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The Existence of Conformationally Labile (Preformed) Drug Binding Sites in Human Serum Albumin as Evidenced by Optical Rotation Measurements

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Abstract—The ability of certain drugs to induce conformational changes in human serum albumin has been examined by differential optical rotation measurements at 233 nm. At drug: protein molar ratios ([D]/[P]) of unity, the optical rotation increased, decreased or remained the same depending on the drug used. The change in the optical rotatory dispersion (ORD) signal was investigated as a function of the drug concentration. Drug-protein interactions were relatively specific. There exists at least one, and possibly more, stable preformed high affinity sites for the binding of drugs to albumin. At low [D]/[P] ratios, the ORD titration curves suggest that the high affinity sites are conformationally labile and that the albumin molecule is flexible.

Certain drugs are associated with simple optical rotatory dispersion (ORD) titration curves and this suggests that the high affinity sites on albumin for the binding of these drugs are equivalent and non-interacting. In such cases, the use of the Scatchard model for the quantitative determination of binding parameters is justified. For other drugs, complex ORD titration curves indicate site-site interactions and the use of Scatchard analysis to calculate binding parameters is invalid. Hence, ORD titrations provide a useful basis for the choice of the mathematical model in the quantitative evaluation of drug binding.

Optical rotation measurements on charcoal-defatted albumin show that the molecule adopts an expanded form in the absence of long-chain free fatty acids (FFA). There is evidence that despite the appreciable conformational changes associated with defatting, albumin retains high affinity drug binding sites and this argues for a degree of configurational stability of the sites. Furthermore, it is evident that FFAs normally associated with human serum albumin are located separately from the high affinity drug binding sites. In general, defatted albumin is found to have greater susceptibility to conformational change than undefatted albumin. The complex ORD titration curves provide further evidence that albumin is extremely flexible and that the interaction of a number of drugs involves a range of new sites that result from the induced conformational changes. The flexibility of albumin and its ad-hoc ability to create a range of low-affinity sites are important factors that enable the protein to perform its physiological function as a carrier of diverse endogenous and exogenous substances.

Human serum albumin (HSA) not only has the function of regulation of colloid osmotic pressure but also is responsible for the translocation of a wide variety of molecules, including fatty acids, metals and vitamins throughout the body. Indeed, many hydrophobic substances bind to albumin including many pharmaceuticals (Jusko & Gretch 1976; Kragh-Hansen 1981; Peters 1985). In principle, albumin can influence the pharmacokinetic profile of drugs although the importance of this phenomenon can be overestimated.

There are a number of drug binding regions on albumin (Müller et al 1984) and in view of the recent reports on the three-dimensional structure of this molecule (Carter et al 1989) we decided to investigate whether the binding of drugs can influence the conformation of albumin.

HSA comprises 585 covalently linked amino acids in a single polypeptide chain. The secondary structure consists of 9 double loops held together by 17 disulphide bridges and the molecule consists of three contiguous domains (Brown & Shockley 1982). Fluorescence energy transfer measurements (Hagag et al 1983; Suzukida et al 1983) and the presence of salt bridges between domains (Bos et al 1989) suggest that albumin has a U-shaped structure. This conformation has recently been confirmed by X-ray crystallography (Fig. 1, Carter et al (1989)) and demonstrates a structure similar to that of the closely homologous α-foetoprotein (Luft & Lörscheider 1983; He & Carter 1992).

Most drugs bind reversibly to a limited number of binding regions and some workers have adopted the view that there are two major preformed stable high affinity sites (Sudlow et al 1976; Fehske et al 1982; Kragh-Hansen 1983). An alternative concept has been presented by Honoré & Brodersen (1984), who suggested that many regions of the albumin molecule are highly flexible and conformationally labile; they further suggest that drug binding sites are induced in the presence of the drug. In the latter case, one might expect to detect large conformational changes in the protein on drug binding, whereas in the former, such changes might be expected to be less marked or even non-existent. In order to investigate these two possibilities, we have studied the influence of 28 drugs on the helical content of albumin at drug: protein molar ratios ([D]/[P]) close to unity where it may be assumed that the interaction with the low affinity sites is minimal. Accordingly, changes in the structural content have been assigned to the interaction of the drug with the major high-affinity sites.

* Arnold Rosen died in December 1988. His death was a sad loss to the Chelsea Pharmacy Department and to his many friends.

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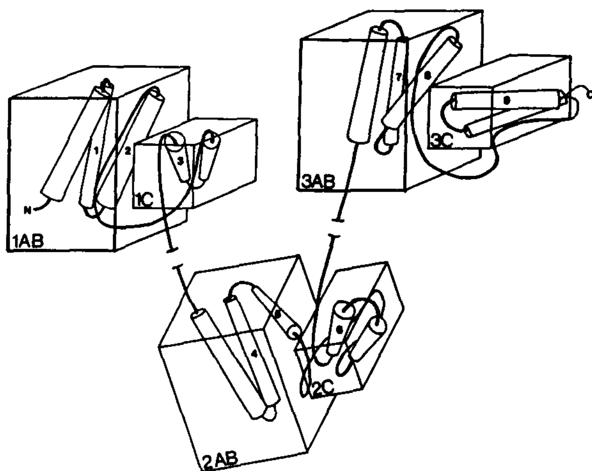

FIG. 1. Schematic representation of human serum albumin based on the tridomain structure initially proposed by Brown (1977) and the more recent X-ray analysis by Carter & He (1990). There are considered to be two major drug binding sites on human albumin. Site I is located between domains 2AB/2C and 3AB; site II is located between 1C and 3AB (Peters 1985). The major free fatty acid binding sites are located in domain 3 and between 2C and 3AB (Peters 1985).

Materials and Methods

Human serum albumin (HSA) of electrophoretic purity 100% and free fatty acid (FFA) content of 0·5 mEq/100 g was obtained from Hoechst (UK) Ltd, Hounslow. HSA was defatted as reported by Chen (1967).

Sucrose, aspirin, salicylic acid, sodium dihydrogen orthophosphate, disodium orthophosphate, sodium hydroxide, and orthophosphoric acid (Analar Grade) were obtained from Fisons, Loughborough, UK; oxyphenbutazone and phenylbutazone, from Ciba-Geigy Pharmaceuticals Ltd, Horsham, UK; flufenamic acid from Parke Davies, Pontypool, UK; benzylpenicillin, cephalexin, flucloxacillin, phenethicillin, cloxacillin and carbenicillin, from Beechams Research, Brentford, UK; phenytoin and bendrofluazide from Boots Co. Ltd, Nottingham, UK; *p*-hydroxydiphenylhydantoin from the Department of Pharmacology, University of Mainz, Germany; *p*-methoxydiphenylhydantoin and the fluorine derivative of diphenylhydantoin from Dr P. Macheras, Department of Pharmacy, King's College, London, UK; ethoin, methoin, dicoumarol and digoxin from Aldrich Chemical Co, Gillingham, UK; chlorothiazide and hydrochlorothiazide from Merck Sharp & Dohme Ltd, Hoddesdon, UK; testosterone and stanazolol from Sterling Winthrop, Surbiton, UK; phenazopyridine from Warner Pharmaceuticals, Pontypool, UK; dequalinium from Allen and Hanburys Ltd, London, UK; methadone from the Wellcome Foundation Ltd, Dartford, UK; chlorpromazine from May and Baker Ltd, Dagenham, UK. The purity of the samples was quoted by the manufacturers to be >99% and the drugs were used without further purification.

Stock solutions of 150 μM HSA and defatted HSA were prepared using filtered M/15 phosphate buffer, pH 7·4 taking the mol. wt of HSA to be 69 000 Da. Standard solutions of drugs were prepared using M/15 phosphate buffer. All solutions were stored at 4–6°C.

Absorption spectrum of the drugs and albumin were recorded using a Unicam SP 1800 Spectrophotometer at 21°C. ORD measurements were carried out using the single beam Polarmatic 62 (Bendix, UK) at 21°C adapted to use fixed pathlength (±0·01 mm) Helma cells. The instrument was calibrated with 1·276% w/v Analar Grade sucrose solution. With the slit width on the optical panel set at 0·2 mm the transmission of light was always kept greater than 40%. At low wavelengths and higher sensitivity, when the signal:noise ratio diminishes, a time-signal scan at constant wavelength was obtained by applying a steady voltage of 10 mV on the x-axis of the instrument x–y plotter. The amplified vertical deflection was averaged to yield the net change in optical rotation, in millidegrees (°m). Accordingly, with the instrument and recorder sensitivity at its optimum, differences in the specific rotation $\Delta\ [\alpha]_{233°m}$ were considered reliable to the nearest 20°m.

The instrument was used in the slow scan speed mode to record the ORD spectra (210–400 nm) of the M/15 phosphate buffer (pH 7·4), drug, HSA, and HSA+drug. HSA produces a characteristic negative maximum at 233 nm, the amplitude of which is influenced by certain drugs (Fig. 2).

The absence of changes in the ORD spectrum of the drug at wavelengths near the absorption band precludes an induced ORD effect in the drug in the presence of the protein. Thus, clearly in most cases, the effect on the ORD at 233 nm can be assigned to changes in the structural content of the albumin. If it is assumed that the $\Delta\ [\alpha]_{233°m}$ value remains constant, irrespective of α-helical segment length, then an estimate of the number of amino acid residues involved in an observed conformational change can be determined.

To determine the effect of increasing the ligand concentration on the ORD of 7·52 μM HSA, solutions containing drug and HSA at [D]/[P] ratios between 0·5 and 6, were freshly prepared using the standard stock solutions. Solutions containing either drug only or HSA only at the same concentration were also prepared. Using the time-signal adaptation, the variation in the optical rotation of HSA at

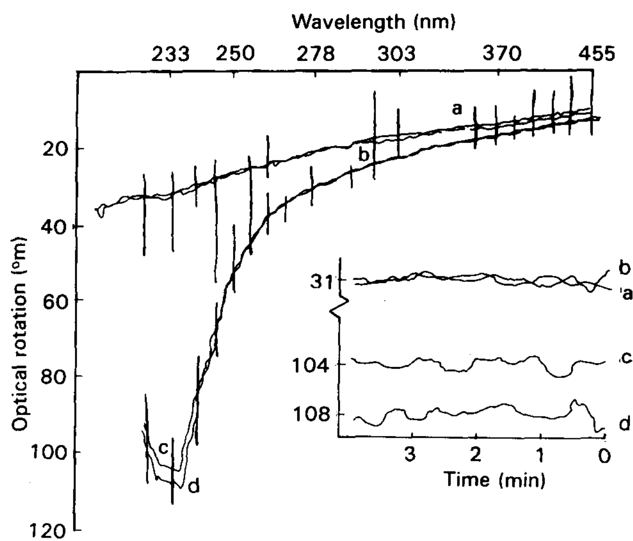

FIG. 2. The ORD spectrum of 7·52 μM HSA, HSA+aspirin 7·52 μM and aspirin 7·52 μM alone in M/15 phosphate buffer, pH 7·4 at 21°C. The inset shows the effect of aspirin on the ORD of albumin at 233 nm, with the instrument set at sensitivity × 20. a, Buffer; b, aspirin; c, HSA+aspirin; d, HSA.

233 nm with increasing [D]/[P] ratios for each of the drugs was determined at 21°C.

Equilibrium dialysis experiments were carried out using preboiled and washed lengths of Visking tubing (Scientific Supplies Ltd, London, UK). Accurately measured quantities of the stock solutions of the chlorothiazide and HSA were transferred to obtain 10 mL 0·5% albumin and chlorothiazide at various [D]/[P] molar ratios between 0·25 and 6 in M/15 phosphate buffer inside the bag and 20 mL chlorothiazide at the same concentration outside the bag. In each case a control dialysis system without albumin was set up to account for the effects of the binding of chlorothiazide to the dialysis bag and the closure. The dialysis tubes were shaken continuously on a rotary system (approx. 12 rotations min^{-1}) for 28 h at 21°C. Equilibrium conditions were ascertained by estimation of the drug at 20, 24, and 28 h. Binding was measured by determining the concentration of chlorothiazide (Baer et al 1959) in the external phase when no changes in the volume occurred. Leakage of albumin was tested spectrophotometrically at 740 nm using the Folin-Ciocalteau reaction. Solutions containing >0·002% w/v of albumin were rejected.

Results

At [D]/[P] ratios close to unity, twelve drugs altered the characteristic negative maximum at 233 nm. Eight drugs decreased the helical content whereas four drugs were associated with an increase (Table 1). The remainder of the drugs produced no measurable changes even when the [D]/[P] ratio was increased to 6. The percentage change was found to vary between −8 and +5%. In the case of the twelve drugs that altered the optical rotation, increase in the [D]/[P] ratio resulted in different types of titration curves (Fig. 3).

An initial survey using charcoal defatted albumin was also undertaken. Drugs that did not alter the ORD of non-defatted albumin did not bring about any measurable changes in defatted albumin. In the case of drugs that induced an ORD change, different titration curves resulted with defatted albumin (Fig. 4).

The linear Scatchard plot for the binding of chlorothiazide to HSA, obtained by equilibrium dialysis experiments, yielded a simple analysis corresponding to two non-interacting sites, with an association constant of $K_a = 3{\cdot}7 \times 10^4\ M^{-1}$ at 21°C. These values are in close agreement with n=2, $K_a = 3{\cdot}07 \times 10^4\ M^{-1}$ at 23°C reported by Breckenridge & Rosen (1970). Accordingly, theoretical binding isotherms for the chlorothiazide albumin system have been calculated using the method of Koch-Weser & Sellers (1976). The resulting theoretical albumin-binding isotherm showed a marked similarity to the experimental ORD titration curve (Fig. 5).

Table 1. The effect of drugs present at [D]/[P] molar ratio 1:1, on the optical rotation at 233 nm of 7·52 μM HSA in M/15 phosphate buffer, pH 7·4 at 21°C. The α_{233}°m value could be measured reliably to 0·2°m.

Drug	$\Delta\alpha_{233}$°m	% change in helical content of 7·52 μM HSA in M/15 phosphate buffer pH 7·4
Aspirin	−4·60	−8·0
Oxyphenbutazone	−1·00	−2·0
Carbenicillin	+1·00	+4·0
Cloxacillin	−0·65	−3·0
Flucloxacillin	−0·65	−3·0
p-Hydroxydiphenylhydantoin	−0·90	−2·0
Bendrofluazide	−0·30	−0·5
Chlorothiazide	−0·55	−1·0
Hydrochlorothiazide	−1·20	−2·5
Dicoumarol	+1·50	+3·0
Stanazolol	+1·60	+3·0
Testosterone	+2·70	+5·0
Salicylic acid	—	
Phenylbutazone	—	
Flufenamic acid	—	
Benzylpenicillin	—	
Cephalexin	—	
Phenethicillin	—	
Phenytoin	—	
p-Methoxydiphenylhydantoin	—	
Fluorodiphenylhydantoin	—	
Ethoin	—	
Methoin	—	
Digoxin	—	
Phenazopyridine	—	
Dequalinium	—	
Methadone	—	
Chlorpromazine	—	

— Signifies no detectable change.

Discussion

Drug binding to non-defatted albumin

Human serum albumin has a characteristic optical rotatory dispersion spectrum and the specific rotation at 233 nm is a reliable indicator of its helical content (Riddiford 1966; Steinhardt et al 1971; Jirgenson 1973). In view of its multidomain structure and the involvement of different subdomains in the formation of drug-binding sites, changes detected in the helical content at low [D]/[P] ratios result from the modification of one or more of the subdomains.

The 1:1 interaction of 16 drugs showed no appreciable changes in the helical content of albumin; the interaction of the remaining 12 drugs is, however, associated with an alteration in the helical content (up to 8%, Table 1). A 3% change in the helical content suggests the rearrangement of approximately 9 amino residues to a less structured form, which corresponds to a segment mol. wt of about 1000 Da. Thus, the binding of these low mol. wt drugs (130–500 Da) influences the structure of an appreciable mass of the protein. However, under the conditions used in these experiments, some of the drugs were incompletely associated with albumin, the degree of association being related to the affinity constant. With, for example, *p*-hydroxydiphenylhydantoin, only 28% of the drug is bound (as determined from binding experiments) under the conditions of the experiment (Walji 1990). Hence with this compound an adjustment to the observed ORD change is necessary in order to compare the induced effect with that induced by other more highly bound drugs such as oxyphenbutazone and dicoumarol. When an allowance is made, the calculated percentage change in helix for those albumin molecules which bind *p*-hydroxydiphenylhydantoin increases to approximately 20%. This corre-

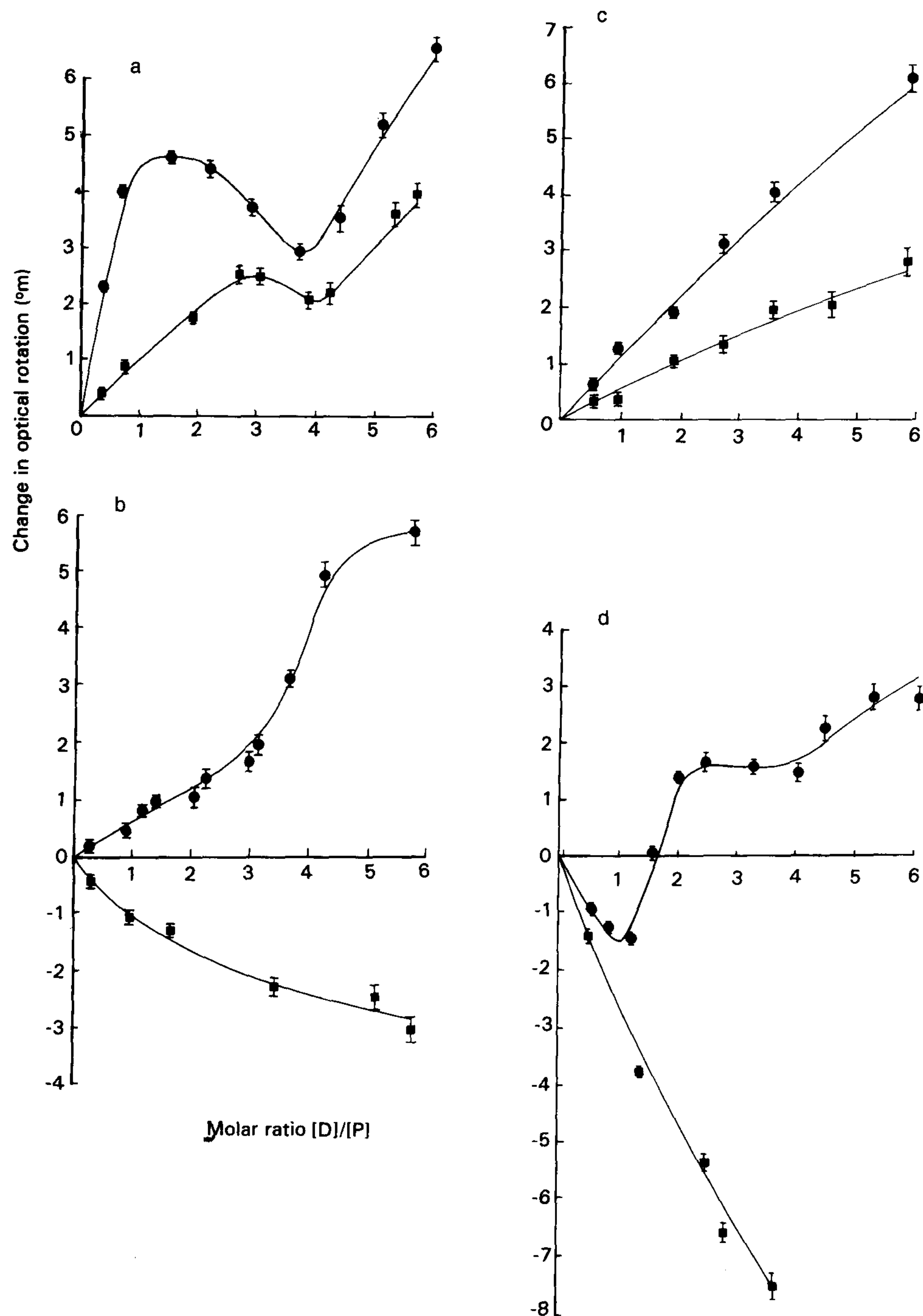

FIG. 3. ORD difference titration curves at 233 nm for the interactions of a range of drugs with 7·52 μM HSA in M/15 phosphate buffer, pH 7·4 at 21°C. a Aspirin (●) and oxyphenbutazone (■); b flucloxacillin (●) and carbenicillin (■); c hydrochlorothiazide (●) and chlorothiazide (■); d dicoumarol (●) and testosterone (■). Each point is the mean of four experiments.

sponds to 60 amino acid residues being affected, which is a major conformational change and might be expected to induce considerable influence on the tertiary structure. These observations add support to the concept that albumin is an extremely flexible molecule (Peters 1985; Bos et al 1989).

Theoretically, at least four different types of ORD titration curves can be predicted for the influence of a drug on the helical content of HSA (Table 2). The binding of many of the drugs used in this study has been previously assigned to one or both of the two major binding regions on HSA (Sudlow et al 1976; Brodersen et al 1977; Müller & Wollert 1979; Fehske et al 1979, 1981; Sjöholm et al 1979) (Fig. 1). Amongst the drugs that alter the helical content, there is no evidence to suggest that interaction with either site is

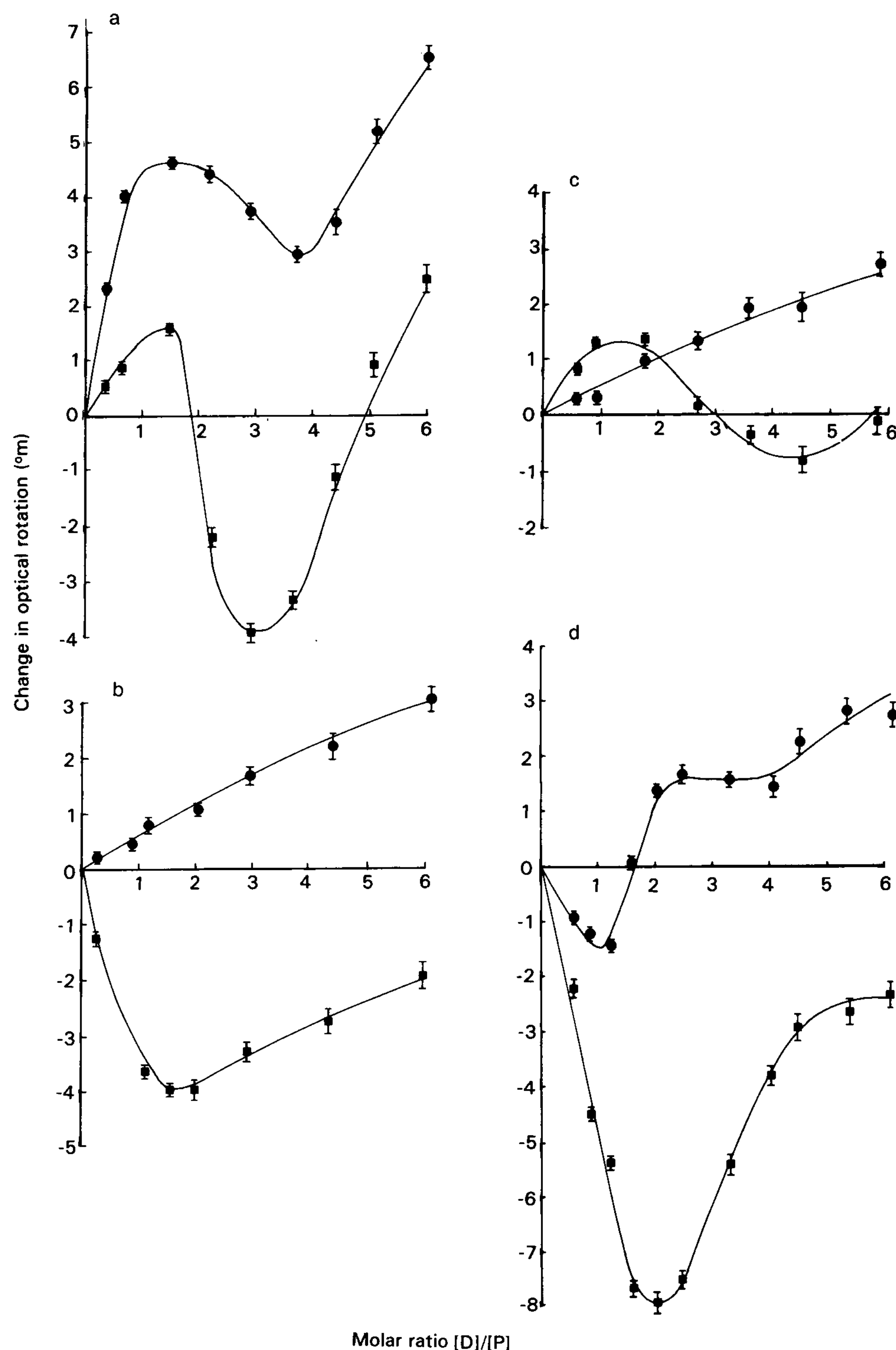

FIG. 4. ORD difference titration curves at 233 nm for the interaction of a range of drugs with 7·52 μM HSA and 7·52 μM defatted HSA in M/15 phosphate buffer, pH 7·4 at 21°C. a Aspirin + HSA (●), Aspirin + defatted HSA (■); b cloxacillin + HSA (●), cloxacillin + defatted HSA (■); c chlorothiazide + HSA (●), chlorothiazide + defatted HSA (■); d dicoumarol + HSA (●), dicoumarol + defatted HSA (■). Each point is the mean of four experiments.

associated with a specific type of ORD change (Table 2). Furthermore, there is no obvious correlation between the mol. wt, pK_a value and the octanol/water partition coefficients of any of the drugs with its relative influence on helical content (Walji 1990). Indeed, drugs of similar structure, for example oxyphenbutazone and phenylbutazone, are found to induce markedly different effects on the secondary structure of albumin (Table 2). This supports the view that drug : albumin interactions may be specific. With the interaction of certain drugs, for example phenylbutazone and salicylic acid, appreciable conformational changes are not induced despite the increase in the amount of complex

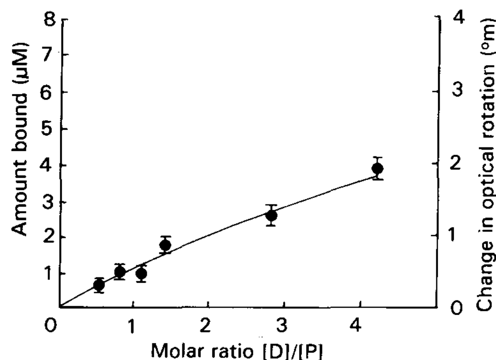

FIG. 5. The calculated binding isotherm (●) and the ORD difference titration curve for the interaction of chlorothiazide with non-defatted HSA, 7·52 μM, in M/15 phosphate buffer, pH 7·4 at 21°C. Each point is the mean of four experiments.

formed at high [D]/[P] ratios. The absence of any appreciable change to the helical content of albumin, as a result of the binding of these drugs to either of the two major high-affinity sites demonstrates that the inter-residue interactions responsible for the helical conformation are not disturbed. Thus, the present findings confirm that the binding of a number of drugs are not associated with extensive conformational changes as previously suggested by Honoré & Brodersen (1984). In view of the extremely flexible nature of albumin, the lack of adjustment of helical content in the presence of drugs such as phenylbutazone and salicylic acid suggests that in both the high-affinity drug binding sites, there are local areas of relatively stable configuration and that these are likely to exist in the absence of the drugs. In contrast, with the binding of other drugs, for example, chlorothiazide and aspirin, titration curves of types C and D are observed. These curves correspond to large changes in secondary structural content.

At low [D]/[P] ratios these changes probably result from interactions with specific functional groups on conformationally labile residues located near the high-affinity binding site. The simple type C titration curves suggest that there is a direct relationship between the amount of drug and the change in the ORD of HSA. This indicates that the sites are equivalent and that site-site interactions are minimal. In contrast, type D titration curves suggest a more complex situation where the loss of helix is not directly proportional to the total drug concentration suggesting the involvement of more than one type of high-affinity drug binding site where site-site interactions are most likely to occur.

At the higher [D]/[P] ratios considered, the effect of ligand binding to the low affinity sites on the ORD of HSA can also be analysed. For the titration curves of type A, binding to the low affinity sites does not involve any measurable change in the secondary structure. For the simple type C curves, drug binding to the high affinity site involves a conformational change but there is no further change in helical content when the low affinity sites are occupied. In contrast, for drugs that yield titration curves of type B and D, binding to the low-affinity sites is associated with appreciable changes in the protein conformation. The interaction of oxyphenbutazone and dicoumarol are examples of the more complicated titration curves, type D. It is apparent that oxyphenbutazone has one high- and at least two distinct low-affinity sites. The dicoumarol titration curve indicates that there are at least four classes of binding sites involved over the range of [D]/[P] ratios investigated (Fig. 3d). These results are in good

Table 2. Predicted titration curves for the influence of a drug on the helical content of HSA. Type A curve shows no measurable changes in the helical content. Type B curve shows no measurable change at lower values of [D]/[P] (abscissa) however, changes in the helical content occur as the [D]/[P] ratio is increased. Type C curve shows changes in the helical content at [D]/[P] ratio < 1 indicating the involvement of one or more high-affinity sites per molecule of albumin with the same affinity constant indicating non-interaction of the sites. Type D curve results when a drug binds to different classes of sites each with its characteristically different effect on the helical content.

A	B	C	D
Salicylic acid—site I and II	Diphenylhydantoin—site II	Cloxacillin—site II	Aspirin—site I and II
Phenylbutazone—site I		*p*-Hydroxydiphenylhydantoin—U	Oxyphenbutazone—site I
Flufenamic acid—site II		Chlorothiazide—U	Flucloxacillin—site I and II
Benzylpenicillin—U		Hydrochlorothiazide—U	Bendrofluazide—U
Phenethicillin—U		Testosterone—U	Stanazolol—U
Cephalexin—U			Dicoumarol—U
p-Methoxydiphenylhydantoin—U			
Methoin—U			
Ethoin—U			
Spiral derivative—U			
Dequalinium—U			
Phenazopyridine—U			
Methadone—U			
Chlorpromazine—U			
Digoxin—digitoxin site			

U-unknown.

agreement with previously reported data by Jusko & Gretch (1976) and Vallner (1977). In the clinical situation however, the [D]/[P] ratios are considerably lower than unity and therefore only the early part of the experimental results are physiologically relevant.

Application of Scatchard plot

The use of the Scatchard model in the mathematical evaluation of binding parameters assumes the absence of site-site interactions (Scatchard 1949). This is often not apparent when simple binding studies are carried out. Indeed, a number of authors have used the Scatchard plot indiscriminately to calculate binding constants (Kermode 1989). ORD titration curves can be used to detect situations where site-site interactions occur and this provides a basis for the adoption of the site-orientated model to determine binding parameters quantitatively. Site-site interactions are minimal for type C titration curves and the use of the Scatchard plot for drugs in this group is justifiable. The marked similarity between the calculated binding isotherm for chlorothiazide and its ORD titration curve confirms that binding parameters can indeed be determined from experimental ORD curves; this independent method is based on a property of the proteins in contrast to conventional methods used in binding studies where bound drug is differentiated from unbound drug.

Drug binding to defatted albumin

Albumin is normally associated with 1 to 2 molecules of free fatty acid (FFA) and the displacement of these molecules can have a modulatory influence on the binding of drugs. There are at least two high-affinity sites for long chain fatty acids which are not shared by any other ligand so far investigated (Spector 1975; Berde et al 1979; Peters 1985; Oida 1986). One of the high-affinity sites exists in the middle of domain 3 where two FFA molecules bind in an anti-parallel fashion. The location of the second high-affinity site is uncertain but it most likely involves subdomain 2C (Fig. 1) (Berde et al 1979). The present study has shown that charcoal defatted albumin is associated with a lower helical content when compared with non-defatted albumin. These changes are most likely on account of the unfolding of parts of the largely helical loops 6–9 which are involved in the high-affinity FFA binding sites. In this state the molecule can be considered to have adopted an expanded form. Accordingly, it can be expected that the conformational changes associated with defatting may well affect the two major sites for the binding of drugs.

At low [D]/[P] ratios, the drugs that did not induce changes in the helical content of undefatted albumin did not bring about any measurable changes in the secondary structure of defatted albumin, giving type A titration curves. These findings suggest that the specific residues that are involved in the interaction of such drugs are not involved in the structural changes associated with defatting, thus implying a degree of configurational stability of the two major high-affinity sites. The absence of an induced ORD effect by these drugs suggests that the drugs in this group do not compete for the same binding location on the albumin molecule as long chain FFA. These findings confirm the well-established view that the molecules of FFA that are normally associated with HSA are located separately from the high-affinity drug binding sites (Spector 1975; Peters 1985).

Drugs that were found to alter the helical content of non-defatted albumin varied in their effect on defatted albumin (Fig. 4). In general, defatted albumin was found to have a greater susceptibility to conformational change than did undefatted albumin. The ORD curves suggest that the interactions involve a new range of sites probably with a reduced affinity, resulting from the larger conformational changes associated with drug binding.

Flexible nature of albumin

The optical rotation measurements reported in this study have shown that the interactions of certain drugs are not associated with appreciable conformational changes and this supports the view that HSA has at least one and possibly more preformed high affinity sites for the binding of drugs. This is consistent with the findings of Birkett & Wanwimolruk (1985) who suggested that the indole-benzodiazepine site, site II, is a structurally well-defined hydrophobic cleft 12–16 Å deep and 6–8 Å wide. There is however, evidence that parts of the high affinity drug binding sites are conformationally labile and this accounts for the observed drug-induced conformational changes observed in this study. The defatting of albumin is also associated with conformational changes; however, in the expanded form, human albumin retains at least one of its high affinity sites. The increased susceptibility to drug-induced conformational changes in the case of defatted albumin provides further evidence that albumin is relatively flexible. This flexibility is probably an important factor in the creation of a range of low affinity binding sites and this enables albumin to perform its physiological role as a non-saturable carrier for diverse endogenous and exogenous substances.

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