiol-17β is, however, much lower and is closer to that of Pearlman et al (1969). However, our values in general show disagreement with the fluorescence quenching data of Attallah and Lata (1968) and those of Romeu et al (1975). Thus the "k_f" values obtained by the fluorescence method for testosterone of 9.7×10^4 ($n.k_f = 58.2 \times 10^4$) and 12.1×10^4 respectively by Attallah and Lata and Romeu et al are much higher than our value, although the n value of 6 obtained by the former

authors is of the same magnitude as ours. The discrepancy in the values is most likely due to the weakness of the fluorescence quenching technique (section 2.e.1.). Our value for testosterone is close to that obtained by Ryan and Hanna (1971) as well as those of other workers (Westphal, 1971a).

Our nk value for dehydroisoandrosterone is much lower than the value obtained by Puche and Nes (1962) for the binding of this steroid to BSA. This may be due to difference in the conditions of binding measurements used by us. Puche and Nes measured binding of dehydroisoandrosterone sulfate in phosphate and Krebs-Ringer bicarbonate buffers. Their results indicate nk value in the former buffer to be about 1.5 times the value in the latter buffer. Thus, the difference in the nature of buffers can explain part of the discrepancy. Further, it may also be due both to difference in the protein concentration and temperature employed by us (0.21 gm%; 25°) and these authors (5 gm%, 5 - 6°). The reciprocal plot for dehydroisoandrosterone sulfate, like ours, was found by these authors to be linear in the same binding range. However, our data given in section 5.e. indicate that this plot is not linear in the higher binding range.

5.a.1.3. Binding and solubility

It may be seen from binding data in table 5.a.1. that the most soluble steroid cortisol (XIII) has the lowest value of binding affinity, whereas the least soluble one,

5α-androstan-3α, 17β-diol (X) has the highest value. This seems to be in keeping with the polarity rule (section 2.e.). It may be pointed out that in the studies cited in section 2.e. the steroid polarity was expressed in terms of the number of polar groups in the molecule. But since all of the C_{19} steroids we have studied contain the same number of polar groups we have compared them on the basis of their solubility in Tris buffer. According to this definition it seems that although the binding of Δ^4-androsten-3, 17-dione (I), testosterone (III), androsterone (VII), progesterone (XIV), 5α-dihydrotestosterone (IX) and 5α-androstan-3α, 17β-diol (X) is also in accord with the polarity rule, i.e. the binding affinity increases as the solubility goes down, it does not hold in the case of the remaining steroids. Thus, while the solubilities of dehydroisoandrosterone (IV) and testosterone (III) are similar, their binding affinities differ by a factor of 2.5. The solubility of 5β-dihydrotestosterone (II) is higher than that of testosterone (III) by about 15% but the binding affinity is also quite markedly higher (by about 112%). Similarly whereas epiandrosterone (V) has a solubility value about 10.8% greater than that of epitestosterone (VI), the binding affinity of the former is higher than the latter by a factor of 2.6. The solubilities of 6-dehydrotestosterone (VIII) and progesterone (XIV) are close but the binding affinities differ by about 46%. These exceptions appear to suggest that the polarity rule proposed for the binding of steroids to

serum albumin (section 2.e.1.) may be a reflection of wider differences in the solubilities of steroids containing a different number of polar groups but that other structural characteristics of the molecule may also be important in deciding the magnitude of binding affinity. However, the contributions due to the latter may be masked by larger contributions of the solubility effect.

Table 5.a.2. shows values for the free energies of binding (see section 2.h.) for various steroids of table 5.a.1. Since, as mentioned earlier in this section, the experimental determination of n may involve large magnitudes of errors we have calculated these free energies as $\Delta G_1^{o'}$ and $\Delta G_2^{o'}$, using nk rather than the k values (see table 5.a.2. for calculations). It can be seen that the $\Delta G_2^{o'}$ value for progesterone (XIV) is about 44% higher than that for cortisol (XIII). The value for 5α-androstan-3α, 17β-diol (X) is 34% and for testosterone (III) about 9% higher than Δ^4-androsten-3, 17-dione. The binding energies of these steroids are therefore suggestive of the validity of the polarity rule. Again this may be so because of the large differences in the polarity and solubility of these steroids. However, a further examination of the data in table 5.a.2. reveals that in the C_{19} Series the $\Delta G_2^{o'}$ values show very little variation for the steroids containing one hydroxyl and one carbonyl group per molecule.

It is evident from table 5.a.2. that the $\Delta G_1^{o'}$ values are much lower compared to the corresponding $\Delta G_2^{o'}$ values

TABLE 5.a.2.

VALUES OF FREE ENERGIES FOR THE INTERACTION OF STEROIDS WITH BSA

	Steroid	$\Delta G_1^{o'}$,(a)	$\Delta G_2^{o'}$,(a)
(i)			
IX	5α-DIHYDROTESTOSTERONE	+0.056	-6.33
X	5α-ANDROSTAN-3α, 17β-DIOL	+0.407	-7.21
XV	ESTRADIOL-17β	+0.22	-6.6
XVI	ESTRONE	+0.77	-6.1
VIII	6-DEHYDROTESTOSTERONE	+1.27	-6.02
(ii)			
XIII	CORTISOL	-0.133	-4.33
XIV	PROGESTERONE	-0.109	-6.24
(iii)			
II	5β-DIHYDROTESTOSTERONE	-0.925	-6.34
IV	DEHYDROISOANDROSTERONE	-0.92	-6.44
V	EPIANDROSTERONE	-0.496	-6.29
III	TESTOSTERONE	-0.396	-5.90
I	Δ^4-ANDROSTEN-3, 17-DIONE	-0.459	-5.38
VII	ANDROSTERONE	-0.263	-6.24
VI	EPITESTOSTERONE	-0.126	-5.73

(a) Derived from data in table 5.a.1. by choosing the crystalline steroid ($\Delta G_1^{o'}$) and the solvated steroid ($\Delta G_2^{o'}$) as the standard states. Calculations: $\Delta G_1^{o'} = -RT \ln nks$, where s is the steroid solubility in Tris buffer; $\Delta G_2^{o'} = -RT \ln nk$. Both $\Delta G_1^{o'}$ and $\Delta G_2^{o'}$ are expressed as kcal/mole protein. Because of the uncertainty involved in the determination of n (see text), these values have been calculated from nk instead of k. They, therefore, represent free energies per n moles of bound steroid. The prime is used in the expression to indicate this.

which clearly suggests that the solubility effect makes up a large contribution to the total binding energy (section 2.h.). More importantly, the $\Delta G_1^{o'}$ values show a much wider variation than the $\Delta G_2^{o'}$ values and markedly dependent on the structure of the steroid.

Thus, it may be noted that $\Delta G_1^{o'}$ values in table 5.a.2. are grouped into three different categories. Steroids in group (i) have positive values. The steroids in this category tend to have a flatter conformation of the steroid nucleus. Thus, the two steroids, 5α-dihydrotestosterone (IX) and 5α-androstan-3α, 17β-diol (X) have an A/B trans conformation. The two estrogenic steroids (XV and XVI) have a planar ring A. 6-dehydrotestosterone (VIII) has a planar arrangement along the C-3, C-4 and C-6 double bonds. Testosterone might also be expected to fall into this category but is in fact in category (iii). Both of the C_{21} steroids exhibit low negative binding energies and widely differing solubilities. Comparison of the values for testosterone (III) and progesterone (XIV) suggest that the two carbon side chain destabilizes the steroid-albumin complex. The steroids in category (iii) have the highest $-\Delta G_1^{o'}$ values. The steroid with A/B cis conformation, 5β-dihydrotestosterone(II) exhibits the highest value. Comparison of this value with the positive value for 5α-dihydrotestosterone (IX) appears to suggest that conformation of the carbon ring is important in the structural

contributions to the binding of the steroids to albumin. It is further seen that the 3β(OH) compound, epiandrosterone (V), has a higher $-\Delta G_1^{o'}$ value than the 3α(OH)-steroid, androsterone (VII). Similarly the 17β(OH)-steroid, testosterone (III), binds more strongly than the 17α(OH)-steroid, epitestosterone (VI), according to these $\Delta G_1^{o'}$ values. Thus it appears that the configuration of the substituent groups also plays a critical role in the structural contributions to steroid binding to albumin and that β is the preferred configuration. Comparison of the values for androsterone (VII) and dehydroisoandrosterone (IV) suggests that introduction of Δ^{5-6} double bond brings about an increase in binding energy. Further it is also seen that reduction of 17-keto group in Δ^4-androsten-3, 17-dione (I) giving rise to testosterone (III) decreases binding energy slightly.

5.a.1.4. Competition

Table 5.a.3. shows binding parameters for the interaction of various ^{3}H-labeled steroids with 32 μM deionized BSA in the absence and presence of a fixed concentration of testosterone-4-^{14}C. This table also shows values of $\bar{\nu}$ for testosterone obtained in these competition experiments. It may be mentioned that in the experiment involving estradiol-17β, the Tris buffer solution of ^{14}C-testosterone used as competitor was half saturated, whereas it was fully saturated in the case of the remaining steroids. It may be seen that only the closely related

TABLE 5.a.3.

VALUES OF BINDING PARAMETERS FOR THE INTERACTION OF VARIOUS ^{3}H-LABELED STEROIDS WITH 32 μM DEIONIZED BSA (LOT 30) IN THE ABSENCE AND PRESENCE OF TESTOSTERONE-^{14}C AS COMPETITOR

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°. Competition experiments were performed as described in section 4.b.10. For the calculation of binding parameters (nk and n) see section 5.a.1.

	Absence of Testosterone		Presence of Testosterone		
Steroid	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n	$\bar{\nu}$ of testosterone[a]
PROGESTERONE	3.5	-	3.52	-	1.02
5α-DIHYDROTESTOSTERONE	4.43	5.5	3.83	-	1.25
DEHYDROISOANDROSTERONE	5.27	10.8	5.27	10.8	1.03
ESTRADIOL-17β	8.6	-	8.6	-	0.56
ANDROSTERONE	3.74	-	3.85	8.0	

[a] Moles of bound testosterone/mole protein.

5α-dihydrotestosterone-1, 2-^{3}H shows slight competition with testosterone-4-^{14}C. In this case the n value remains unchanged but the nk value is lowered by about 13.5% in the presence of testosterone-4-^{14}C (see also fig. 5.a.1.). However, the remaining steroids do not show any competition--the n and nk values are almost identical in the absence and presence of testosterone-4-^{14}C.

The data in table 5.a.4. show that the nk values for the binding of progesterone-1,2-^{3}H and androsterone-1, 2-^{3}H to BSA containing 5 moles lauric acid/mole protein in the presence of competing steroid are significantly lower compared to the values in its absence. The inhibited values in the presence of fatty acid are, however, higher than in the absence of fatty acid. It is further evident in the case of androsterone that even though the n values were not obtained, the inhibition is more marked with the more closely related dehydroisoandrosterone than with the less similar testosterone. This structural dependence appears to suggest that the mechanism of inhibition in the presence of fatty (5 moles lauric acid/mole protein) may be competitive in nature.

5.a.2. Discussion

Our data in general indicate that the solubility of steroids increases on oxidation of 3(OH) and 17(OH) groups. The introduction of double bond into the steroid has marked influence on aqueous solubility which varies with their location in the molecule. There are wide differences in the

FIGURE 5.a.1.

COMPETITION BETWEEN 5α-DIHYDROTESTOSTERONE ($-^{3}H$) AND TESTOSTERONE ($-^{14}C$) FOR BINDING TO 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°. Procedure details are described in section 4.b.10. Data presented in the form of reciprocal plots.

Binding of 5α-dihydrotestosterone ($-^{3}H$) in the absence of testosterone ($-^{14}C$).

Binding of 5α-dihydrotestosterone ($-^{3}H$) in the presence of testosterone ($-^{14}C$).

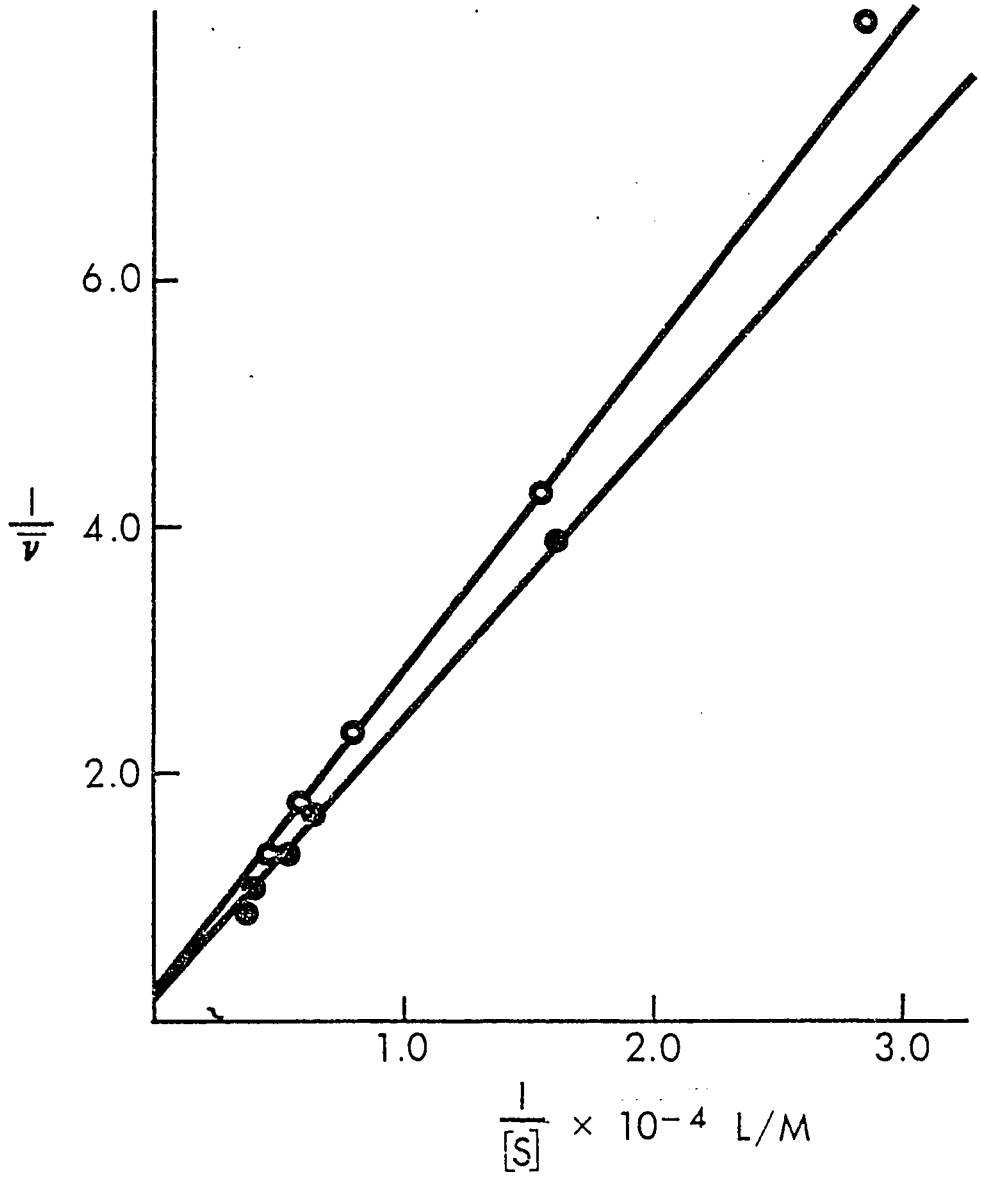

6.0
4.0
2.0
$\frac{1}{v}$
1.0
2.0
3.0
$\frac{1}{[S]} \times 10^{-4}$ L/M

TABLE 5.a.4.

VALUES OF BINDING PARAMETERS FOR THE INTERACTION OF ^{3}H-LABELED STEROIDS WITH 32 μM DEIONIZED BSA (LOT 30) CONTAINING 5 MOLES LAURIC ACID/MOLE PROTEIN IN THE ABSENCE AND PRESENCE OF A COMPETING STEROID (^{14}C-LABELED)

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°. Fatty acid was added to the BSA by the alcohol method (see section 4.b.3.). Refer to table 5.a.3. for more details.

	Absence of Competing Steroid		Presence of Competing Steroid		
Steroid	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n	$\bar{\nu}$ max (c)
PROGESTERONE	12.1	4.2	6.55 (a)	5	1.47-2.02
ANDROSTERONE	14.32		9.48 (a)		
ANDROSTERONE	14.32		5.91 (b)		1.75-2.34

(a) Testosterone ($^{14}_{-C}$) as the competing steroid.

(b) Dehydroisoandrosterone ($^{14}_{-C}$) as the competing steroid.

(c) Range of molar binding ratio of the competing steroid.

solubilities of the two configurational isomers, 5α-dihydrotestosterone and 5β-dihydrotestosterone and those differing in the configuration of 3(OH) and 17(OH) groups. Yet the dipole moments of these steroids do not follow any consistent pattern in relation to the variations in the solubility values. These findings seem to suggest that the structural features of the steroid molecule such as the nature, distribution and configuration of functional groups as well as the conformation of the steroid ring do not necessarily affect their water solubilities in the same fashion as they affect their dipole moments. In some cases the structural effects on one of the two properties may far exceed those on the latter so that no direct correlation between them is evident. It appears that some structural characteristics of the steroid molecule may be important in determining a proper fit and interaction of the steroid with the liquid water structure as proposed by Warner (1965). It is evident from our data that the compounds with flatter conformation of the rings A and B, and the A/B trans form of the saturated compounds have much lower solubilities than one would expect from values of their dipole moments, whereas A/B cis compound has a much higher value. According to Warner's model displacement of water oxygens by steroid oxygens at C-3 and C-17 will allow dipolar interactions with the frozen tetrahedral structure in the case of planar steroids. However, on the basis of this model we are unable to explain the very high solubility of 5β-dihydrotestosterone

as compared to the 5α-isomer. Furthermore, as previously mentioned, the water molecules interact independently with the substituent dipoles. However studies on larger groups of steroids will be necessary to establish these relations more definitely.

Our binding data (nk values) indicate structural influences on binding which are in general accord with the findings of other studies employing spectrophotometric and fluorescence quenching techniques. However, the data suggest that the C_{19} steroids containing the same number and types of polar groups per molecule, i.e. containing one hydroxyl and one carbonyl group, do not fully conform to the polarity rule when their nk values are compared on the basis of their solubilities in Tris buffer. The deviations from the polarity rule can be attributed to interactions other than hydrophobic (see section 2.h.).

Thus, while there are only slight differences in the values of $\Delta G_2^{o'}$ (see section 2.h.) calculated according to the reaction

$$n\ [\text{Steroid}](aq) + \text{Protein}\ (aq) \rightarrow [\text{Steroid}]_n\text{- Protein}\ (aq) \quad \text{------ (1)}$$

binding energies calculated as $\Delta G_1^{o'}$ according to Eik-Nes et al (1954)

$$n\ [\text{Steroid}](\text{crystalline}) + \text{Protein}\ (aq) \rightarrow [\text{Steroid}]_n\text{- Protein}\ (aq) \quad \text{------ (2)}$$

do not involve energy of transferring the steroid from the aqueous phase to the non-polar regions of the protein (section 2.h.) and show sufficient specific variations which must be indicative of structure dependent interactions between the steroid and protein, although these may also involve differences in the crystal free energies of steroids. The structure dependent interactions may include hydrogen-bonding and dipole-dipole interactions between the steroid and albumin (Ryan and Gibbs, 1970a; Romeu et al, 1975).

Comparison of the $\Delta G_1^{o'}$ values suggest that both the conformation of the steroid ring and the configuration of the substituent groups are important in the binding to the BSA. The data indicate that the steroids having flatter conformation are bound least tightly and that 5β- is the preferred conformation. Further, compounds with β-orientation of C-3 hydroxyl groups are bound more strongly compared to those with the α- configuration. This is contrary to what considerations based solely on the nk values suggest.

The conclusions drawn above from the $\Delta G_1^{o'}$ values must be accepted with certain reservations. Firstly, the number of steroids investigated is rather small. Secondly, since $\Delta G_1^{o'}$ values are only a very small fraction (10 - 20%) of the $\Delta G_2^{o'}$ values, they are subject to imprecision associated with the determination of solubility (s) and nk values. In order that these correlations be taken as valid, similar studies must be undertaken with larger numbers of

different types of steroids and the errors related to the determination of nk and S values must be taken into account.

The data in this section further suggest that while deionized BSA shows no competition between the binding of testosterone and progesterone, androsterone, dehydroisoandrosterone or estradiol-17β, in the absence of fatty acid, there is competition between testosterone and progesterone, testosterone and androsterone and dehydroisoandrosterone and androsterone in the presence of 5 moles of lauric acid per mole of protein. It should be recalled that in their one-point data obtained with HSA, Sandberg et al (1957) could not observe competition between testosterone and progesterone and a number of other steroid pairs. It appears that in the absence of fatty acid these steroids are not bound to the same sites on the albumin molecule. This would tend to suggest that there may be specific sites for the binding of each type of steroid and that the binding process itself may be specific. However, our results do show slight competition between testosterone and 5α-dihydrotestosterone even in the absence of fatty acid. This may be so because these two steroids are structurally more similar and therefore may be bound to the same sites. In this connection it may be recalled (section 2.f.) that competition has been demonstrated between corticosterone and cortisol (Slaunwhite et al, 1963) and between deoxycorticosterone and progesterone (Westphal and Harding, 1971b) in their binding to the HSA (delipidated). It appears that fatty acid must have a very

special influence on the BSA to produce such an apparent change in the binding site specificity. This might be brought about by the fatty acid-induced changes in the conformation of the protein (Soetewey et al, 1972). Our data in section 5.d. have provided some insight into the nature of the fatty acid effect on the binding of testosterone and progesterone to BSA and HSA.

5.b. The effect of steroid-BSA interaction on the chromophoric spectra as examined by means of difference spectroscopy

Difference spectra recorded on a Cary 15 for the interaction of sixteen different steroids with a single lot of deionized BSA (lot 30) at 32 μM protein concentration are presented in figs. 5.b.1., 5.b.2. and 5.b.3. The steroid concentrations at which these difference spectra were determined and the corresponding $\bar{\nu}$ values obtained in equilibrium dialysis are also given in the legend to these figures. Each of these figures also includes a difference spectrum for the interaction of fatty acid (3 moles of palmitic/mole protein or 5 moles of lauric/mole protein) with deionized BSA at the same protein concentration. All of these difference curves were measured under the same set of experimental conditions, i.e., in Tris buffer, pH 8.0, I = 0.1 at room temperature.

The steroid difference spectra were measured at one or more steroid concentrations; in certain cases they were measured at six different concentrations. At low steroid concentrations the amplitudes or aromatic difference extrema

FIGURE 5.b.1.

DIFFERENCE SPECTRA FOR THE INTERACTION OF STEROIDS LACKING A Δ^4-3-KETOGROUP AND FATTY ACID WITH 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, room tenperature.

A, 9.1 μM estrone ($\bar{\nu}$ = 0.12); B, 7.3 μM estradiol-17β ($\bar{\nu}$ = 0.2); C, 34.7 μM androsterone; D, 47 μM epiandrosterone ($\bar{\nu}$ = 0.69); E, 67 μM dehydroisoandrosterone; F, 86.5 μM 5β-dihydrotestosterone ($\bar{\nu}$ = 1.2); G, 108.6 μM 5α-dihydrotestosterone; H, 5α-androstan, 3α, 17β-diol ($\bar{\nu}$ = 1.47); I, 3 moles palmitic acid/mole protein.

The difference spectrum E was measured in rectangular tandem cells having a path length of 0.438 cm/compartment. Other difference spectra were obtained in either cylindrical tandem or regular cells (see below) with an optical path length of 1 cm/compartment. The difference spectra G and H were measured as follows: An aliquot of 32 μM protein solution was saturated with steroid by adding a concentrated solution of steroid in ethanol with constant and gentle shaking. The precipitated steroid was removed by centrifugation. An equivalent amount of ethanol was added to a second aliquot of the

protein solution. The difference spectra were recorded in matched rectangular (regular) cells of 1 cm path length by placing the steroid-protein mixture in the reference beam and the protein solution containing ethanol alone in the sample beam. Fatty acid difference spectrum (I) was also obtained similarly. Procedure details for the other difference spectra are the same as described in section 4.b.11. $\bar{\nu}$ values were derived from binding data determined by equilibrium dialysis (section 4.b.9.) performed under the conditions employed in measuring the difference spectra. The base line in each of the difference spectra is represented by the spectral region between 320 and 350 mμ. The values above this base line are positive with reference to the reference cell (blue-shifts) and those below are negative (red-shifts).

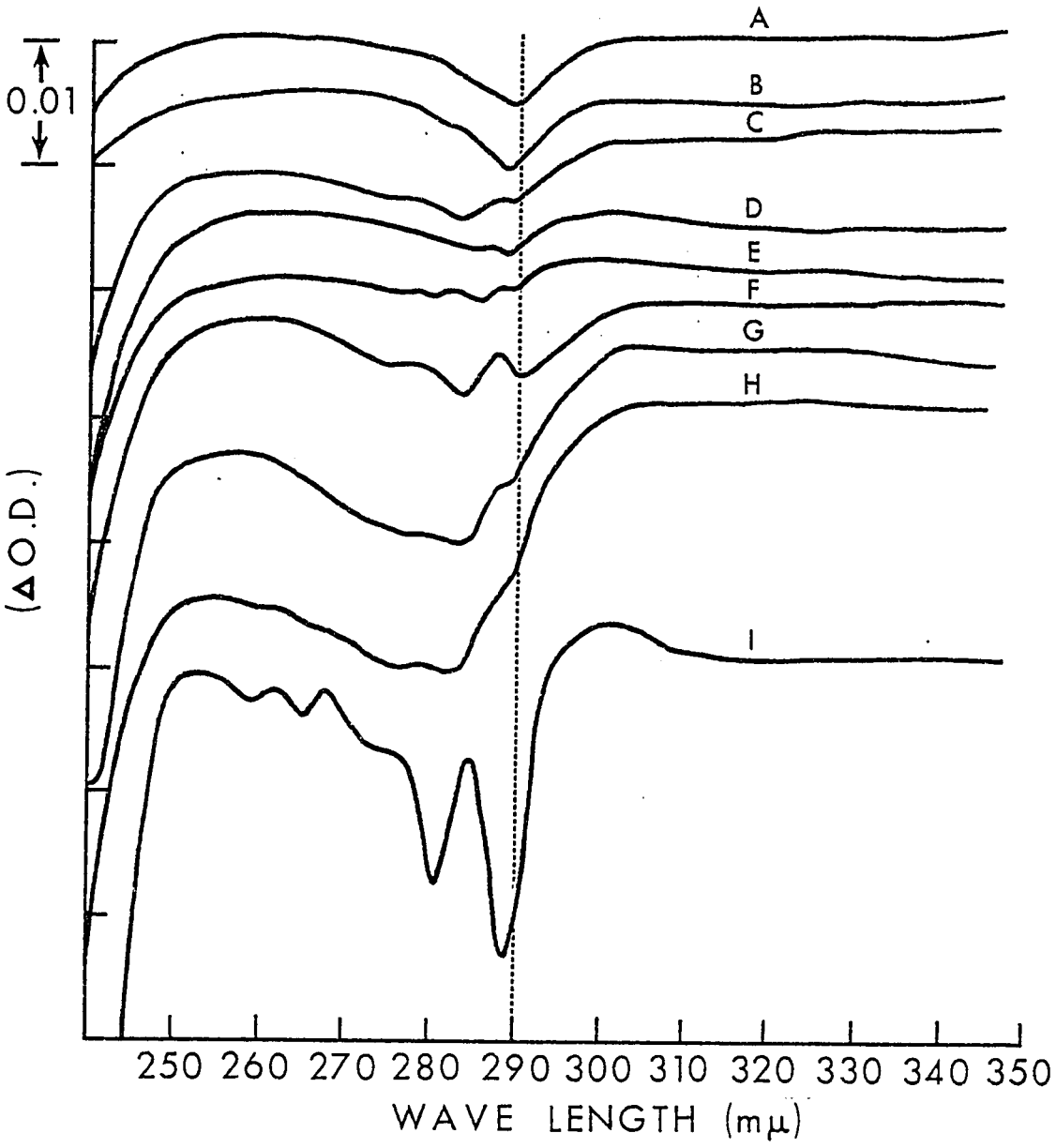

A
B
C
D
E
F
G
H
I
0.01
(ΔO.D.)
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

FIGURE 5.b.2.

DIFFERENCE SPECTRA FOR THE INTERACTION OF Δ^4-3-KETOSTEROIDS AND FATTY ACID WITH 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, room temperature.

J, 332 μM cortisol ($\bar{\nu}$ = 0.3); K, 10.9 μM progesterone ($\bar{\nu}$ = 0.184); L, 5 moles lauric acid/mole protein; M, 33 μM Δ^4-androsten-3, 17-dione ($\bar{\nu}$ = 0.33); N, 25.4 μM testosterone ($\bar{\nu}$ = 0.30).

Difference spectrum J was measured in rectangular tandem cells (0.438 cm path/compartment). K, M and N were recorded with cylindrical tandem cells (1 cm path length/ compartment). Procedure details for these difference spectra are the same as described in 4.b.11. Refer to fig. 5.b.1. for the measurement of $\bar{\nu}$ values and fatty acid difference spectrum (L) and for an explanation of the base line.

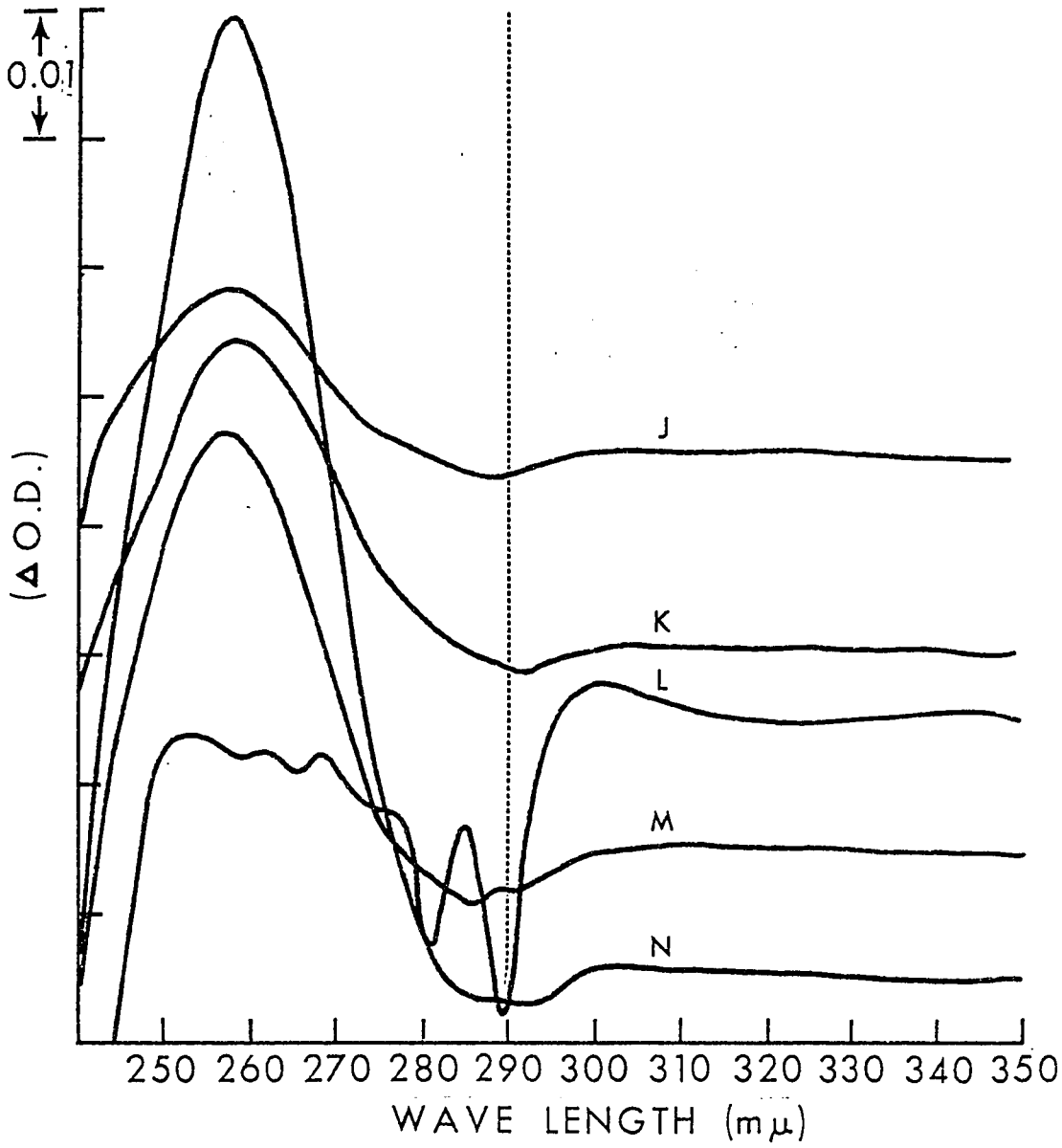

0.01
(Δ O.D.)
J
K
L
M
N
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

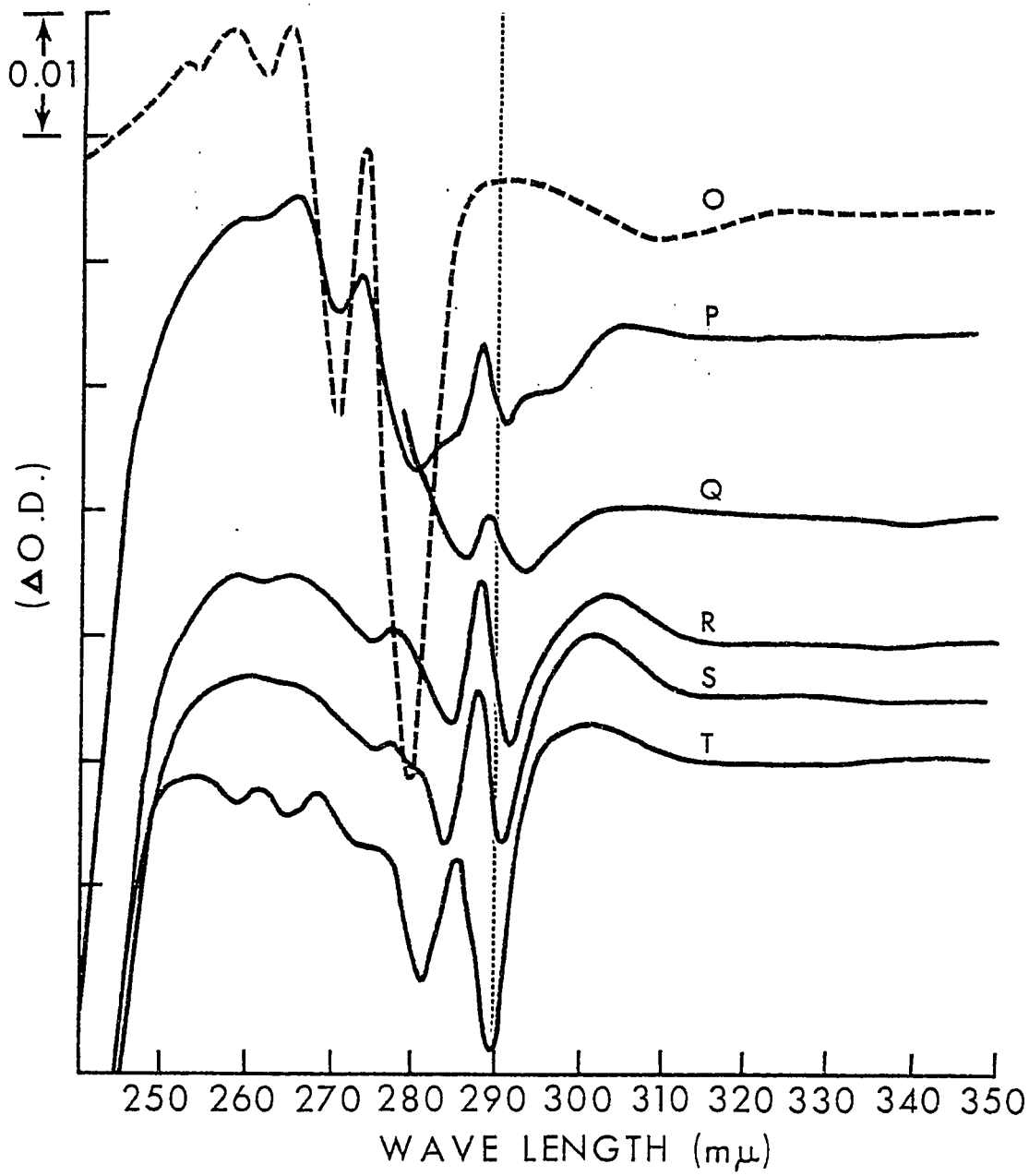
FIGURE 5.b.3.

DIFFERENCE SPECTRA FOR THE INTERACTION OF STEROID SULFATES AND FATTY ACID WITH 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, room temperature.

O, solvent perturbation difference spectrum for 230 μM estrone sulfate in ethanol and Tris buffer; P, 192 μM estrone sulfate ($\bar{\nu}$ = 0.425); Q, 36.7 μM testosterone sulfate; R, 190 μM dehydroisoandrosterone sulfate ($\bar{\nu}$ = 3.76); S, 157.4 μM androsterone sulfate ($\bar{\nu}$ = 3.33); T, 3 moles palmitic acid/mole protein.

The difference spectrum O was obtained in rectangular tandem cells (0.438 cm path length/compartment). P, Q, R and S were measured in cylindrical tandem cells (1 cm path length/compartment). The procedure details for these difference spectra are the same as described in sec. 4.b.11. Refer to fig. 5.b.1. for the measurement of $\bar{\nu}$ values and fatty acid difference spectrum (T) and for an explanation of the base line.


0.01
(ΔO.D.)
O
P
Q
R
S
T
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

for some steroids, especially non-sulfated, were so low that they could hardly be identified. Further, some of the non-sulfated steroids had very low solubility in Tris buffer so that when their difference spectra were measured by mixing the saturated solution of steroid in Tris buffer with the protein (see section 4.b.11.) they were found to be similar to the base lines. In such cases the difference spectra were obtained at higher steroid concentrations by adding a concentrated solution of the steroid in ethanol to the protein solution (see legend to fig. 5.b.1.). The final concentration of ethanol in these difference spectra was maintained very low (0.138%) and an equivalent amount of ethanol was added to the reference solutions. As already mentioned in the previous section such low concentrations of ethanol are not expected to alter the binding behavior significantly (Ryan et al, 1977).

The steroids for which difference spectra are presented in figs. 5.b.1. to 5.b.3. are of three types: (1) Steroids lacking a Δ^4-3-keto group, (2) Steroids containing this group, and (3) Steroids sulfated at either C-3 or C-17 positions. The difference curves for these steroids appear in figs. 5.b.1., 5.b.2. and 5.b.3. respectively.

Since, with the exception of estrone and estradiol-17β, none of the steroids in category (1) absorb strongly in the near ultraviolet, the difference spectra for the interaction of these with deionized BSA shown in fig. 5.b.1.

arise entirely due to perturbation (red-shift) of the protein spectrum (Ryan, 1968). However, although estrone and estradiol-17β have significant absorption in the 240 - 350 mμ region, the concentrations at which the difference spectra for these could be obtained (because of their very poor solubility) are so low that contributions due to perturbation of the steroid spectrum would be almost negligible. The difference spectra for the interaction of Δ^4-3-ketosteroids in fig. 5.b.2. contain two spectral effects, one arising out of perturbation (blue-shift) of the steroid band at 248 mμ giving rise to a difference peak at 258 mμ and another due to perturbation (red-shift) of the protein spectrum with the appearance of difference minima in the 280 - 295 mμ range (section 2.1.). Category (3) contains both non-absorbing (androsterone sulfate and dehydroisoandrosterone sulfate) and UV-absorbing (estrone sulfate and testosterone-sulfate) steroids. Their difference spectra (fig. 5.b.3.) therefore result from binding-induced alterations in the spectral properties of either the protein alone or both steroid and protein depending upon the type of steroid-sulfate. Thus difference spectra for dehydroisoandrosterone sulfate (curve R) and androsterone sulfate (curve S), like those of their corresponding non-sulfated analogues, contain contributions due only to perturbation of the protein bands. Since estrone sulfate exhibits marked absorption around 280 mμ, its difference spectrum (curve P) contains in addition to the

effects arising out of perturbation of the protein spectrum various other small peaks or minima which by comparison with the solvent perturbation (Tris buffer/ethanol) difference spectrum of estrone sulfate (curve O) can be assigned to binding-induced changes in the steroid spectrum. The difference spectrum for testosterone sulfate (curve Q) resembles that of the non-sulfated testosterone (curve N) in regard to positions of both the steroid difference peak (not shown) and the aromatic difference minima and therefore contains effects resulting from mutual alterations of both protein and steroid spectra.

In figure 5.b.1. the difference minima for androsterone (curve C), 5α-dihydrotestosterone (curve G) and 5α-androstan-3α, 17β-diol (curve H) are located at 282.5 - 283.5 mμ and 289 - 290 mμ. The positions of these minima are similar to those (282 and 289 mμ) observed by Ryan and Gibbs (1970a) for the interaction of dehydroisoandrosterone with HSA and suggest perturbation of tryptophan and tyrosine residues in a ratio of approximately 1:10. However, it is evident from figure 5.b.1. that in the case of epiandrosterone (curve D) and dehydroisoandrosterone (curve E), while the position of second difference minimum is the same (289 - 289.5 mμ) the first difference minima appear at higher wavelengths, 285 and 286 mμ respectively. Further, in the case of 5β-dihydrotestosterone (curve F) both minima are shifted to slightly higher wavelengths, 284 and 290.5 mμ. It seems the interaction of these three

steroids involves perturbation of tryptophan relative to tyrosine in a ratio greater than 1:10. Binding data (see section 2.1.) in the last section shows that these steroids also have higher $-\Delta G_1^{o'}$ values compared to those of the three mentioned above, i.e. androsterone, 5α-dihydrotestosterone and 5α-androstan, 3α, 17β-diol. It therefore seems that, in the interaction of steroids of category 1 with BSA, increased perturbation of tryptophan relative to tyrosine is associated with greater free energy of binding due to non-hydrophobic interactions.

The appearance of perturbations of the tryptophan and tyrosine residues in the difference spectra for steroid-BSA interaction appears to suggest that these residues may be involved in the binding. Other studies (sections 2.k. and 2.l.) have also proposed that tryptophan residues may participate in the binding of steroids to serum albumin. The greater perturbation of tryptophan relative to tyrosine associated with higher $-\Delta G_1^{o'}$ values, as noted above, might be taken to mean that binding of the steroid to BSA involves specific interactions with the tryptophyl groups. However, our results in section 5.c. will indicate that tryptophans may not be directly involved in steroid binding to the BSA and that tryptophan perturbations seen in the difference spectra could arise as a result of decrease in the exposure of tryptophan residues brought about by steroid-induced conformation change in the protein. Further, results in that section suggest that steroid-BSA interaction involves

H-bonding of the steroid carbonyl to the tyrosine residues. But since there is a negative rather than positive correlation between tyrosine perturbation and $\Delta G_1^{o'}$ values, in order to account for this free energy change it seems necessary to consider the interaction of the steroid which may also involve other amino acid residues. Swaney and Klotz (1970) have suggested that arginine and lysine residues both can serve as good proton-donor groups in the steroid-albumin interactions. The results of Romeu et al (1976) have further indicated that arginine groups may play a significant role in steroid binding to the BSA (section 2.k.). The steroid-binding domains in the BSA which we have suggested in section 5.c. as loops 3 and 4 (according to the model of Brown, fig. 2.a.1.) contain a number of arginine and lysine residues. It appears that the interaction of the steroid in these two binding regions may be determined by the spatial orientation and distribution of these charged residues. This may explain the variation of $\Delta G_1^{o'}$ with structure of the steroid (section 5.a.) and is supported by our finding that fatty acid, which alters the location of positively and/or negatively charged groups in these loops (see below) affects steroid-binding to the BSA very markedly (section 5.d.).

It may be noted that the positions of our difference minima in the case of dehydroisoandrosterone differ from those observed by Ryan and Gibbs (1970a) for the interaction of this steroid with HSA. This could be due to difference

in the nature of binding sites for dehydroisoandrosterone in the two proteins, i.e. BSA and HSA.

It is further seen from fig. 5.b.2. that in the case of Δ^4-androsten-3, 17-dione (curve M) and testosterone (curve N) the aromatic difference minima are located at 285 - 286 and 291.5 - 293 mμ. However the difference spectra for progesterone (curve K) and cortisol (curve J) exhibit a broad single trough extending between 280 - 295 mμ. This suggests (see section 2.1.) far greater involvement of tryptophan in the aromatic perturbations accompanying the interaction of testosterone and Δ^4-androsten-3, 17-dione with BSA than that of progesterone and cortisol. Again, the data in section 5.a. reveals that $-\Delta G_1^{o'}$ values for the former two steroids are significantly higher than the latter two. However, while tryptophan involvement in the case of Δ^4-androsten-3, 17-dione and testosterone is also seen to be greater than that in the case of steroids of category (1) (lacking a Δ^4-3-keto group), the $-\Delta G_1^{o'}$ values of the former two steroids are lower than many of the steroids of the latter category. Since, of the two possible steroid binding domains in the BSA (section 5.c.2.), one (loop 3) is rich in tyrosine while the other (loop 4) contains no tyrosine (fig. 2.a.1.), it is probable that Δ^4-androsten-3, 17-dione and testosterone may be bound in loop 4 whereas steroids of category (1) may interact in loop 3, as may also be the case for progesterone and cortisol. This would be in keeping with the results obtained in section 5.a. for competition

studies performed with deionized BSA.

Each of the steroids in fig. 5.b.2. also shows a difference peak at 258 mμ. Studies of Ryan and Gibbs (1970a) indicate that the height of this peak (expressed as $\Delta\varepsilon_{258}^{\bar{\nu}=1}$) may be affected by hydrogen bonding between the steroid and the protein (section 2.1.). It is therefore interesting to compare the $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ values for these steroids (see table 5.b.1.). These values were obtained from plots of $(\Delta O.D.)_{258}$ versus $\bar{\nu}$ shown in fig. 5.b.4. by dividing slopes of their initial (linear) portions by protein concentration (Ryan and Gibbs, 1970a). It may be seen from table 5.b.1. that the values for testosterone and progesterone are significantly lower than those for these steroids in Tris buffer and ethanol which may be due to hydrogen bonding between the steroid and the protein (Ryan and Gibbs, 1970a; section 2.1.). The progesterone value is much lower compared to the value we have obtained for the interaction of this steroid with defatted BSA (section 5.d.). The reason for this difference is not clear. Although, because of the low solubility of progesterone, some error is encountered in the determination of this value, such a large difference (about 30%) cannot be simply due to that. Further the values for testosterone and Δ^4-androsten-3, 17-dione are rather close while that for progesterone is much smaller. This might be due to the suggestion made above that the two types of steroids are bound respectively in loops 4 and 3. Since loop 3 contains large numbers of tyrosines, hydrogen bonding of the steroid

TABLE 5.b.1.

VALUES OF SPECTRAL MOLAR PERTURBATION

OF Δ^4-3-KETOSTEROIDS

Steroid	Spectral Molar Perturbation $\Delta\varepsilon_{258}^{\bar{\nu}=1}.10^{-3}$ (a)	$\Delta\varepsilon_{258}.10^{-3}$ (b)
TESTOSTERONE	5.25	7.3
PROGESTERONE	3.78	7.48 (c)
Δ^4-ANDROSTEN-3, 17-DIONE	4.94	

(a) Values for the interaction of steroids with deionized BSA (lot 30). Calculated from slopes of curves in fig. 5.b.4. (see text).

(b) Values for the transfer of steroids from Tris buffer to alcohol.

(c) Value of Ryan and Gibbs (1970a).

with these (section 5.c.1.) can explain the very low value for progesterone.

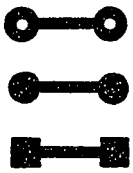
It is evident from fig. 5.b.4. that while the plots for progesterone and Δ^4-androsten-3, 17-dione are linear over the binding ranges covered in the difference spectra, that for testosterone is linear only up to binding level of about 0.6 and shows departure from linearity at a higher value of $\bar{\nu}$. It may be mentioned that Ryan and Gibbs (1970a) found a linear relationship between $(\Delta O.D.)_{258}$ and $\bar{\nu}$ for the interaction of testosterone with HSA, but their plot for cortisol was not linear.

The fatty acid difference spectra (curves I, L and T) show two very sharp minima at 281 and 289 mμ and one broad peak around 300 mμ. These curves are qualitatively similar to the difference curves published by Steinhardt and coworkers (Polet and Steinhardt, 1968; Steinhardt et al, 1972) for the interaction of octanoate and dodecanoate with BSA. The positions of minima seen in our fatty acid difference spectra are also similar to those observed by Zakrzewski and Goch (1968) in the interaction of dodecanoate with HSA, although the peak at 300 mμ was not observed in the difference spectrum for HSA. The positions of difference minima in the fatty acid difference spectra are attributed to a red-shift of the tyrosyl spectrum (Zakrzewski and Goch, 1968). However, the peak at 300 mμ results from a blue shift of a tryptophan minor band due either to increase in positive charge or decrease in negative charge in the vicinity of

FIGURE 5.b.4.

PLOTS OF $(\Delta O.D.)_{258}$ VS. $\bar{\nu}$ FOR THE INTERACTION OF Δ^4-3-KETOSTEROIDS WITH 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°.

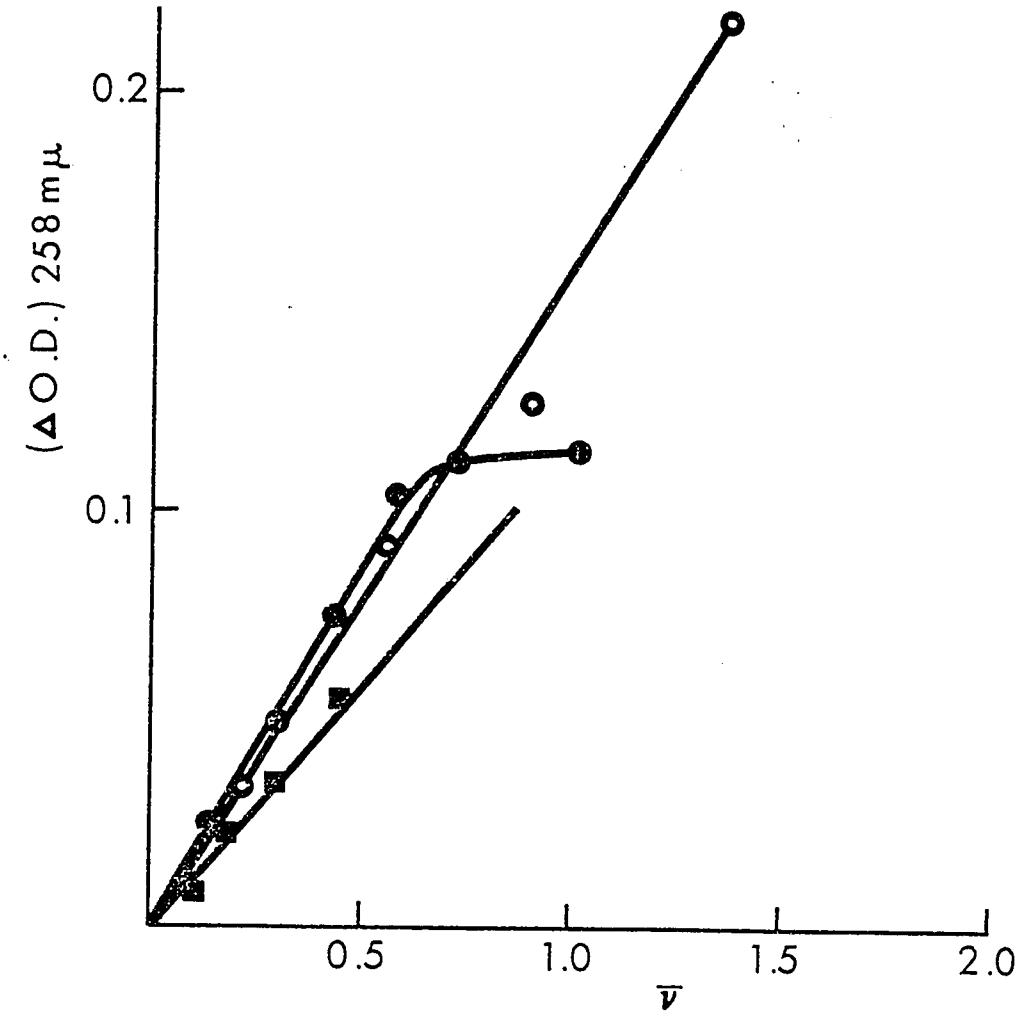


Δ^4-androsten-3, 17-dione

Testosterone

Progesterone

$(\Delta O.D.)_{258}$ values for each curve were derived from difference spectra (see fig. 5.b.2.) measured at different steroid concentrations and the corresponding $\bar{\nu}$ values determined as mentioned in fig. 5.b.1.


0.2
0.1
(ΔO.D.) 258 mμ
0.5
1.0
1.5
2.0
ν̄

tryptophyl residues (Ananthanaryanan and Bigelow, 1969a, b). Since fatty acids are negatively charged ions at pH 8.0, the perturbation of tryptophan cannot be due to direct interaction. It seems that the binding of fatty acid involves some conformation change in the BSA (Soetewey et al, 1972) which results in either removal of negatively charged groups from or transfer of positively charged residues into the neighborhood of tryptophan. However, since the 300 mμ peak is not observed in the case of HSA (Zakrzewski and Goch, 1968; our difference spectrum not shown), it seems that interaction of fatty acid with this protein involves no alteration in the distribution of charged amino acid residues in the vicinity of tryptophan. This difference between the interactions of fatty acid with BSA and HSA is quite noteworthy. Since it has been suggested that the positively charged residues may be involved in the binding of steroids to albumin, as noted, above, and our data in section 5.c.2. indicate that the steroid binding sites in albumin are most probably located in the neighborhood of tryptophyl groups, it can explain why the stimulatory effect of fatty acid which we have observed on the interaction of testosterone and progesterone is much more marked in the case of BSA than in the case of HSA (see section 5.d.).

Two very sharp minima observable in the difference spectra for steroid sulfates shown in fig. 5.b.3. are located in the 280 - 295 mμ region. The positions of these minima are found to be similar for the three steroid-3-sulfates

and are seen at 283.5 - 284.5 and 290.5 - 291.5 mμ. In the case of testosterone-17-sulfate the two minima are located at 286 and 293 mμ. This suggests that while the interaction of steroid-3-sulfate involves perturbation of a mixture of tryptophan and tyrosine residues, that of testosterone-17-sulfate involves almost exclusively tryptophan (section 2.1.), again most probably due to their binding in different loops in the BSA, i.e. in loops 3 and 4 respectively. Further, in the difference spectrum for testosterone-17-sulfate a peak is observed (not shown) at 260 mμ which is due to perturbation (blue-shift) of the steroid band. It might be questioned that the appearance of aromatic difference minima at longer wavelengths in the case of testosterone-17-sulfate compared to their positions in the case of steroid-3-sulfates might result from the effect of steroid difference band at 260 mμ on the aromatic difference spectrum. However, this does not appear to be so since Ryan and Gibbs (1970a) have shown that in the interaction of testosterone with HSA, the positions of aromatic minima are not altered after correction to the difference spectrum in the aromatic region. Such a correction was found to affect only the depth of the two difference minima.

The curve for each of the steroid-3-sulfates also shows a peak around 300 mμ. This peak is similar to the difference peak found at 300 mμ in the interaction of fatty acid with BSA and most likely arises due to perturbation of the tryptophan minor band, again probably resulting

from binding induced conformation change in the protein affecting the charge environment of tryptophan. It is interesting to note that 300 mμ peak is not observed in the interaction of testosterone-17-sulfate. Although the exact cause of this difference cannot be given it is at least a further indication of binding to two different binding domains in the case of the sulfates as well as the free steroids, i.e. loops 3 and 4.

5.c. The effect of steroid-albumin interaction on the aromatic amino acid exposure

5.c.1. Solvent perturbation difference spectroscopy

Solvent perturbation difference spectra of BSA obtained with three different perturbants, 20% glycerol, 90% deuterium oxide (D_2O) and 20% ethylene glycol, in the absence and presence of dehydroisoandrosterone sulfate are shown in fig. 5.c.1. Perturbation difference spectra determined with two different perturbants, 20% ethylene glycol and 90% D_2O, in the absence and presence of testosterone are presented in fig. 5.c.2. The spectral effects in these difference spectra in the 275 - 300 mμ region arise as a result of perturbation of the spectra of exposed (partially or fully) tyrosyl and tryptophyl residues by the perturbant (Herskovits and Laskowski, 1960). Glyercol and ethylene glycol cause red-shifts in the aromatic spectra. These were recorded as positive peaks by placing the protein solution containing perturbant in the sample beam (for other details

FIGURE 5.c.1.

EFFECT OF DEHYDROISOANDROSTERONE SULFATE ON THE SOLVENT PERTURBATION DIFFERENCE SPECTRA OF 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, room temperature. A, 20% glycerol; B, 90% D_2O; C, 20% ethylene glycol. ------, in the absence of dehydroisoandrosterone sulfate; _____, in the presence of dehydroisoandrosterone sulfate (A, 770 μM; B, 698 μM; C, 765 μM).

Difference spectra A and C were measured in cylindrical tandem cells of 1 cm path length/compartment, whereas difference spectra B were obtained with rectangular tandem cells having a path length of 0.438 cm/compartment. The blue shifts caused by 90% D_2O were recorded as positive difference spectra by placing protein solutions containing perturbant in the reference beam. Other details are as given in section 4.b.12. In each of the difference spectra the base line is represented by the spectral region 320-350 mμ. Values above this base line are positive (red-shifts in the case of A and C and blue-shift in the case of B) and those below are negative.

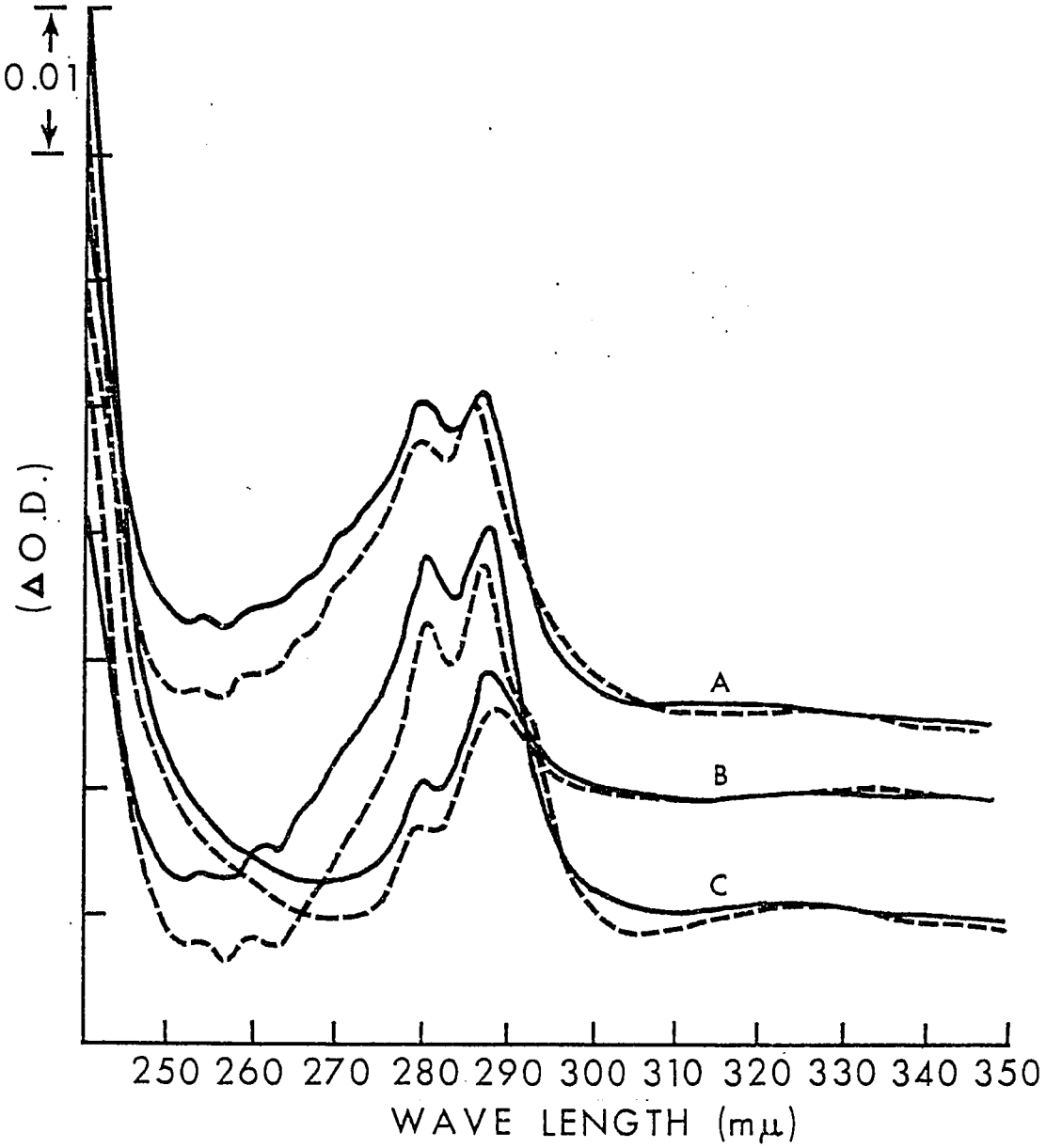
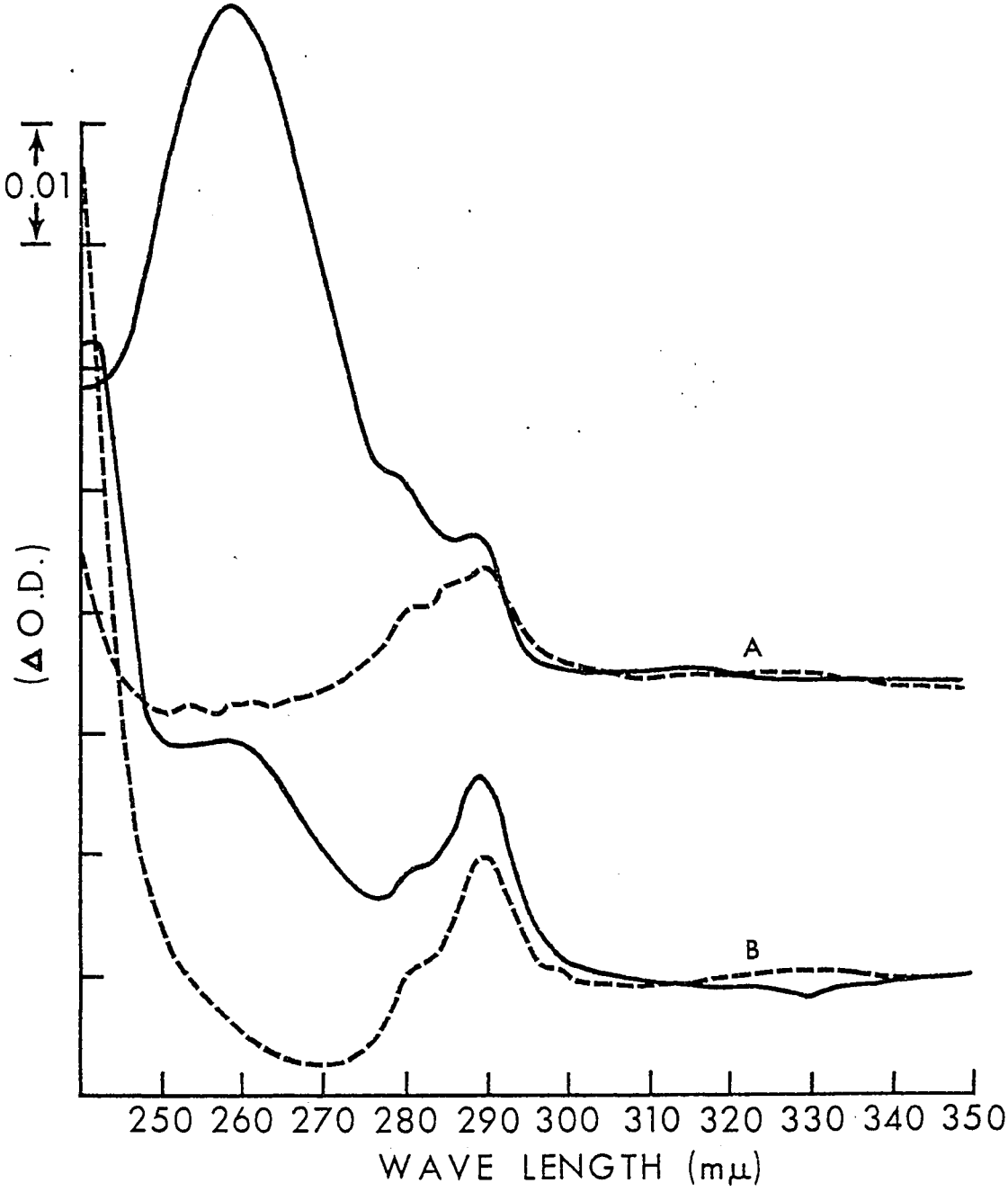

0.01
(ΔO.D.)
A
B
C
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

FIGURE 5.c.2.

EFFECT OF TESTOSTERONE ON THE SOLVENT PERTURBATION DIFFERENCE SPECTRUM OF 32 μM DEIONIZED BSA (LOT 30) CONTAINING 5 MOLES LAURIC ACID/MOLE PROTEIN

Conditions: Tris buffer, pH 8.0, room temperature. A, 20% ethylene glycol; B, 90% D_2O. ------, in the absence of testosterone; _____, in the presence of testosterone (A, 68.3 μM; B, 101.9 μM). All these difference spectra were measured with rectangular tandem cells having a light path of 0.438 cm/compartment. Although the spectral shifts produced by ethylene glycol and D_2O are of opposite sign (see text), these were recorded, for convenience, as positive peaks for both these solvents by choosing the appropriate arrangements of the sample and reference cells (refer to the procedures in section 4.b.12.). For an explanation of the base lines refer to the legend of fig. 5.c.1.


0.01
(ΔO.D.)
A
B
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

see section 4.b.12). However, D_2O produces blue-shifts but these were also obtained as positive peaks by measuring the difference spectra with cells containing a mixture of protein and perturbant in the reference rather than the sample beam. The difference peaks seen in these spectra at 279 - 281 and 286 - 289 mμ are due to perturbations of the tyrosyl residues (section 2.1.). Since bovine albumin is a tyrosine-rich protein, the tryptophyl perturbations in most of these difference spectra are masked by the larger magnitude of the tyrosyl perturbations. However, difference spectra obtained with 20% glycerol and 20% ethylene glycol exhibit slight shoulders (more clearly marked in the original recordings) in the 291 - 293 mμ range which can be attributed to perturbation of the exposed tryptophan residues. Such a shoulder is also evident in the solvent perturbation difference spectrum of BSA obtained by Herskovits and Sorensen (1968) with 20% ethylene glycol as perturbant at pH 5.6. Except in the presence of testosterone, perturbations of the fine structure of phenylalanine spectrum are also seen in the region below 270 mμ in the solvent perturbation difference spectra obtained with ethylene glycol and glycerol as the perturbants. However, these perturbations are not observed with D_2O even in the absence of testosterone.

The magnitudes of perturbation peaks represent measures of the extent of exposure of the tyrosyl and tryptophyl residues (Herskovits and Laskowski, 1962). It is evident from the solvent difference spectra in figures 5.c.1.

and 5.c.2. that the heights of the tyrosine peaks are increased by the presence of testosterone or dehydroisoandrosterone-sulfate. In the presence of testosterone, a peak at 258 mμ is observed in the solvent perturbation of BSA with both 90% D_2O and 20% ethylene glycol (fig. 5.c.2.). Such a peak is not found in the absence of testosterone and is also absent in the solvent perturbation difference spectra measured in the presence of dehydroisoandrosterone sulfate (fig.5.c.1). It seems that this peak arises because of the effects of solvent on the difference spectra for the testosterone-protein interaction. Figure 5.c.3. shows the difference spectra obtained for the interaction of testosterone with BSA in the absence and presence of 20% ethylene glycol. It can be seen that the height of the steroid difference peak at 258 mμ is decreased by about 66% in the presence of 20% ethylene glycol. The 258 mμ difference peak in the ethylene glycol perturbation difference spectrum in fig. 5.c.2. therefore results from decrease in the binding of testosterone caused by the solvent. It may be mentioned that decreased interaction of testosterone in the presence of solvent will tend to lower rather than increase the height of the aromatic peaks in the solvent perturbation.

The effect of D_2O on the difference spectrum for testosterone-BSA interaction was not examined but it appears that the 258 mμ peak in the D_2O perturbation difference spectrum arises due to increased interaction (note the arrangements of cells employed in recording the difference spectra in

FIGURE 5.c.3.

EFFECT OF 20% ETHYLENE GLYCOL ON THE DIFFERENCE SPECTRUM FOR THE INTERACTION OF 68.3 μM TESTOSTERONE WITH 32 μM DEIONIZED BSA (LOT 30) CONTAINING 5 MOLES LAURIC ACID/MOLE PROTEIN

Conditions: Tris buffer, pH 8.0, room temperature.

------, in the absence of ethylene glycol, _____, in the presence of 20% ethylene glycol. Both difference spectra were obtained in rectangular tandem cells (0.438 cm path length/compartment), using the same solutions as those employed for measuring the solvent perturbation difference spectra A in fig. 5.c.2. Refer to fig. 5.b.1. for an explanation of the base line.

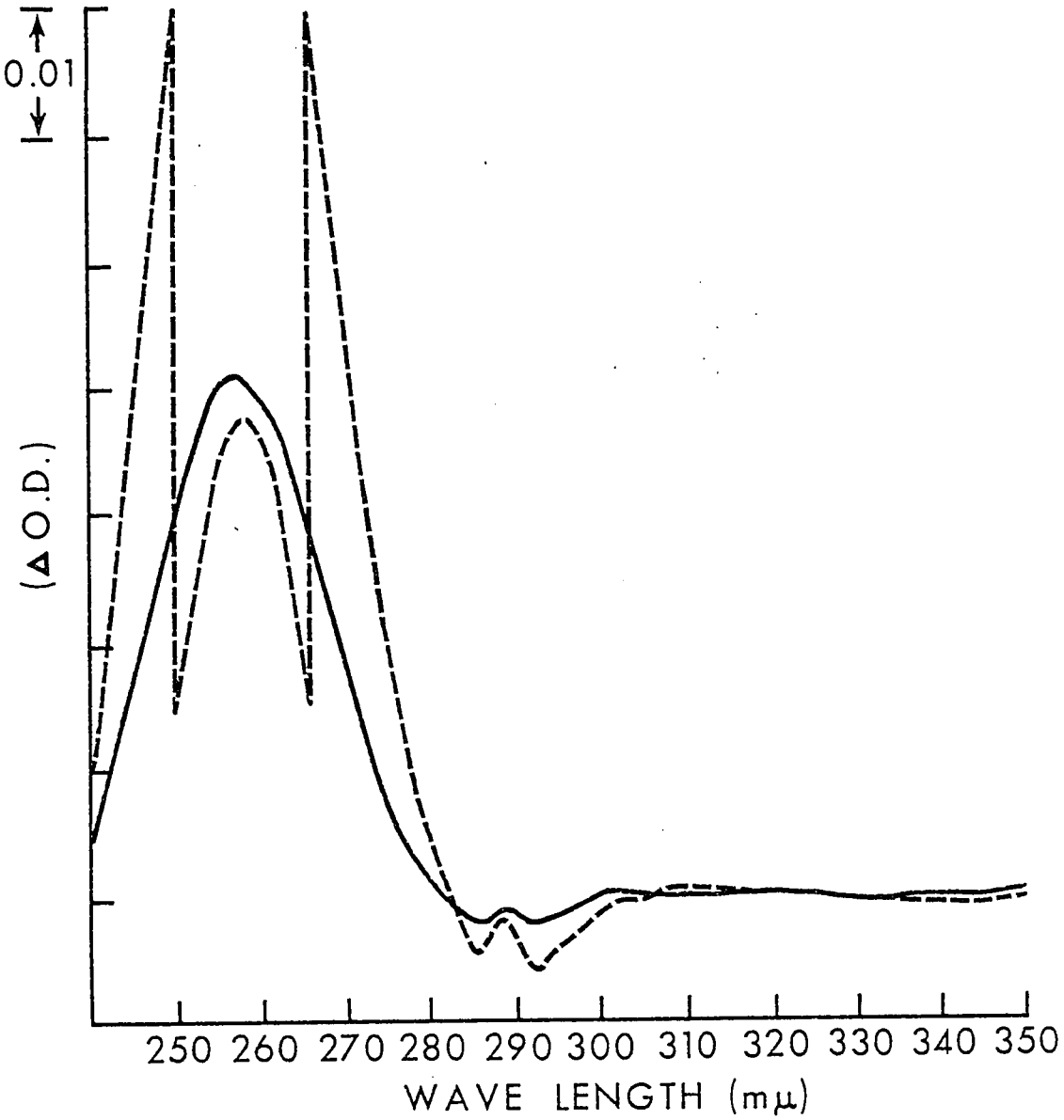

0.01
(Δ O.D.)
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

fig. 5.c.2.) of testosterone with BSA. In this connection it may be mentioned that D_2O has been shown to be a stabilizer for a number of proteins (Maybury and Katz, 1956; Berns, 1963) and it is possible that such an effect on the BSA is responsible for increased binding of the steroid.

Since ethylene glycol and glycerol have been shown to be the safest perturbants for solvent perturbation studies (Herskovits and Laskowski, 1962; Williams et al, 1965), it seems that the decreased binding of testosterone in the presence of ethylene glycol is probably due to simple effect of solvent on steroid solubility rather than due to its effect on protein conformation affecting the interaction (see also below).

Since the height of tyrosyl peaks is increased in the presence of testosterone or dehydroisoandrosterone sulfate in the solvent perturbation performed with three different kinds of perturbants, it seems quite evident that the tyrosyl exposure is increased by the binding of these steroids to the BSA. It may be recalled that the tyrosyl groups are known to be involved in steroid-binding to the albumin (section 2.k.). It is therefore probable that those tyrosine red-shifts seen in the interaction of steroids with albumin (section 5.b.) could arise due to direct interaction of the tyrosyl groups with the steroid rather than due to decrease in exposure. The nature of this interaction is most likely hydrogen-bonding since Chignell and Gratzer (1968) have reported red-shifts in the transfer of phenolic compounds into hydrogen-bonding solvents. The increase in exposure of

other tyrosyl residues is apparently brought about by some steroid-induced conformation change in the protein. Such conformation changes have been recognized in the interaction of serum albumins with testosterone and other steroids (section 2.m.).

It is evident from fig. 5.c.1. that in the solvent perturbation of BSA with 20% glycerol and 20% ethylene glycol, a tryptophan shoulder (at 291 - 293 mμ) seen in the absence of steroid disappears when dehydroisoandrosterone sulfate is present. Although this suggests that tryptophan exposure is decreased in the interaction of this steroid with the albumin the evidence is not very strong. Firstly, the change observed is quite small and secondly it may also be caused by a change in the binding of dehydroisoandrosterone sulfate due to the presence of 20% solvent. Although we have not determined the effect of solvent on the interaction of dehydroisoandrosterone sulfate with BSA (because longer contact with the solvent may affect the protein), difference spectra for HSA-testosterone sulfate interaction measured in the absence and presence of 20% glycerol (fig. 5.c.4., curves B) suggest that the solvent causes a decrease in binding affinity between the protein and the steroid sulfate. It can be seen that both the height of the steroid difference peak at 258 mμ and the depth of the aromatic difference minima are decreased by approximately the same magnitudes in the presence of solvent, although the effect is much less marked compared to that of 20% ethylene glycol on the

FIGURE 5.c.4.

A. EFFECT OF TESTOSTERONE SULFATE (147.6 μM) ON THE SOLVENT PERTURBATION DIFFERENCE SPECTRUM OF 32 μM DEIONIZED HSA (LOT 33) CAUSED BY 20% GLYCEROL

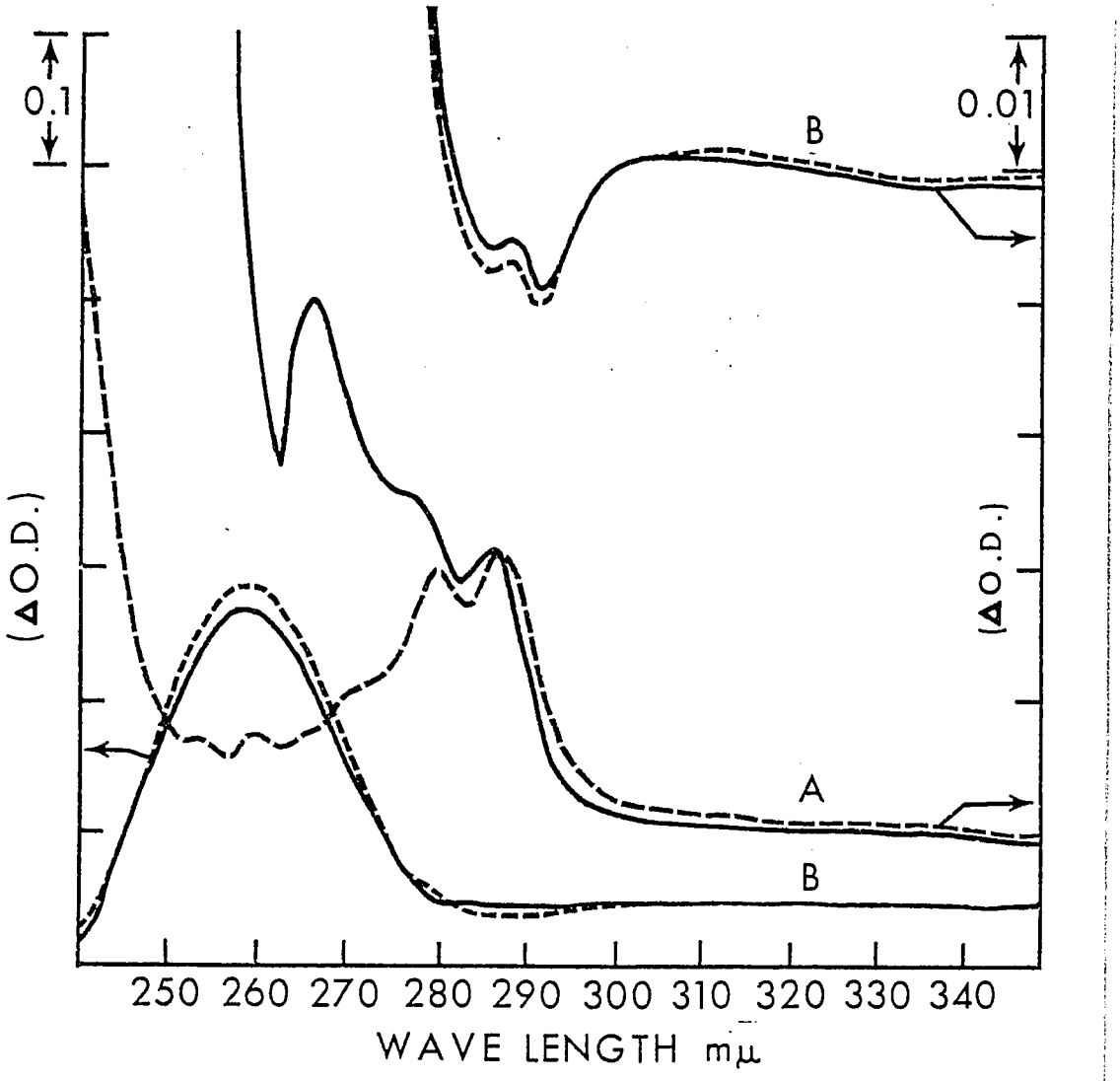
Conditions: Tris buffer, pH 8.0, room temperature.

------, in the absence of testosterone sulfate,

_____, in the presence of 147.6 μM testosterone sulfate. Both difference spectra were recorded with cylindrical tandem cells having a path length of 1 cm/compartment. Refer to fig. 5.b.1. for an explanation of the base line.

B. EFFECT OF 20% GLYCEROL ON THE DIFFERENCE SPECTRUM FOR THE INTERACTION OF 147.6 μM TESTOSTERONE SULFATE WITH 32 μM DEIONIZED HSA (LOT 33)

------, in the absence of glycerol, _____, in the presence of 20% glycerol. These difference spectra were measured using rectangular tandem cells (path length 0.438 cm/compartment) with the same solutions as those employed for obtaining the solvent difference spectra A. Refer to fig. 5.b.1. for an explanation of the base line.


0.1
0.01
B
(ΔO.D.)
(ΔO.D.)
A
B
250 260 270 280 290 300 310 320 330 340
WAVE LENGTH mμ

testosterone-BSA interaction (fig. 5.c.3.). It seems that the effect of solvent on binding is simply due to decrease in the polarity of the aqueous medium rather than due to change in the structure of the protein so that the affinity decreasing effect of solvent is much more marked in the case of the less polar steroid testosterone than in the case of the more polar steroid testosterone sulfate. From this it appears that the effect of solvent on the interaction of dehydroisoandrosterone sulfate with BSA may be quite small so that decrease in the height of the shoulder at 291 - 293 mμ brought about by the binding of this steroid sulfate in the solvent perturbation difference spectrum (fig. 5.c.1.) may still be significant. On the basis of this tenuous and fragmentary evidence it seems that binding of dehydroisoandrosterone sulfate to BSA may be accompanied by decrease in the exposure of the tryptophyl residues. This decrease in the tryptophan exposure may be brought about either because of covering of this residue by the steroid (direct interaction if the binding site is located in a narrow crevice) or because of burying of this amino acid into the hydrophobic interior of the protein due to conformation change caused by steroid-binding.

Our results of solvent perturbation for the interaction of testosterone with BSA do not provide clear-cut answers. Because of the marked influence of solvent, ethylene glycol, on testosterone interaction with BSA (fig. 5.c.3.) solvent perturbation was studied with binding

of testosterone sulfate to HSA. Fig. 5.c.4. (curves A) shows results which apparently give no clue as to whether or not tryptophan exposure is altered in the interaction because the difference peak (or shoulder) at 291 - 293 mμ was not distinctly seen even in the absence of steroid. This may be so because HSA contains only one tryptophan, whereas BSA contains two (section 2.a.).

5.c.2. NBS-oxidation of tryptophyl residues

Due to the inconclusiveness of the above results, a further attempt was made to decide about the origin of red-shifts accompanying the interaction of Δ^4-3-ketosteroids with BSA. Thus, the effect of modification of tryptophyl groups on the interaction was examined, using NBS as the oxidizing reagent, so that if direct interaction is involved it would be markedly affected (inhibited) by such a treatment. First of all titration of BSA was performed in acetate buffer, pH 4.0 according to the method (section 4.b.14) described by Spande and Witkop (1967). The results of this showed that out of the two tryptophan residues in BSA, 1.4 are exposed in the native protein under the above mentioned conditions. The value obtained by Herskovits and Sorensen (1968), 1 - 1.5 (50 - 70%) exposure (section 2.a.), by means of solvent perturbation with 20% glycerol is in agreement with ours. Table 5.c.1. shows data for testosterone-binding to untreated and NBS-treated preparations of BSA. It may be mentioned that the NBS-treated preparations were exhaustively dialyzed against deionized water before the determination of

binding values (section 4.b.14). The values in table 5.c.1., in fact, indicate that binding affinity of testosterone to BSA is increased after treatment of the protein with NBS. The increase is more marked with 10 moles of NBS/mole protein than with 5 moles.

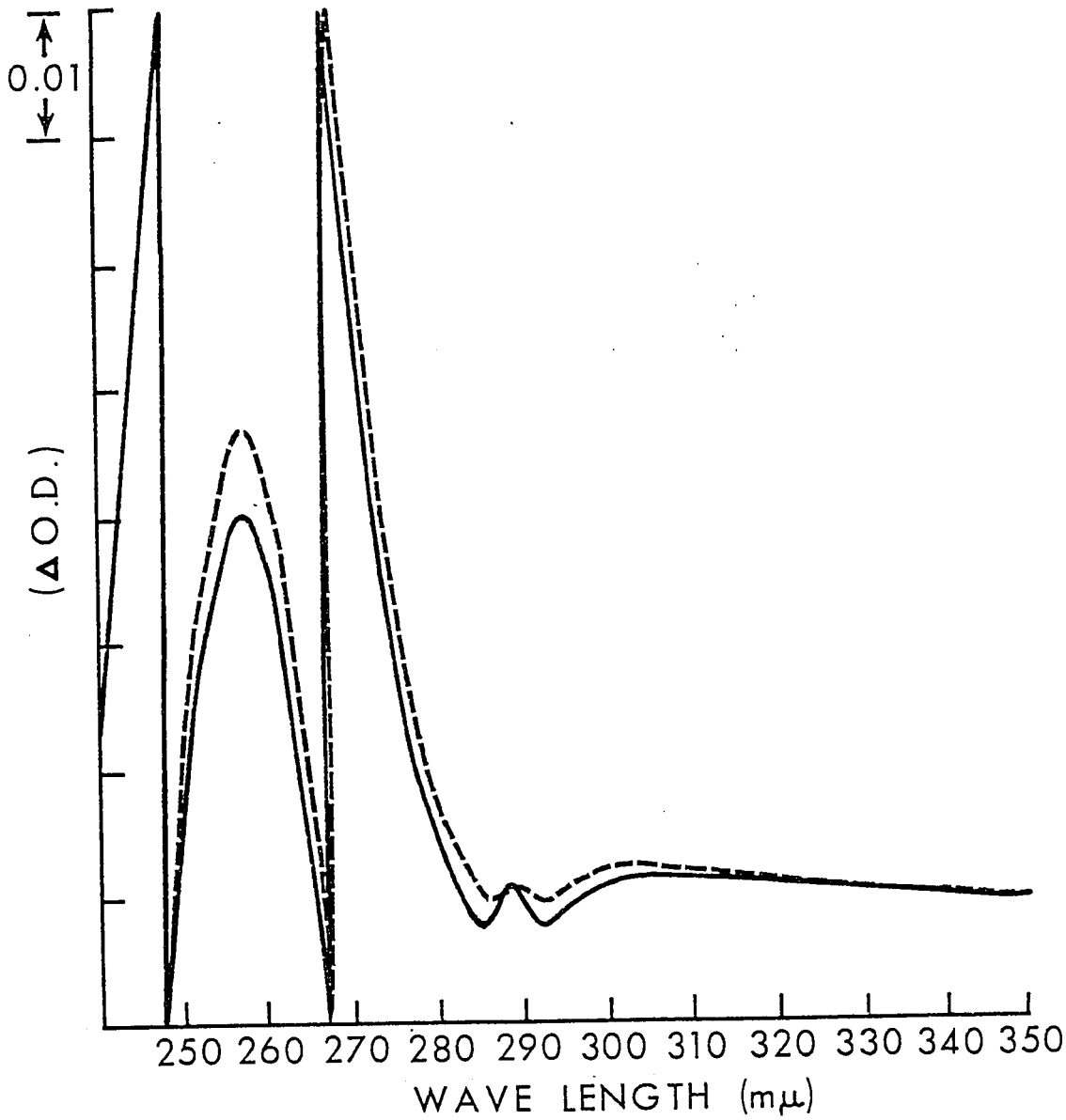
Difference spectra determined for the interaction of testosterone with untreated (dialyzed) and treated BSA preparations (not shown) showed that the magnitude of the steroid difference peak at 258 mμ is increased after oxidation of the protein with NBS. This provides additional support for increase in binding due to NBS treatment. As the tryptophan perturbations are not well marked in the testosterone-BSA (non-fatted) interactions, as found in section 5.b., the effect of NBS-oxidation was further examined with testosterone sulfate (which gives stronger aromatic perturbations) and results thereby obtained are represented in fig. 5.c.5. One can see that tryptophan perturbations are markedly reduced--though still evident--with NBS-treatment, but the height of the steroid difference peak is increased significantly in magnitude.

Increased binding of testosterone to BSA after NBS treatment appears to indicate that binding does not involve any direct interaction between the steroid and the tryptophan residues. It further suggests that the unmodified tryptophans in the native protein may actually inhibit steroid binding. This inhibition may be brought about in some manner by the influence of these residues on the protein structure

FIGURE 5.c.5.

EFFECT OF OXIDATION OF TRYPTOPHYL RESIDUES WITH NBS ON THE DIFFERENCE SPECTRUM FOR THE INTERACTION OF 32 μM BSA (LOT 30) WITH 116 μM TESTOSTERONE SULFATE

Conditions: Tris buffer, pH 8.0, I = 0.1, room temperature. See "Experimental" for details of procedures. _____, untreated BSA, ------, NBS-oxidized BSA. Both difference spectra were obtained in rectangular tandem cells (path length 0.438 cm/compartment). Refer to fig. 5.b.1. for an explanation of the base line.

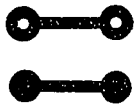

0.01
(ΔO.D.)
250 260 270 280 290 300 310 320 330 340 350
WAVE LENGTH (mμ)

at the binding sites. Further, this also suggests that binding sites for the steroid may be present in the region of the protein containing the tryptophanyl residues, i.e. in loops 3 and 4. This, however, does not merely mean that other parts of the protein are not involved in binding but rather it suggests that if in fact they are involved, they must be closely associated with the tryptophan residues in the tertiary structure of the protein. We have suggested in section 5.c.1. that tyrosine residues are directly involved in steroid binding and these are most likely the tyrosyl residues of loop 3 as our data in section 5.d. seems to indicate. But other studies (Pearlman and Fong, 1972; Feldhoff and Peters, 1975; Lantz and Pearlman, 1977) have clearly suggested that the tyrosine rich loop 6 (section 2.a.) must also be involved in the binding of steroids to the BSA. Since the binding affinities (nk values) of the loop 6 containing peptic fragment of BSA isolated by Pearlman and Fong (1972), (designated by the authors as fragment KL) are only small fractions of those for the native protein, loop 6 must contain some residues which either constitute separate binding sites in the intact protein or constitute a binding domain. The latter, however, would mean that in the native BSA-steroid complex loop 6 must be situated in the neighbourhood of loops 3 and 4. A similar possibility has also been suggested by Reed et al (1975) in the case of bilirubin binding to the BSA. These workers have shown that loop 6 perturbs the spectrum of bilirubin bound in loop 4 in the native BSA. The same would most likely also hold true in the case of steroid binding to this protein.

FIGURE 5.c.6.

PLOTS OF $(\Delta O.D.)_{258}$ VS. $\bar{\nu}$ FOR THE INTERACTION OF TESTOSTERONE WITH 32 μM UNTREATED AND NBS-TREATED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°.



Untreated BSA

NBS-treated BSA (5 moles NBS/ mole protein)

$(\Delta O.D.)_{258}$ values for each curve were derived from difference spectra for testosterone-BSA interaction measured at different testosterone concentrations and the corresponding $\bar{\nu}$ values determined from binding data obtained by means of equilibrium dialysis (see section 4.b.9. for procedure details). NBS treatment was performed as described in section 4.b.14.

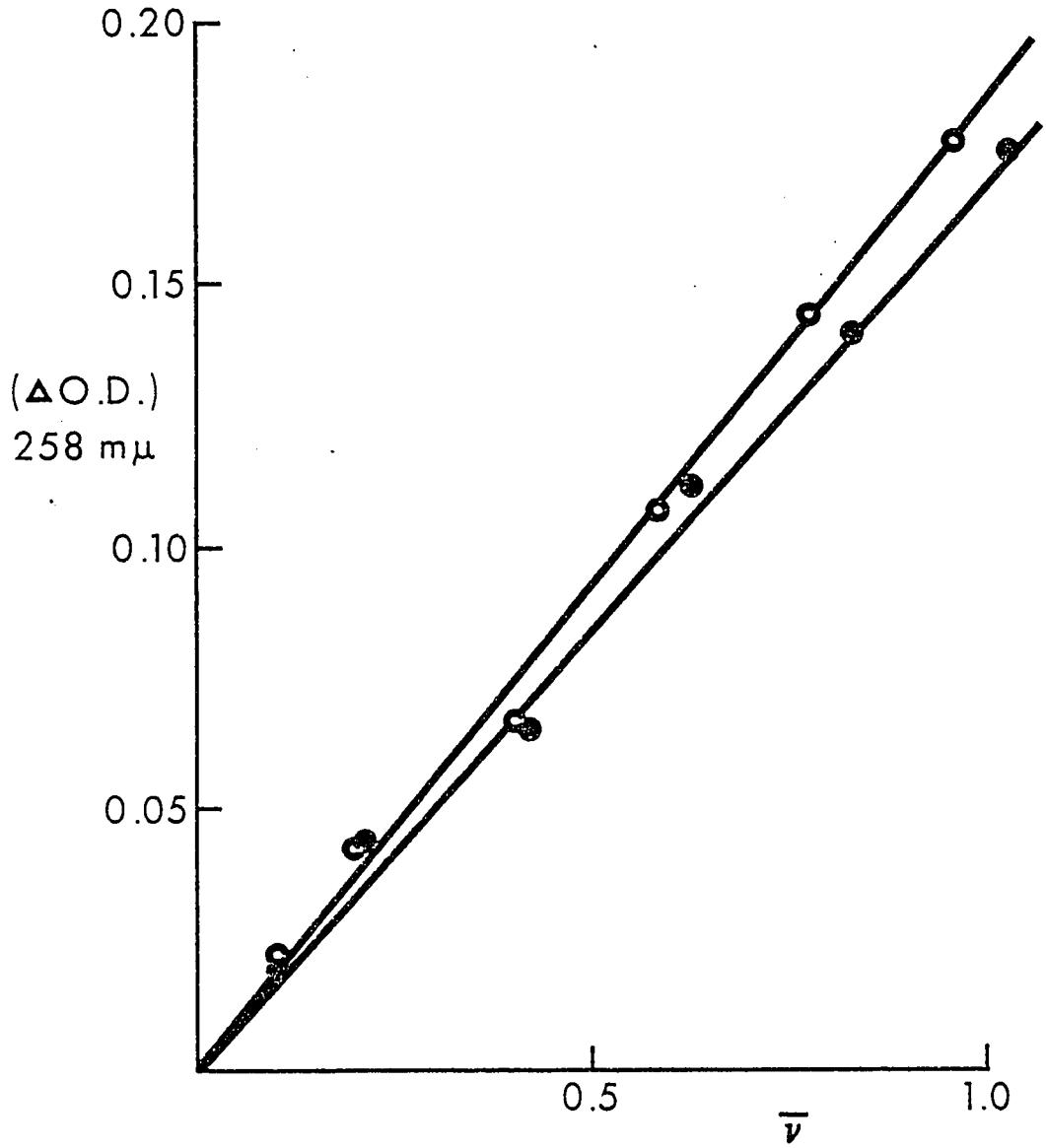

0.20
0.15
(ΔO.D.)
258 mμ
0.10
0.05
0.5
1.0
$\overline{\nu}$

TABLE 5.c.1.

EFFECT OF NBS-OXIDATION OF TRYPTOPHYL RESIDUES ON THE VALUES OF TESTOSTERONE-BSA (LOT 30) INTERACTION PARAMETERS

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°. Binding was determined by equilibrium dialysis. For details of procedures see sections 4.b.9. and 4.b.14.

Protein Concentration and Treatment	Interaction Parameters[a] $nkx10^{-4}$ (M^{-1})	n	$\Delta\varepsilon_{259}^{\bar{\nu}=1} \cdot 10^{-3}$[b]
32 μM untreated BSA[c]	1.90	14.0	5.86
32 μM oxidized BSA (5 moles NBS/mole protein)	2.20	7.0	5.20
32 μM untreated BSA[c]	2.12	9.0	
32 μM oxidized BSA (10 moles NBS/mole protein)	2.51	7.0	

[a] Determined from equilibrium dialysis data using reciprocal plots (section 5.a.).

[b] Values derived from slopes of curves in fig. 5.c.6. by dividing by protein concentration (32 μM).

[c] Control preparations which were dialized (prior to the binding measurements) together with the corresponding treated preparations.

It may be noted that although our data suggest increased binding of testosterone to BSA after NBS-oxidation of tryptophyl residues, those of Romeu et al (1975) indicate lowering of binding affinity of deoxycorticosterone after modification of these residues with 2-nitrophenyl-sulfonyl chloride (section 2.k.). This may be due to difference in the nature of two types of reagents. Further, studies of Abelson et al (1960) and those of Attallah and Lata (1968) have provided experimental evidences of a direct interaction between L-tryptophan and the steroid in aqueous solutions. However, the free energy values determined for these complexes by the latter workers are so low in relation to the values for the protein that they can hardly be considered as a proof of a direct interaction in the protein.

In an attempt to determine the cause of increase in binding affinity after NBS-treatment we further examined the effect of this treatment on the $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ value (section 5.b.) for the testosterone-BSA interaction. The data were first obtained as plots of $(\Delta O.D.)_{258}$ versus $\bar{\nu}$ (fig. 5.c.6.). It can be seen that these plots are linear both for the untreated and the NBS-treated proteins. The $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ values calculated from the slopes of these curves (see calculations in section 5.b.) are presented in table 5.c.1. The value for the untreated BSA and testosterone is higher than the values we have found for the interaction of this steroid and deionized (section 5.b.) and defatted and refatted (section 5.d.) albumins. The data in table 5.c.1. further suggest that

$\Delta\varepsilon_{258}^{\bar{\nu}=1}$ value for the NBS-treated BSA is significantly lower than that for the untreated protein. This appears to indicate that hydrogen-bonding of the steroid may be increased after treatment of the protein with NBS (refer to section 2.1 and 5.b). Since binding affinity of the steroid for the protein is also increased with this treatment, it suggests that this increase may be brought about by increase in hydrogen bonding between the steroid and the protein.

5.d. The effect of fatty acid on steroid-albumin interaction

5.d.1. Results

5.d.1.1. Fatty acid effect on binding

Table 5.d.1. shows the effect of palmitic acid (3 moles per mole protein) on the binding of testosterone to 32 μM defatted BSA and HSA. It can be seen that palmitic acid causes about 30% increase in binding affinity in the case of HSA, but a much greater increase of about 200% in the case of bovine albumin. The latter increase brings the nk value of BSA close to that of HSA. Palmitic acid causes no effect on the value of n in BSA, but it decreases the already high n value for HSA to a value which is closer to that for BSA. It may be noted that, at two different protein concentrations, nk values for untreated HSA are slightly higher than the corresponding values for deionized HSA. This confirms the earlier observations of Ryan and Gibbs (1970b) and is in agreement with the fatty acid effect. Both nk values for HSA are, however, higher than those of Ryan and Gibbs.

Binding data in table 5.d.2. shows that fatty acid has a stimulatory effect also on the binding of progesterone.

TABLE 5.d.1.

EFFECTS OF DEIONIZATION, DEFATTING AND REFATTING (3 MOLES PALMITIC ACID/MOLE PROTEIN) OF BSA AND HSA ON THE VALUES OF BINDING PARAMETERS FOR THEIR INTERACTION WITH TESTOSTERONE

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°. Binding measurements were performed by means of equilibrium dialysis, (see section 4.b.9. for procedure details) and the values (nk and n) determined using reciprocal plots (see section 5.a.1.).

Protein and Concentration (μM)	Untreated		Defatted		Defatted-Refatted (a)		Deionized	
	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n
Lot 31 Human Albumin: 32	5.92	9	5.4	7.5	6.64	5.3	-	-
			5.48	6.8	6.5	5.9	-	-
			5.47	7.7	6.6	6	-	-
Lot 32 Human Albumin: 32	5.46	10	-	-	-	-	5.26	7.7
			-	-	-	-	5.36	8.3
91	5.34	10	-	-	-	-	4.95	11
Lot 22 Bovine Albumin: 32			2.78	5.5	6.0	5.5	-	-

(a) Refatting was done by the Celite method (see section 4.b.3.).

TABLE 5.d.2.

EFFECT OF FATTY ACID ON THE VALUES OF BINDING PARAMETERS FOR THE INTERACTION OF PROGESTERONE WITH 32 μM BSA AND HSA

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°. Binding values (nk and n) were determined as mentioned in table 5.d.1.

Protein	Untreated		Defatted		Refatted[a] 3 Moles Palmitic		Refatted[a] 5 moles Lauric	
	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n	$nkx10^{-4}$ (M^{-1})	n
Lot 31 Human Albumin			11.05	5.9	14.2[i]	3.8		
			10.4	5	13.3[b]	4	14.8	3
					13.9[b]	4		
Lot 32 Human Albumin	10.16[c]	4					12.58[c]	2.7
Published data of Westphal and Harding (1971a) for Human Albumin	9.0	2	41[d,e]	3			20[d]	2
			19[f,e]	4.2			3[g,d]	2
							11[f,h]	2
Lot 22 Bovine Albumin			2.55	25	8.6	5	12.3	3.2
			2.46	15				
Lot 30 Bovine Albumin	2.9	>20					12.4[g]	3.2

[a] Fatty acid was added to the protein by alcohol method. [b] Baker palmitic acid was used instead of Sigma product. [c] Equilibrium dialysis performed under the conditions of Westphal and Harding (1971a) in phosphate buffer, pH 7.6, 4°. [d] Data of Westphal and Harding (1971a) on an unspecified protein preparation. [e] Protein was defatted with chloroform/methanol. [f] Data of Westphal and Harding (1971a) on an unspecified preparation. [g] Lauric acid was added to untreated protein. [h] 5 moles myristic acid instead of lauric acid. [i] Palmitic acid was added using the Celite method (see section 4.b.3.); no ethanol was added to the defatted protein.

This table also includes the data of Westphal and Harding (1971a) on the inhibitory effect of fatty acid on the binding of progesterone to HSA.

Because of the contradiction between our results and findings of these authors, we examined the fatty acid effect with different lots of BSA and HSA and used two different methods (celite method and alcohol method, section 4.b.3.) for the addition of fatty acids to the two proteins. In order to further prevent doubt, the fatty acid effect was studied with fatty acid samples derived from two different sources (Baker and Sigma). Finally the experiments were also performed under the conditions used by Westphal and Harding, i.e. in phosphate buffer, pH 7.4 and 4^{o}. It can be seen from table 5.d.2. that the fatty acid effect is invariably stimulatory in all these experiments.

The data in table 5.d.2. further indicates that, as in the case of testosterone binding, the stimulatory effect of fatty acid is more marked on the binding of progesterone to BSA than its binding to HSA. Thus 3 moles of palmitic acid caused 33% increase in binding affinity and reduction of n value from approximately 6 to 4 in the case of HSA and 244% increase in nk and a decrease in n value from approximately 20 to 5 in the case of BSA. From these data it is also evident that the effect of fatty acid is greater on the binding of progesterone than on the binding of testosterone. Data in table 5.d.2. also suggests that the stimulatory effect on the binding of

progesterone is greater with 5 moles of lauric acid than with 3 moles of palmitic. It is further seen that nk and n values in the presence of 5 moles of lauric acid are almost identical for BSA and HSA. It may be pointed out that in spite of the contradiction between our data and that of Westphal and Harding (1971a), our nk value for the untreated HSA is reasonably close to the value observed by these authors for the same protein. It may be also mentioned that although our reciprocal plots for progesterone binding to both defatted and refatted HSA (not shown) were linear over the binding range we covered, deviation from linearity is reported in the data of Westphal and Harding over the same binding range.

5.d.1.2. Fatty acid effect on difference spectra

Figures 5.d.1. and 5.d.2. show the effects of 3 moles of palmitic acid/mole protein on the difference spectra for the interaction of testosterone with BSA and progesterone with BSA and HSA. In all of the difference spectra the steroid difference peak (section 5.b.) is located at 258 mμ and its location is thus unaffected by the presence of fatty acid. However, the height of this peak is increased after addition of fatty acid to the protein, the increase being more marked in the case of BSA than in the case of HSA. This behavior is in keeping with the greater stimulatory effect of fatty acid observed on the nk value for BSA than for HSA.

FIGURE 5.d.1.

EFFECT OF PALMITIC ACID (3 MOLES/MOLE PROTEIN) ON THE DIFFERENCE SPECTRUM FOR THE INTERACTION OF TESTOSTERONE WITH 32 μM BSA (LOT 22)

Conditions: Tris buffer, pH 8.0, I = 0.1, room temperature. Palmitic acid was added to the protein by celite method (see section 4.b.3.). Difference spectra were obtained as described in section 4.b.11.

------, 16 μM testosterone and defatted protein, _____, 16 μM testosterone and refatted protein. Insert: ------, 68 μM testosterone and defatted protein, _____, 31.4 μM testosterone and refatted protein.

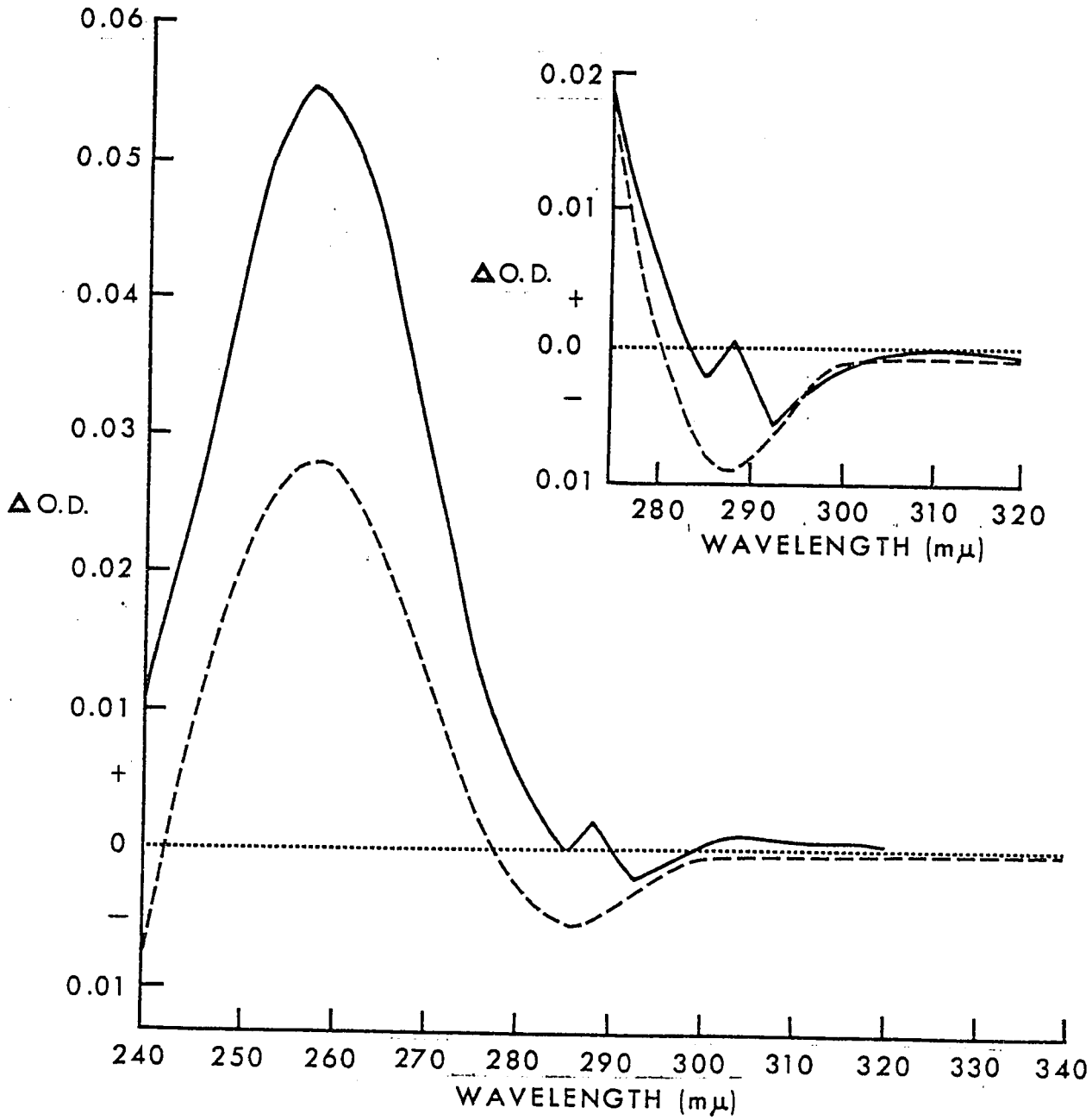

0.06
0.05
0.04
0.03
0.02
0.01
+
0
−
0.01
ΔO.D.
240 250 260 270 280 290 300 310 320 330 340
WAVELENGTH (mμ)
0.02
0.01
ΔO.D.
+
0.0
−
0.01
280 290 300 310 320
WAVELENGTH (mμ)

FIGURE 5.d.2.

EFFECT OF PALMITIC ACID (3 MOLES/MOLE PROTEIN) ON THE DIFFERENCE SPECTRUM FOR THE INTERACTION OF PROGESTERONE WITH 32 μM BSA AND HSA

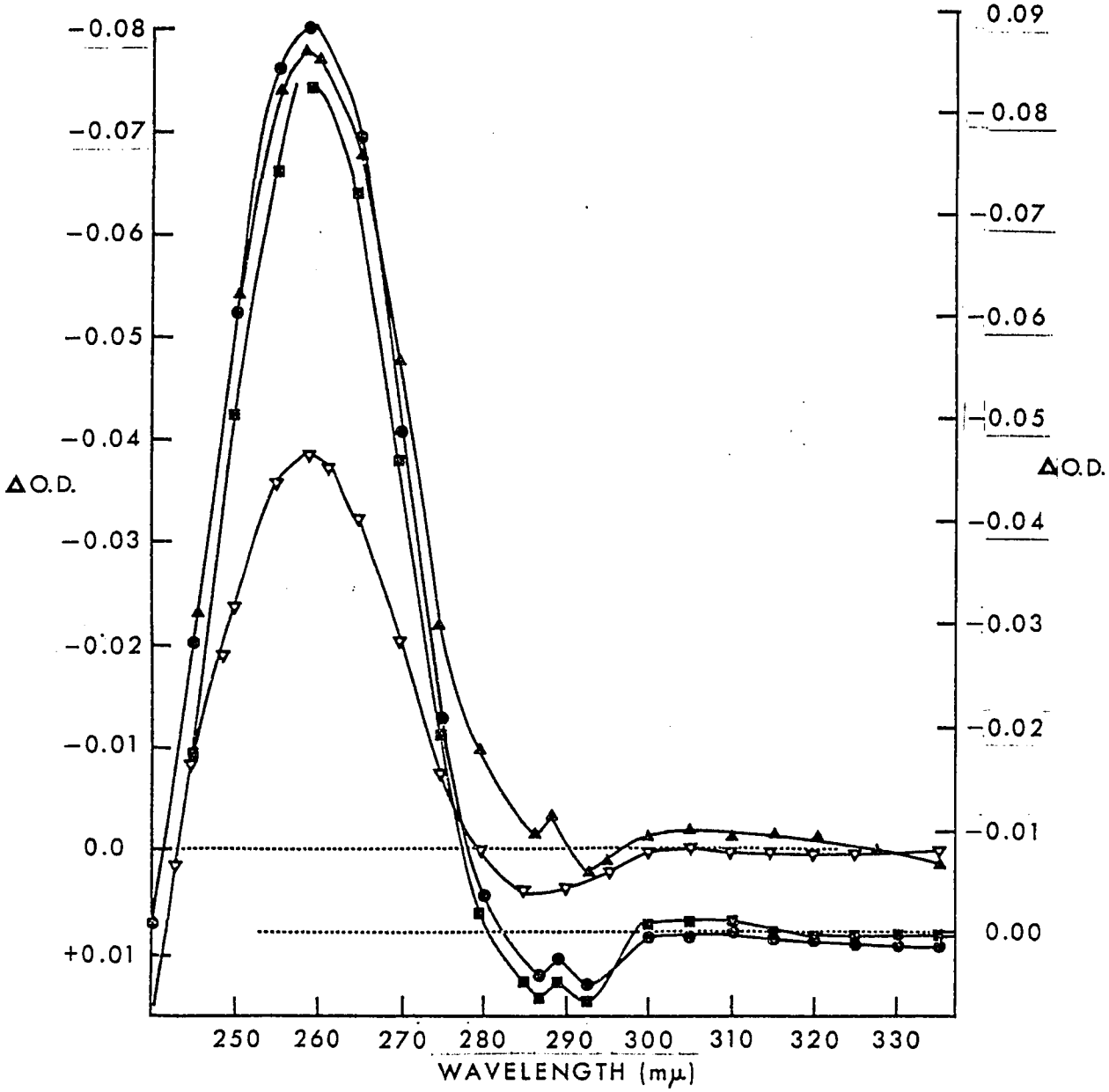
Conditions: Tris buffer, pH 8.0, I = 0.1, room temperature. For procedures see sections 4.b.3. and 4.b.11.

■——■ 21.7 μM progesterone and defatted HSA (lot 31)

●——● 21.7 μM progesterone and refatted HSA (lot 31)

△——△ 20.9 μM progesterone and defatted BSA (lot 22)

▲——▲ 20.9 μM progesterone and refatted BSA (lot 22)


−0.08
−0.07
−0.06
−0.05
−0.04
ΔO.D.
−0.03
−0.02
−0.01
0.0
+0.01
0.09
−0.08
−0.07
−0.06
−0.05
ΔO.D.
−0.04
−0.03
−0.02
−0.01
0.00
250 260 270 280 290 300 310 320 330
WAVELENGTH (mμ)

The difference spectrum in the aromatic region for the interaction of progesterone with defatted HSA shows two minima which are located at 286.5 and 292.5 mμ. The positions of these minima are identical in location to these observed by Ryan (1968) for the interaction of progesterone with deionized HSA. It can be seen from fig. 5.d.2. that the fatty acid shows no significant effect on either the location or the depth of these minima in the case of HSA. However, the aromatic difference spectra in the case of defatted BSA are seen as single broad troughs each located around 286 mμ. After addition of fatty acid this difference trough is shifted towards higher wavelength side and simultaneously split into two minima which are identical in location to those seen for the defatted and refatted HSA. This is evident even at 1mole fatty acid (not shown).

Table 5.d.3. shows the effect of fatty acid on the spectral molar perturbation of bound steroid for HSA and BSA. These values were derived from plots (not shown) of $(\Delta O.D.)_{258}$ versus $\bar{\nu}$ in the manner described in section 5.b. All of these plots, except that for testosterone-defatted HSA interaction, were linear over the binding range covered in the difference spectra. In the case of testosterone-HSA the data could be drawn as two intersecting straight lines. These, therefore, gave two values of spectral molar perturbation which are given in table 5.d.3. along with their corresponding binding ranges. It can be seen that $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ values are increased after addition of fatty acid to the

TABLE 5.d.3.

EFFECT OF ADDITION OF PALMITIC ACID (3 MOLES/MOLE PROTEIN) TO 32 μM DEFATTED BSA AND HSA ON THE SPECTRAL MOLAR PERTURBATION OF BOUND STEROID

Conditions: Tris buffer, pH 8.0, I = 0.1, 25°.

Interaction	$\Delta\varepsilon_{258}^{\nu=1}$ [a]	
	Defatted	Refatted [b]
TESTOSTERONE-BSA (Lot 22)	4.1	5.55
PROGESTERONE-BSA (Lot 22)	4.9	5.60
PROGESTERONE-HSA (Lot 31)	4.85	5.07
TESTOSTERONE-HSA (Lot 31)	5.16 (0 - 0.6) [c] 4.16 (0.6 - 1.6) [c]	5.40

[a] Values determined in the same manner as described in table 5.b.1.

[b] Celite method (section 4.b.3.) was used for the addition of fatty acid to the protein.

[c] Range of $\bar{\nu}$ values determined by equilibrium dialysis.

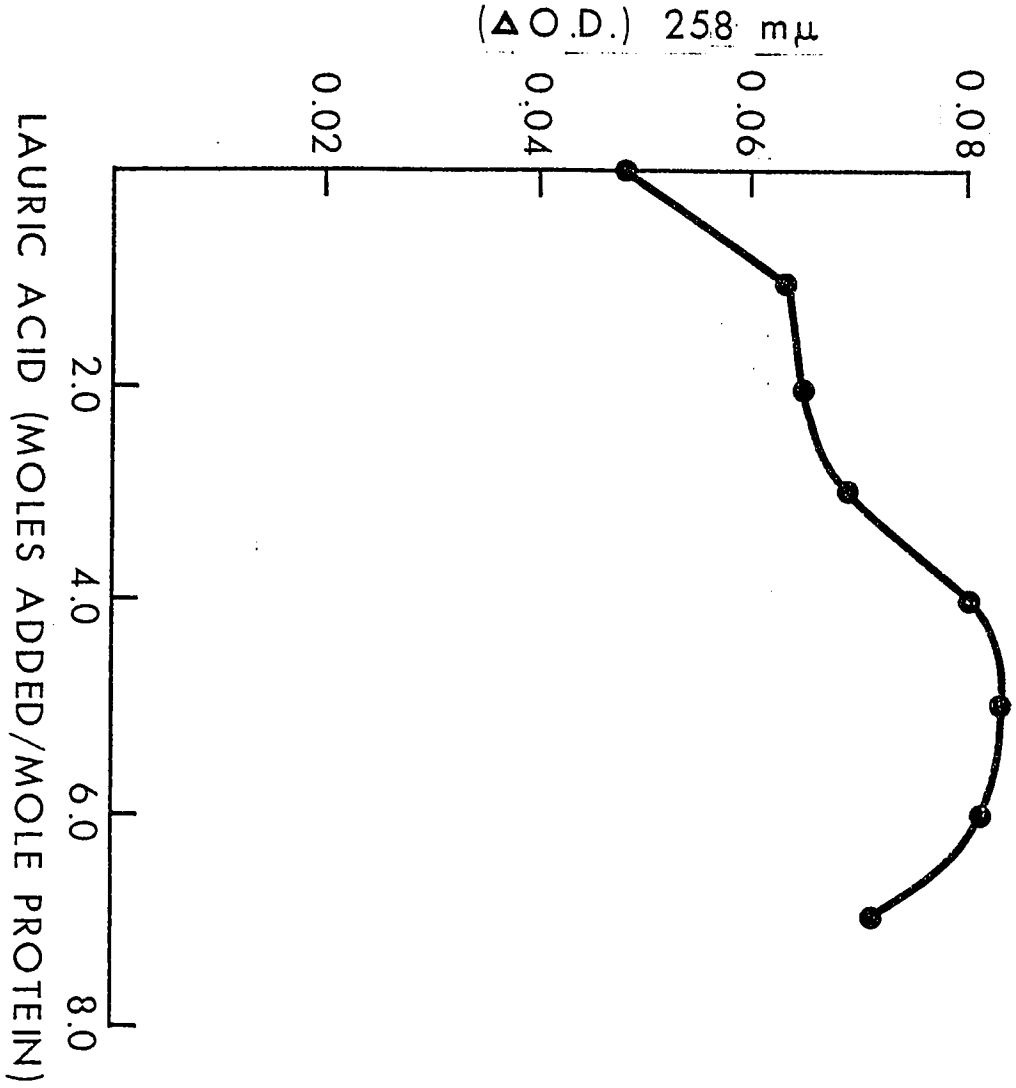
protein, although the effect is quite small in the case of progesterone-HSA interaction. Since the plot for testosterone-HSA was linear after the addition of fatty acid, it gave a single value of $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ which is higher than either of the two for the defatted protein. From these values it appears that fatty acid has a greater effect in the higher binding range than in the lower binding range. Further, the values for the defatted and refatted proteins in the case of testosterone are closer to the value obtained by us in the case of deionized BSA (section 5.b.). However, the value for the interaction of progesterone with deionized BSA in section 5.b. is much lower.

Figure 5.d.3. shows the plot of $(\Delta O.D.)_{258}$ versus moles of lauric acid added to BSA per mole protein. From this curve it is evident that fatty acid causes an increase in binding between testosterone and BSA and that the effect is strongest for the first mole. The second and third moles have very small effects. Soltys and Hsia (1977) found a similar but not identical effect of fatty acid levels on the binding of GABA-DNP-SL to HSA. Then the effect is increased sharply for the fourth mole and subsequently stays constant until the sixth mole has been added. Addition of the seventh mole of fatty acid causes lowering of the $(\Delta O.D.)_{258}$ value which suggests inhibition of steroid binding. It should be mentioned that the points on the curve in fig. 5.d.3. are values of single determinations.

FIGURE 5.d.3.

VARIATION OF THE HEIGHT OF STEROID DIFFERENCE PEAK $[(\Delta O.D.)_{258}]$ WITH NUMBER OF MOLES OF LAURIC ACID ADDED PER MOLE PROTEIN IN THE INTERACTION OF 23.3 μM TESTOSTERONE WITH 32 μM DEIONIZED BSA (LOT 30)

Conditions: Tris buffer, pH 8.0, I = 0.1, room temperature. Fatty acid was added by alcohol method (section 4.b.3.). $(\Delta O.D.)_{258}$ values were determined from difference spectra measured in the manner described in section 4.b.11. Each point on the curve is a single value determination.


(Δ O.D.) 258 mμ
0.02
0.04
0.06
0.08
2.0
4.0
6.0
8.0
LAURIC ACID (MOLES ADDED/MOLE PROTEIN)

5.d.2. Discussion

Our data show a stimulatory effect of fatty acid on the binding of testosterone and progesterone to the two serum albumins. This effect is contrary to the inhibitory influence of fatty acid reported in the literature on the binding of a variety of other ligands to serum albumin (Spector et al, 1973; Peters, 1975; Spector, 1975). As mentioned earlier the contradiction is also found with the results of Westphal and Harding (1971a) who have shown lowering of both nk and n values in the binding of progesterone to human albumin. Our results indicate that the latter disagreement is not due to difference in the experimental conditions used in the measurement of binding. However, it could be due to difference in the methods used for defatting the proteins. Westphal and Harding defatted HSA by treatment with chloroform/methanol at 4^{o}. This treatment appears to be more drastic compared to Chen's defatting procedure used by us. The Chen procedure is considered to be the mildest available (Soetewey et al, 1972), although protein defatted by this treatment also shows slightly different binding behavior compared to the deionized protein (Steinhardt et al, 1972). However, our results (Ryan and Chopra, 1976) find some support in the recent observations of Soltys and Hsia (1977) showing a stimulatory effect of fatty acid on the binding of GABA-DNP-SL to HSA. The increase in the height of the difference peak at 259 mμ caused by fatty acid provides additional support in favor of the fatty acid induced stimulation of steroid binding.

Our data indicate that the increase in steroid-binding

affinity produced by fatty acid is much more marked in the case of BSA than in the case of HSA so that, in fact, after addition of fatty acid to the defatted BSA, the values of binding affinity and number of sites almost approach those for the refatted HSA. Thus fatty acid appears to eliminate the difference in the steroid-binding properties of the two proteins which are already known in the literature (section 2.e.1.). Data in section 5.c.2. have indicated that binding sites for the steroids are located in the regions of BSA containing the tryptophyl residues. Further, discussion in section 5.b. revealed that interaction of fatty acid with BSA may result in movement of positively charged residues from other parts of the molecule into the neighborhood of the tryptophyl groups. Since the positively charged groups appear to be involved in steroid-BSA interaction (Swaney and Klotz, 1970; Romeu et al, 1976), it seems probable that the greater stimulatory effect in the case of BSA might result from increase in the interaction of steroid with positively charged residues at the binding sites. However, since the interaction of fatty acid with HSA involves no alteration in the charge environment of tryptophyl residues (section 5.b.), the fatty acid effect in this case (and any additional effect in the case of BSA) must involve some other type of change (see below).

Since the position of steroid difference peak (at 258 mμ) is unaffected by the addition of fatty acid in the case of both proteins, it seems that fatty acid does not affect the hydrophobicity of steroid-binding sites.

Increase in the value of spectral molar perturbation of bound steroid brought about by fatty acid appears to suggest that binding of the steroid at hydrophobic sites may be increased and/or its binding at the non-hydrophobic sites may decrease (see Ryan and Gibbs, 1970b). It seems that, in the case of BSA, fatty acid may increase binding affinity at the hydrophobic sites by facilitating the interaction of the steroid with positive residues at these sites (as noted above) so that fraction of the steroid which is hydrophobically bound is increased. Similarly in the case of HSA the fatty acid may bring about an increase in hydrogen-bonding of the steroid with some uncharged residues of the protein other than tyrosines (see below) at the hydrophobic sites. Because of the blue shift associated with transferring of the steroid from the aqueous into the hydrophobic phase (Ryan, 1968), the magnitude of steroid difference peak will be greater and therefore $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ values will be higher when more steroid is bound at hydrophobic sites than at non-hydrophobic sites. Our data suggest that the addition of fatty acid to the BSA or HSA results in decrease in the number of steroid-binding sites. It seems that some (or all) of the non-hydrophobic sites may actually disappear after addition of fatty acid as this will further account for the fatty acid induced increase in the $\Delta\varepsilon_{258}^{\bar{\nu}=1}$ values. Thus the stimulation of binding in the case of both BSA and HSA may result from increased hydrogen-bonding of the steroid at the hydrophobic sites.

Difference spectra in the aromatic region for the interaction of testosterone and progesterone with defatted BSA (a broad trough at 286 mμ) are indicative of perturbations of mainly tyrosine residues, whereas those for the interaction of the two steroids with refatted BSA (two minima at 286.5 and 292.5 mμ) suggest perturbations characteristic of the tryptophyl groups (see sec. 2.1.). The tyrosyl perturbations in the case of defatted BSA most likely arise due to hydrogen-bonding of the steroid with tyrosyl residues (section 5.c.1.) in loop 3 (section 5.c.2.) which is rich in tyrosine (fig.2.a.1.). However, tryptophan perturbations in the refatted BSA may result from decrease in the exposure of tryptophan induced by the steroid (section 5.c.1.) in either loop 3 or 4 (section 5.c.2.). Thus two explanations can be offered for the alterations in aromatic perturbations brought about by the fatty acid: (1) Binding of the steroid takes place in loop 3 before and after the addition of fatty acid. In this case fatty acid may inhibit hydrogen-bonding between the steroid and tyrosyl residues and thereby bring about decrease in the tyrosyl perturbations. Since affinity of the binding sites is increased after addition of fatty acid, as noted above, steroid-binding at the higher affinity sites may result in greater decrease in tryptophan exposure giving rise to enhancement of tryptophan perturbations. (2) Steroid binding is shifted from loop 3 to loop 4. According to this view, fatty acid may completely destroy binding of the steroid

in loop 3 and initiate its binding in loop 4. Since loop 4 contains no tyrosine residue (fig. 2.a.1.), tyrosyl perturbations will not arise. The tryptophan perturbations in loop 4 will arise in the same manner as described above in the first explanation. It may be noted that fatty acid can bring about these changes in the BSA as a result of binding-induced conformation changes in the protein. Evidence for the fatty acid induced conformation changes in the BSA has been presented by Soetewey et al (1972).

Out of the two possibilities suggested above the second one seems more plausible. It may be recalled that in section 5.b. difference spectroscopic results for the interaction of steroids with deionized BSA have suggested that in this protein binding of progesterone involves loop 3 and that of testosterone loop 4. This conclusion was in keeping with the results of competition study in section 5.a. for the interaction of the two steroids with the deionized BSA. Since deionized BSA is known to contain traces (up to one mole) of fatty acid (section 4.b.4.; Polet and Steinhardt, 1968), the latter may be responsible for the initiation of testosterone binding in loop 4. However, the amount of residual fatty acid in the deionized BSA may be too small to abolish binding of progesterone in loop 3. That after addition of fatty acid binding of both steroids takes place in the same loop is suggested by competition data obtained in section 5.a. with BSA in the presence of 5 moles of lauric acid. The strongest evidence

in support of the binding of both steroids in loop 4 in the refatted BSA is that the binding parameters of both BSA and HSA have nearly the same values after the addition of 5 moles of lauric acid. Since the lone tryptophan in HSA is present in loop 4, the binding to this protein will probably take place only in that loop (section 5.c.2.) and the same would also be true in the case of the refatted BSA. It is evident from fig. 2.a.1. that while the amino acid sequences of loop 4 in the two proteins are almost identical, those of the loop 3 are very dissimilar. It may be pointed out that after addition of fatty acid the positions of the aromatic minima observed in the interaction of testosterone and progesterone with the two proteins are also similar (286.5 mμ and 292.5 mμ). It seems probable that similar binding behaviors will result from binding of the steroid in identical loops, i.e. in loop 4.

Our data suggest that the first mole of fatty acid has the largest effect on steroid-BSA interaction, i.e. on $(\Delta O.D.)_{258}$. Goodman (1958) showed that fatty acid binding sites in the BSA are heterogenous and that the first mole is bound with highest affinity (sec. 2.b.). The discussion in this section has indicated that the stimulatory effect of fatty acid on steroid-binding is exerted through a fatty acid induced conformation change in the protein. The studies of Reed et al (1975) indicate that the highest affinity site (first site) for fatty acid is located in BSA in the region of loops 7 and 8. However, in view of the linear model of

BSA proposed by Anderson and Weber (1969), it is difficult to imagine how the conformation change induced by fatty acid in this region would traverse a long distance along the length of the molecule (particularly across the connections between the globular regions) to affect steroid binding in loops 3 and 4. The second and third fatty acid sites in BSA are located in the regions involving residues 239 - 306 (containing the entire loop 5 and a part of the loop 4) and 307 - 377 (containing loop 6) respectively (Reed et al, 1975). But our data in fig. 5.d.3. suggest that fatty acid binding at these sites has no significant influence on testosterone binding to the BSA. The locations of other fatty acid sites in the BSA are not yet known. An elucidation of these in the future might explain the shape of the curve in fig. 5.d.3. in the region of higher fatty acid levels, particularly if these sites are found in the region of loop 3.

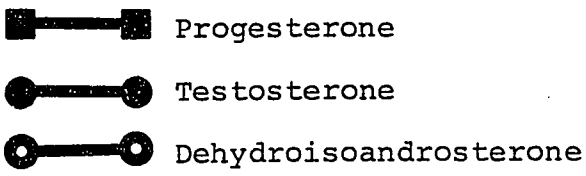
5.e. Effect of steroids on tryptic hydrolysis of BSA

In figs. 5.e.1. and 5.e.3. are presented plots of % inhibition or stimulation (in the case of progesterone) of tryptic hydrolysis of BSA against steroid concentration for testosterone, progesterone, dehydroisoandrosterone (fig. 5.e.1.) and dehydroisoandrosterone sulfate (fig. 5.e.3.). Comparison of these curves indicates quite clearly that there are marked qualitative and quantitative differences in the influence of the four steroids on tryptic digestibility of bovine albumin, It has been shown

FIGURE 5.e.1.

DEPENDENCE OF % INHIBITION OR STIMULATION OF TRYPTIC HYDROLYSIS OF 91 μM DEIONIZED BSA (LOT 30) ON STEROID CONCENTRATION

Values derived from rates of hydrolysis determined for 10 min. reaction in the pH - stat in 0.1M NaCl at pH 8.5 and 25° and 25 mg BSA and 1.6 mg trypsin (1.25% ethanol) in the absence and presence of each steroid concentration (see section 4.b.13. for procedure details). $[S]_t$ means total steroid concentration.



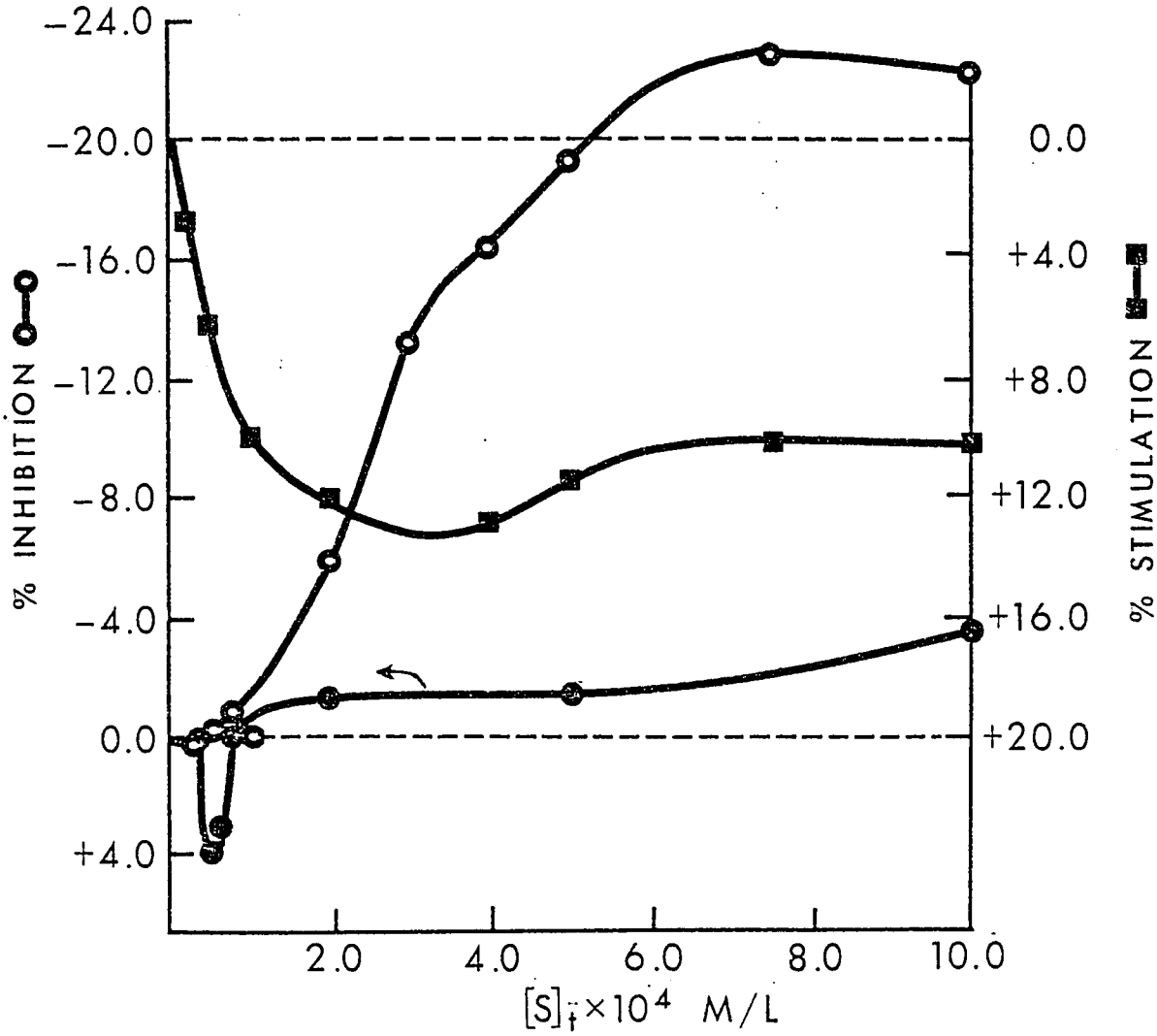
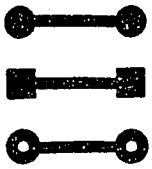

-24.0
-20.0
-16.0
-12.0
-8.0
-4.0
0.0
+4.0
% INHIBITION
0.0
+4.0
+8.0
+12.0
+16.0
+20.0
% STIMULATION
2.0
4.0
6.0
8.0
10.0
[S]$_i$×10^4 M/L

FIGURE 5.e.2.

VARIATION OF % INHIBITION OR STIMULATION OF TRYPTIC HYDROLYSIS OF 91 μM DEIONIZED BSA (LOT 30) WITH LEVEL OF BOUND STEROID ($\bar{\nu}$)

Rates of hydrolysis were determined with pH - stat (refer to fig. 5.e.1.) and $\bar{\nu}$ values obtained from binding measurements performed under the conditions of pH - stat in the absence of trypsin and presence of 5% Tris buffer, pH 8.5 by means of equilibrium dialysis (see section 4.b.9.).



Testosterone

Progesterone

Dehydroisoandrosterone

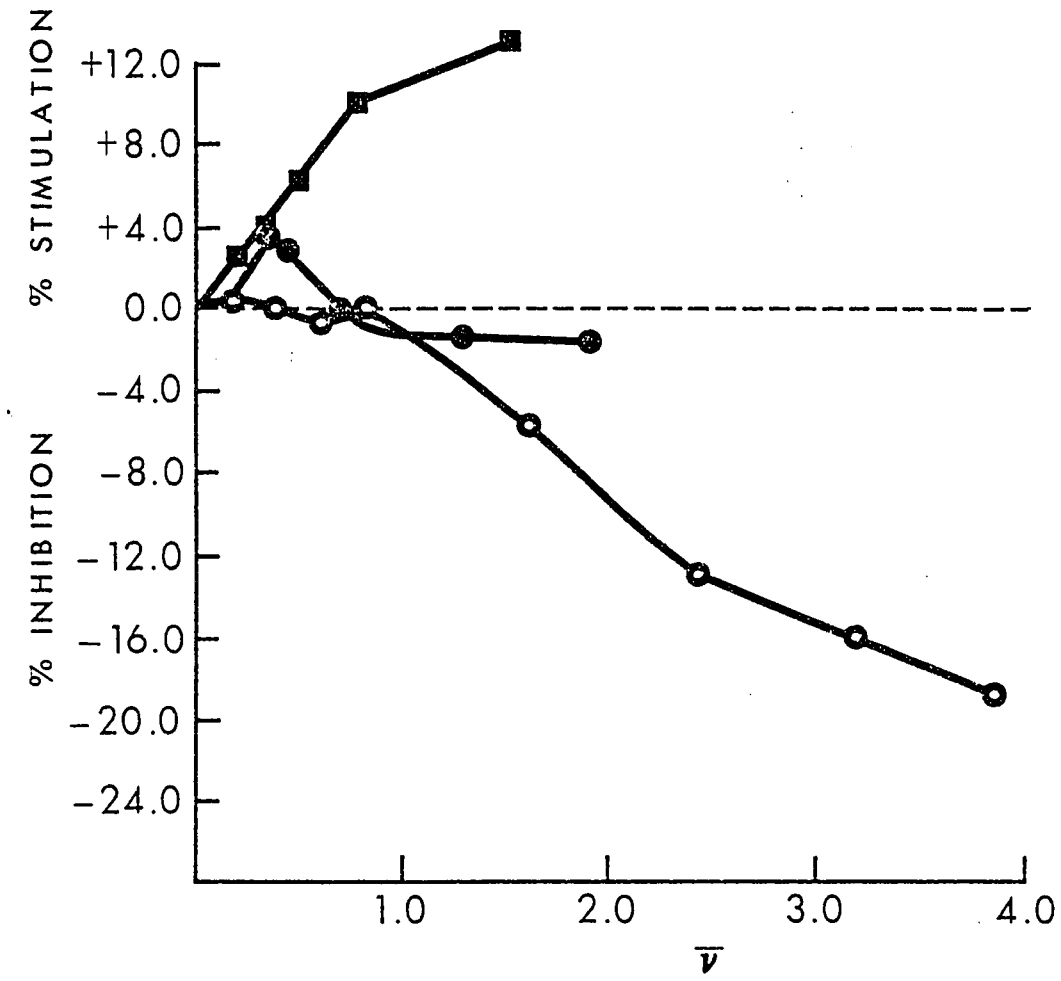
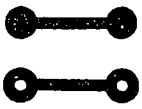

% STIMULATION
% INHIBITION
+12.0
+8.0
+4.0
0.0
-4.0
-8.0
-12.0
-16.0
-20.0
-24.0
1.0
2.0
3.0
4.0
$\overline{\nu}$

FIGURE 5.e.3.

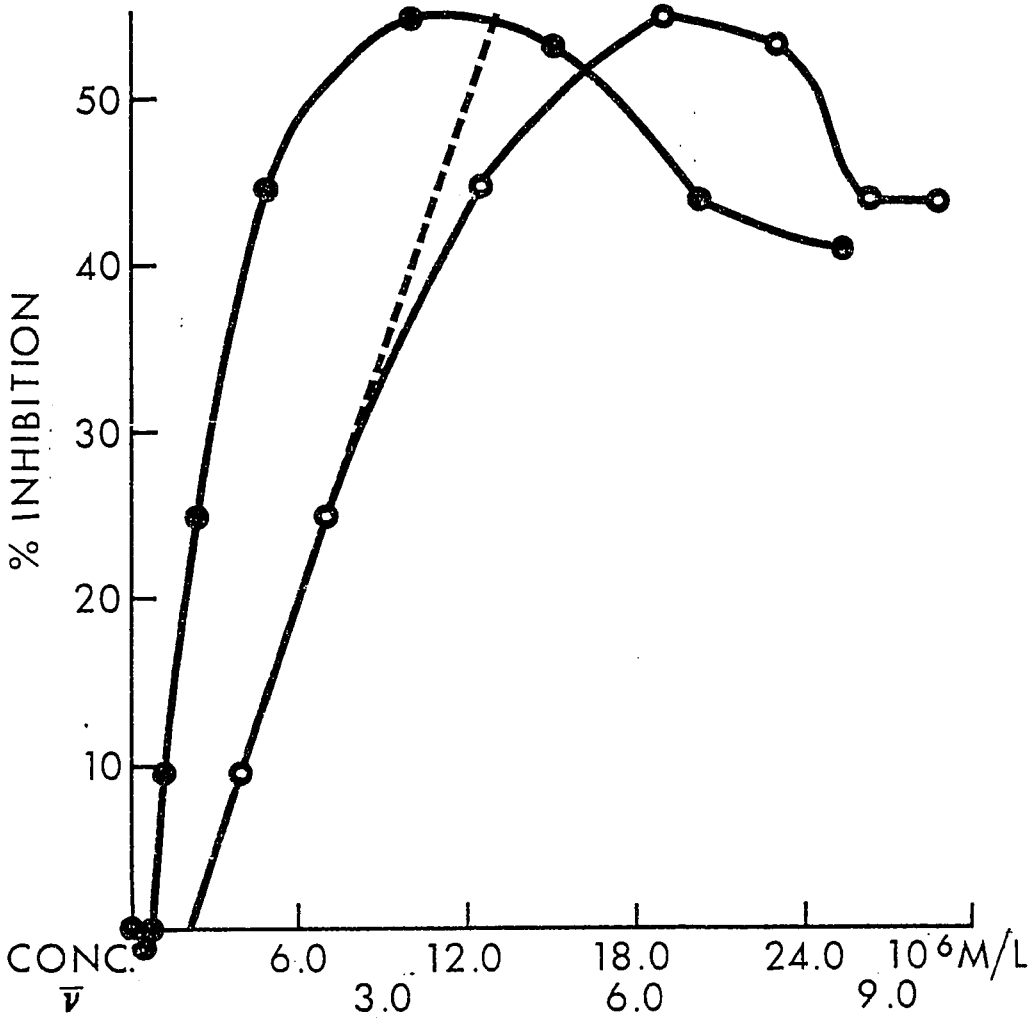
DEPENDENCE OF % INHIBITION OF TRYPTIC HYDROLYSIS OF 91 μM DEIONIZED BSA (LOT 30) ON STEROID CONCENTRATION AND LEVEL OF BOUND STEROID ($\bar{\nu}$) FOR DEHYDROISOANDROSTERONE SULFATE

The values of % inhibition and $\bar{\nu}$ were determined as mentioned respectively in figs. 5.e.1. and 5.e.2. $[S]_t$ means total steroid concentration.



Steroid concentration

Level of bound steroid ($\bar{\nu}$)


% INHIBITION
50
40
30
20
10
CONC.
$\bar{\nu}$
6.0
12.0
18.0
24.0
10^6M/L
3.0
6.0
9.0

previously by Ryan (1973) that the three steroids, testosterone, progesterone and dehydroisoandrosterone exert no significant effect on the activity of trypsin in assays using synthetic ester, Tos-Arg-oMe, instead of albumin as the substrate for the enzyme. A very low concentration of trypsin was used in the Tos-Arg-oMe assays. However, for the determination of trypsin activity towards albumin we have used much higher concentrations of trypsin (200 times), so that any minor influences on the specific activity of the enzyme are considerably diluted out (Ryan, 1973). Since the concentration of albumin relative to enzyme in these assays was very low, the reaction was essentially substrate limited. The results presented in figs. 5.e.1. and 5.e.3. showing influences on the apparent activity of trypsin determined under these assay conditions are therefore mainly attributable to the binding of steroids to albumin.

In our experiments we have also measured the binding of the four steroids under the conditions of the pH - stat by means of equilibrium dialysis and the results obtained are presented in table 5.e.1. It can be seen that the binding affinities of these steroids are markedly different. Since Ryan et al (1977) found that the steroid-albumin complexes are unstable under the pH - stat conditions (unbuffered solutions), the binding values, except for those given in the parentheses, were determined in the presence of 5% Tris buffer, pH 8.5.

TABLE 5.e.1.

BINDING PARAMETERS FOR THE INTERACTION OF STEROIDS WITH 91 μM DEIONIZED BSA (LOT 30)

Binding was measured by equilibrium dialysis under the conditions of pH - stat (0.1 NaCl, pH 8.5, 25°, 1.25% ethanol) in the absence of trypsin and in the presence of 5% Tris buffer, pH 8.5.

	Binding Parameters [a]			
Steroid	$nkx10^{-4}$ (M^{-1})	n	$\Delta G_1^{o'}$ [b] (kcal/mole)	$\Delta G_2^{o'}$ (kcal/mole)
TESTOSTERONE	1.74 (1.457)	12.0 (4.0)	-0.276	-5.73
PROGESTERONE	2.90 (1.732)	32.0 (26)	-0.055	-6.08
DEHYDROISO-ANDROSTERONE	4.17	10.0	-0.778	-6.30
DEHYDROISOANDRO-STERONE SULFATE	6.76 0.85	8.0 15.0		

[a] Values for testosterone, progesterone and dehydroisoandrosterone were calculated as described in section 5.a. and those for dehydroisoandrosterone sulfate were determined from a Scatchard plot (see fig. 5.e.4.). Values in parentheses represent those measured in the absence of Tris buffer.

[b] Calculated using steroid solubility values of table 5.a.1. determined in Tris buffer, pH 8.0, I = 0.1.

It is evident from table 5.e.1. that the values for testosterone and progesterone determined in the presence of 5% Tris buffer are higher than those determined in its absence. For this reason only the binding data determined in the presence of Tris buffer were employed to correlate the effects on the rates of tryptic hydrolysis with the level of bound steroid (see below). It may be pointed out that all the values of binding affinity given in table 5.e.1. are lower than those obtained in section 5.a. (table 5.a.1.) for the binding of these steroids to 32 μM deionized BSA in Tris buffer pH 8.0 in the presence of almost negligible concentration of ethanol or no ethanol. This may be due to difference in the experimental conditions of binding measurements. It has been reported that binding affinity of steroid-albumin interaction decreases with increasing protein concentration (section 2.j.4.) and in the presence of 1.25% ethanol (Ryan et al, 1977).

Although it might appear so, the differences observed among the four steroids in figures 5.e.1. and 5.e.3. are not merely due to their different binding affinities for BSA. This is evident from plots of % inhibition (or stimulation) versus $\bar{\nu}$ for these steroids shown in figs. 5.e.2. and 5.e.3. Clear-cut differences are evident in these plots among the four steroids. The curve for testosterone shows stimulation in the lower binding range ($\bar{\nu}$ = 0 - 0.70) and slight inhibition (less than 2%) in the upper binding range. Dehydroisoandrosterone shows almost

no influence on tryptic hydrolysis up to $\bar{\nu} = 0.8$ and dehydroisoandrosterone sulfate only slight stimulation up to $\bar{\nu} = 0.25$ and marked inhibition in the upper binding range. Progesterone shows only stimulation over the entire binding range and the plot also goes through the origin. The curve for dehydroisoandrosterone appears like a saturation type of curve and resembles in shape to the plot of % inhibition versus $\bar{\nu}$ obtained by Ryan (1973) for the effects of testosterone and dehydroisoandrosterone on tryptic hydrolysis of HSA. However, the reported value of maximum % inhibition for dehydroisoandrosterone and HSA (about 47%) is much higher than our value (22.5%) for the same steroid and BSA (fig. 5.e.l.). Further, although the two steroids, testosterone and dehydroisoandrosterone showed similar values of maximum inhibition in the case of HSA, our values for the three steroids in the case of BSA are appreciably different--1.2% for testosterone, 22.5% for dehydroisoandrosterone and 53% for dehydroisoandrosterone sulfate.

It should be pointed out that the curves for none of the steroids reported by Ryan (1973) passed through the origin. Later, however, Ryan and Baraff (unpublished results) made more precise measurements for testosterone and HSA in the low binding range and found slight stimulation (rather than inhibition) in that range. In this respect our results for testosterone and BSA in the low binding range agree with those of these authors for testosterone and HSA. It is thus evident that differences between the behavior of the two

proteins are more clearly seen in the upper binding range.

It is further apparent from fig. 5.e.3. that in the case of dehydroisoandrosterone sulfate inhibition first increases with increasing level of bound steroid to the maximum value at $\bar{\nu} = 6.5$ and then drops gradually with further increase in $\bar{\nu}$ to a somewhat constant value of 44% at $\bar{\nu} = 9.0$ and above. The relationship between % inhibition (or stimulation) and $\bar{\nu}$ is also not linear for the remaining steroids (see fig. 5.e.2.).

Binding data in table 5.e.1. shows that the number, n, of binding sites for dehydroisoandrosterone is 10. As noted above the maximum % inhibition found for this steroid is 22.5% (fig. 5.e.1.). Extrapolation of the linear portion of the curve (fig. 5.e.2.) to this maximum gives a value of 4.0 for the average number of sites at which inhibition will be directly related to $\bar{\nu}$. Above the binding level of 4.0 other factors may be involved (see below). Interestingly, in the case of dehydroisoandrosterone sulfate this value is also approximately 4.0 and in the case of progesterone it is 1.0. The total number of binding sites for these two steroids are respectively 23 and 32.

Although the above findings suggest that not all the binding sites are linked to stimulation or inhibition of BSA hydrolysis for the four steroids, we found that the reciprocal plots (not shown) for testosterone, progesterone and dehydroisoandrosterone were linear over the entire binding range we covered in the equilibrium dialysis

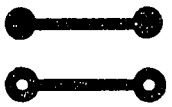
performed under the conditions of pH - stat (and in the presence of 5% Tris buffer). However we found that the reciprocal plot in the case of dehydroisoandrosterone sulfate was not linear. The data for dehydroisoandrosterone sulfate was analyzed by means of Scatchard plot (see fig. 5.e.4.) and revealed two sets of binding sites. It can be seen from fig. 5.e.4. that the first set of sites have higher affinity than the second set of sites. Although this could explain the nature of the plot (fig. 5.e.3.) for dehydroisoandrosterone sulfate, the number of sites in the first set does not correspond to the tryptic data.

For the assessment of anomalies in the binding data for the three steroids, testosterone, progesterone and dehydroisoandrosterone, we have also analyzed them by means of Hill plots which are shown in fig. 5.e.5. The plot for dehydroisoandrosterone is essentially a straight line having a slope of approximately 1.0, which suggests simple binding. However, this plot for progesterone is linear in the lower binding range, having a slope of less than 1.0, but departs from linearity in the higher binding range with increasing value of the slope. Although this appears to suggest a complex binding behavior, it must be pointed out that some variations in the values of slope and deviations from linearity of the Hill plots can also arise depending on the values of n, the number of binding sites, used for constructing these plots. This is evident from two plots for testosterone shown in fig. 5.e.5., one obtained with the

FIGURE 5.e.4.

SCATCHARD ANALYSIS (SCATCHARD ET AL, 1957) OF THE BINDING DATA FOR THE INTERACTION OF DEHYDRO-ISOANDROSTERONE SULFATE WITH 91 μM DEIONIZED BSA (LOT 30)

For experimental details refer to table 5.e.1.



Experimental data

Data obtained after subtracting $\bar{\nu}$ values corresponding to the first set of sites from the experimental $\bar{\nu}$ values.

First set of sites: n_1 = intercept of the asymptotic tangent to the curve on the x-axis = 8.4 ≃ 8.0; $n_1k_1^2$ = product of the asymptotic slope and intercept on the y-axis, $n_1k_1 = 6.76 \times 10^4$ L/M. Second set of sites: n_2k_2 = intercept of curve on the y-axis = 0.85×10^4 L/M and n_2 = intercept of curve on the x-axis = 15.0.

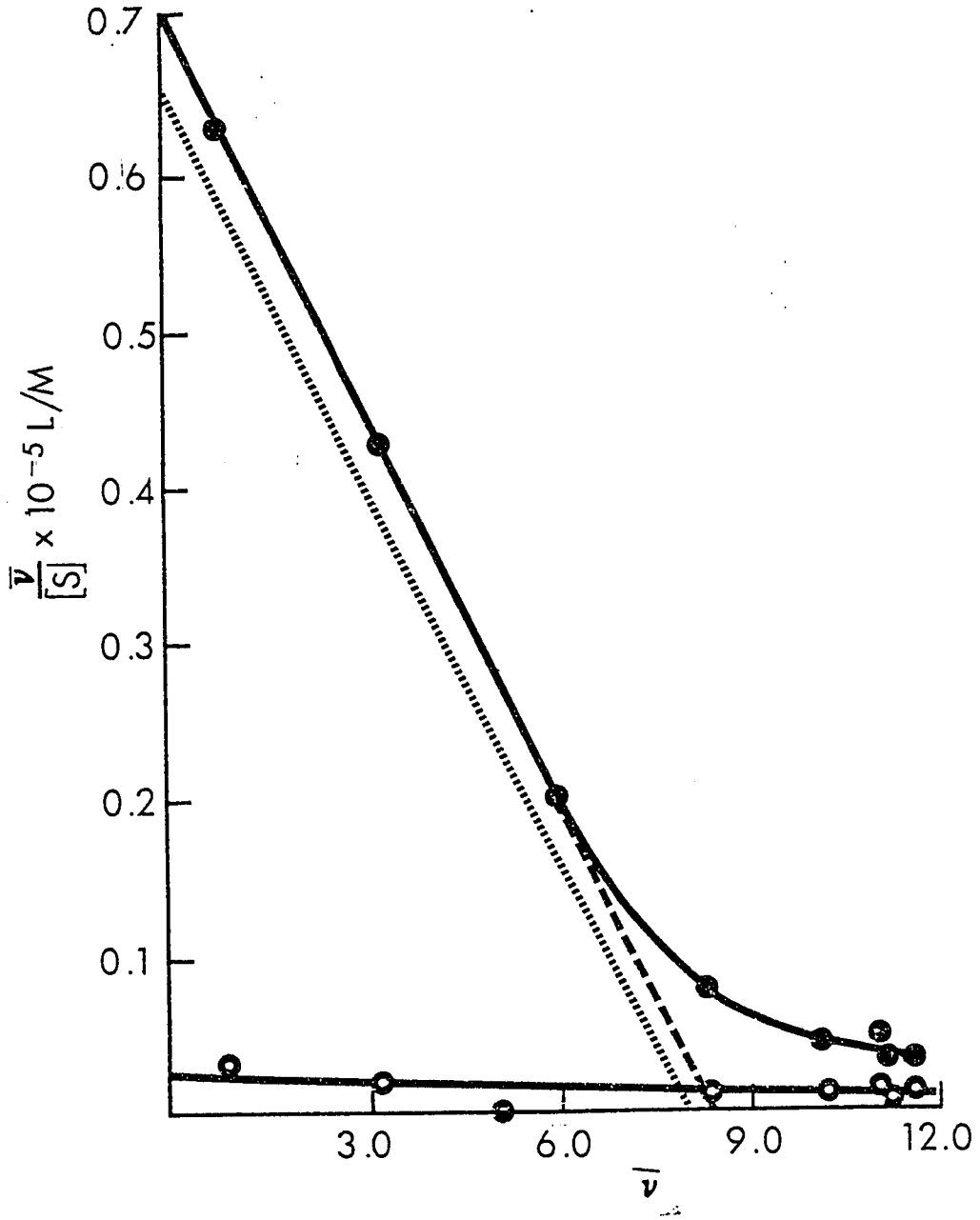
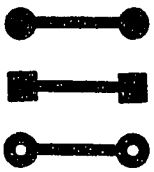

0.7
0.6
0.5
0.4
0.3
0.2
0.1
$\frac{\bar{\nu}}{[S]} \times 10^{-5}$ L/M
3.0
6.0
9.0
12.0
$\bar{\nu}$

FIGURE 5.e.5.

HILL PLOTS OF THE BINDING OF STEROIDS TO 91 μM DEIONIZED BSA (LOT 30)

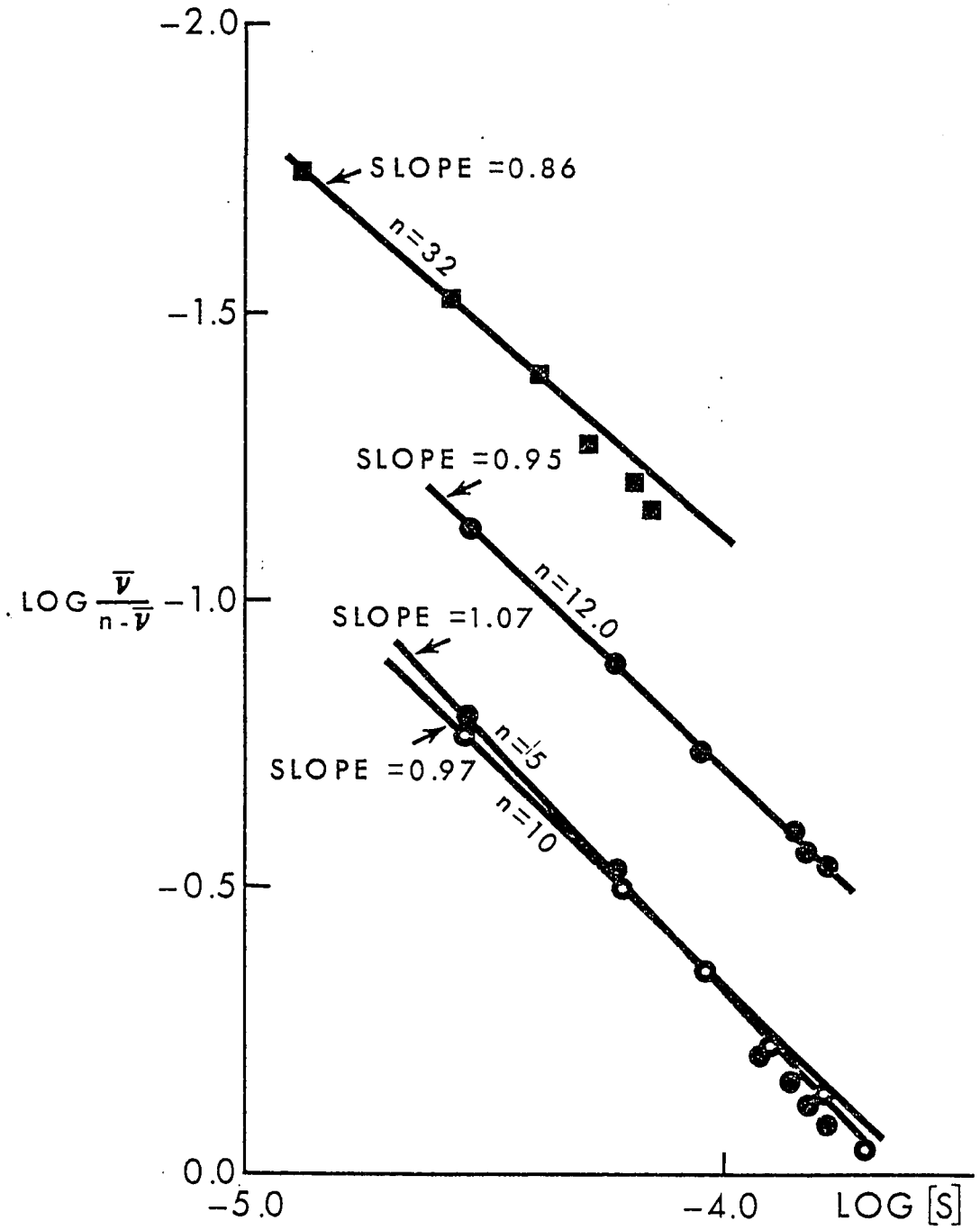
Conditions: 0.1M NaCl, pH 8.5 (5% Tris buffer, pH 8.5), 1.25% ethanol, 25°. Other details are the same as mentioned in table 5.e.1.



Testosterone

Progesterone

Dehydroisoandrosterone


-2.0
-1.5
-1.0
-0.5
0.0
LOG $\frac{\bar{\nu}}{n-\bar{\nu}}$
SLOPE =0.86
n=32
SLOPE =0.95
n=12.0
SLOPE =1.07
n=5
SLOPE =0.97
n=10
-5.0
-4.0
LOG [S]

actual experimental value of n = 12 and the other with the more usual value of 5.0 for the testosterone-BSA interaction (Ryan and Hanna, 1971; Ryan et al, unpublished results). Since the determination of n values for steroid-albumin interaction involves considerable uncertainty (see section 5.a.), it seems that precise predictions about the binding behaviors cannot be made using the Hill plots. It may be pointed out that Ryan (1973) reported cooperative effects in the binding of testosterone and dehydroisoandrosterone to HSA using these plots, however, later studies by Ryan et al (1977) showed that such effects could in part also be due to instability of the protein under the conditions (pH - stat) employed for measurement of binding. We are unable to provide definite answers to the anomalies in our tryptic hydrolysis data.

Since, as noted above, the differences in the behavior of the four steroids cannot be attributed to their different binding affinities (see fig. 5.e.2.), these may arise due to differences in the nature of interaction involved. Analysis of binding data in section 5.a. has revealed that in addition to the hydrophobic bonding, the binding of the three steroids, testosterone, progesterone and dehydroisoandrosterone also involves nonhydrophobic interactions with the BSA. This analysis was made with $\Delta G_1^{o'}$ values determined in Tris buffer, pH 8.0, $\mu = 0.1$ at 32 μM protein concentration. However, we have also calculated the $\Delta G_1^{o'}$ values for the three steroids using nk

values determined under the conditions of pH - stat. These values can be found in table 5.e.1. It can be seen that these values again show almost the same type and magnitude of variation in $\Delta G_1^{o'}$ as that observed in section 5.a.

From fig. 5.e.2. it is evident that progesterone which has the lowest value of $-\Delta G_1^{o'}$ shows stimulation of hydrolysis over the whole range of experimental $\bar{\nu}$. Testosterone, which has a higher value than progesterone shows stimulation only in the very low binding range and dehydroisoandrosterone with highest value shows almost no stimulation and very marked inhibition over the entire range. Since it has been suggested that the effects of steroids on tryptic hydrolysis are due to steroid-induced conformation changes in the protein (Ryan, 1973), it would appear that stimulation of hydrolysis will result from destabilization of protein structure and inhibition from its stabilization. It thus seems that steroids with higher energy of nonhydrophobic interactions (i.e. with higher values of $-\Delta G_1^{o'}$) stabilize the protein whereas those with lower value of this parameter tend to destabilize the albumin structure. Although $\Delta G_1^{o'}$ value for dehydroisoandrosterone sulfate could not be determined, it would appear that the electrostatic interactions involved in the binding of this steroid to BSA (Puche and Nes, 1962, section 2.g.) would have very large contribution to the nonhydrophobic interactions. The data in both sections 5.a. and 5.b. have revealed that the nonhydrophobic interactions between the steroid and BSA may be dependent on the structure

of the interacting steroid. From the above discussion it is evident that the steroid-induced conformation changes in the BSA are related to the strength of these interactions. It therefore seems that structure-dependent (nonhydrophobic) interactions between the steroid and BSA may be important in determining the magnitude and type of steroid induced conformation changes in this protein.

The differences in the behaviors of the four steroids may also arise due to the existence of separate binding sites for these on the BSA. Our competition data in section 5.a. and difference spectroscopic results in section 5.b. indicate that the binding sites on the BSA for testosterone and progesterone, and testosterone and dehydroisoandrosterone are not identical. Since data in section 5.b. suggest that, in the deionized BSA, progesterone binding involves loop 3 and testosterone binding loop 4, it may explain the difference between progesterone-stimulation and testosterone-inhibition. It is possible that the regions of loops 3 and 4 in which binding sites for these two steroids are located (section 5.c.2.) may differ in the susceptibility of protein structure to the binding of the steroid. However if the binding sites for some steroids, e.g. dehydroisoandrosterone and progesterone are similar, it may mean that the interactions involved are so different that one (dehydroisoandrosterone) results in inhibition (stabilization of protein) and the other (progesterone) stimulation (destabilization of protein) of tryptic hydrolysis.

Further, our results on the influence of steroids on tryptic hydrolysis obtained with BSA differ from those obtained by Ryan (1973) with HSA for the same steroids. This appears to suggest that the natures of the binding sites in the two proteins may be different. Such differences have also been emphasized by Ryan in a comparison of the two proteins (section 2.m.). It is interesting to note that our results in section 5.d. have indicated that conformation changes produced by addition of fatty acids can abolish the difference in the binding behaviors of the two proteins.

It must be pointed out that the above discussion of our results is based entirely on the assumption (made by Ryan, 1973) that the effects of steroids on tryptic hydrolysis of albumin are due to conformation changes in the protein. Although controversy exists in the literature as to the influence of testosterone binding on the conformation of BSA (section 2.m.) our results of solvent perturbation studies in section 5.c.1. do suggest such changes in the binding of testosterone as well as of dehydroisoandrosterone sulfate to this protein. However, it would be necessary to demonstrate these changes by means of other techniques of conformation analysis such as ORD, viscosity and deuterium-hydrogen exchange measurements. Further, studies of the effects of other appropriate steroids on tryptic hydrolysis will be required for the precise understanding of the relationship between steroid structure and binding induced conformation changes in the albumin.

6. SUMMARY AND CONCLUSIONS

The results of the present study have revealed that there are marked differences in the Tris buffer solubilities of C_{19} steroids containing the same number and type of polar groups but differing in other structural features such as their orientation and distribution, the absence and presence of double bonds and their locations in the carbon skeleton. Comparison of the solubility values of these steroids with the published values of their dipole moments suggest that in many cases structural contributions to one of these properties may far exceed those of the other. These solubility differences are attributed to specific interactions of the substituent groups with the tetrahedral structure of the liquid water, according to the Warner's model and the solubility values are used in calculating the $\Delta G_1^{o'}$ values for the steroids.

One of the aims of this study was to attempt to define the contributions which the specific structural elements in the steroid molecule might play in the apparently non-specific operation of the polarity rule in interactions with serum albumin. Measurement of the $\Delta G_1^{o'}$ values have provided evidence that there are such contributions--constituting no more than 10% of the total binding energy--which are paralleled by a certain degree of structural specificity in the steriod-induced aromatic red-shifts of the difference spectra as well

as in the effect of steroid on susceptibility of albumin to tryptic hydrolysis. In the latter case a large value of $\Delta G_1^{o'}$ is associated with marked inhibition of hydrolysis and a small value with marked stimulation.

In the attempt to clarify the role which aromatic chromophores might play in binding, evidence has been provided from solvent perturbation studies that tyrosine red-shifts are indeed probably due to direct interaction (hydrogen bonding), as has been suggested in the literature, while the tryptophan red-shifts are probably due to a steroid-induced change in tryptophan exposure. There is also a steroid-induced change in tyrosine exposure. However the results of NBS oxidation suggest that while tryptophan is not directly involved in steroid binding to bovine albumin, it is probably near the binding sites and hence strongly involved in steroid-induced aromatic perturbations.

It is difficult readily to locate steroid binding sites on the basis of chromophore involvement in difference spectra alone, in spite of the existence of only two tryptophans in the albumin molecule. However, the evidence from competition data in the absence and presence of fatty acid, the existence of structure-related, tyrosine-dominated and tryptophan-dominated red-shifts, as well as the influence of fatty acid on these accompanied by dramatic stimulation of binding affinity, the difference between the spectra for steroid-3-sulfates and a 17-sulfate in interactions with albumin, suggest that there are at least two sets of steroid

binding sites. The data mentioned above make it plausible that these could be located in tyrosine-rich tryptophan containing loop 3, the tyrosine-free tryptophan containing loop 4 and the tyrosine-rich tryptophan free loop 6. The established location of the binding sites for tryptophan and bilirubin would favor this, nevertheless, such a suggestion remains only tentative in view of the difficulty of interpreting difference spectra in such a large molecule as albumin. Thus, in spite of the location of tryptophan in loops 3 and 4 only, the spectral effects might be produced by a conceivable, though less likely, steroid-induced conformation change at a site far removed from the aromatic chromophores. Thus the first fatty acid site is located in loop 7, yet occupancy of this site markedly strengthens the steroid induced perturbation of the tryptophan residue in loop 3 or 4, and significantly enhances testosterone interaction with BSA. An influence of fatty acid induced conformation change has been suggested by Spector et al (1973) in connection with drug binding and it is obvious that our data for the effect of 3 moles of fatty acid on both steroid competition and binding affinity indicate that fatty acid must produce a drastic rearrangement of the steroid binding sites which has been explained as a shifting of the binding sites from loop 3 to 4. However, conclusive evidence for these suggestions can only be obtained when further binding studies have been performed on isolated albumin fragments similar to the measurements made by Pearlman and Fong (1972) in fragment KL.

The comparison of $\Delta G_1^{o'}$ values with difference spectral data has suggested that residues other than tyrosine may be involved in steroid binding to albumin and the suggestion of Romeu et al (1976) and Swaney and Klotz (1970) that these may be arginines and lysines finds some support in the charge effect observed on tryptophan perturbation observed in these studies in interactions of steroid-3-sulfates with serum albumin.

It is thus believed that the studies reported here have provided for the first time a consistent set of data--based on direct binding measurement--on a wide selection of steroid types describing the relationship between structure and values of binding parameters on a single batch of deionized albumin. The results thus provide information as to the role of functional groups in the steroid and residues in the protein which are involved in interaction, the possibility of the existence of different sets of binding sites for different steroid types as well as structure-related effects of site occupancy on induced conformation change in the protein molecule. The data provide suggestions as to where steroid binding sites may be located--and the influence of fatty acid in this--which may act as a useful guide in future studies seeking to locate intact binding sites in the albumin fragments.

Although for the sake of achieving valid experimental data, these in vitro studies had to be performed under the non-physiological conditions, the results might have important

biological significance. Thus the surprising stimulation of steroid binding to the BSA brought about by even small amounts of fatty acid (one mole/mole protein) appears to suggest that at least in the case of bovine species the levels of free fatty acids in the normal plasma (0.5-2.0 moles) may play a fundamental role in the regulation of steroid hormone action and metabolism (refer to section 2.c.). In this connection it is noteworthy that although it has been generally considered that the physiological concentration of plasma free fatty acids do not generally affect the binding of other ligands to serum albumin (section 2.b.), it is currently being realized that in fact they do so, depending on the structure of the ligand (Spector, 1975; Soltys and Hsia, 1977).

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ABBREVIATIONS

AAG	α_1-acid glycoprotein (orosomucoid)
BLG	β-lactoglobulin
BSA	Bovine serum albumin
CBG	Corticosteroid-binding globulin
DEAE	Diethylaminoethyl
HSA	Human serum albumin
kcal	Kilocalories
mμ	Millimicrons
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NBS	N-bromosuccinimide
PBG	Progesterone-binding globulin
POPOP	1,4-Bis-[2(5-phenyloxazolyne)-benzene]
PPO	2,5-diphenyloxazole

SYMBOLS

$E_{279}^{1\%}$	Absorbance of 1% solution of the protein at 279 mμ
I	Ionic strength
n	Number of ligand binding sites on the native protein
nk	Binding affinity
r	Average number of moles of steroid bound per mole protein
R	Gas constant
s	Steroid (aqueous) solubility
[S]	Unbound steroid concentration
$[S]_t$	Total steroid concentration
T	Absolute temperature
ΔG_1^o	Change in free energy accompanying steroid binding with reference to the crystalline steroid as the standard state
ΔG_2^o	Change in free energy accompanying binding with reference to the solvated steroid as the standard state
ΔO.D.	Change in optical density
ΔH^o-	Change in enthalpy
$-\Delta\varepsilon$	Decrease in the molar extinction coefficient of steroid resulting from protein interaction (spectral molar perturbation)

$\Delta\varepsilon_{258}$	Spectral molar perturbation of steroid at 258 mμ
$\Delta\varepsilon_{258}^{\overline{\nu}=1}$	Spectral molar perturbation of bound steroid at 258 mμ
λ_{max}	Wavelength of maximum light absorption
μ	Dipole moment
$\overline{\nu}$	Average number of moles of bound steroid/mole protein (level of bound steroid)
o	Degree centigrade