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BINDING OF DRUGS TO HUMAN SERUM ALBUMIN—I. CIRCULAR DICHROISM STUDIES ON THE BINDING OF SOME ANALGESICS, SEDATIVES AND ANTI-DEPRESSIVE AGENTS

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Abstract—The possibility of using circular dichroism measurements as a method for studying drug-protein binding has been investigated with 52 analgesics, sedatives and antidepressive agents. The binding to human serum albumin (HSA) in 0·1 M KCl at pH 7·4 and 25–27° has been studied. New extrinsic Cotton effects have been obtained from HSA-complexes with 24 different drugs. Of the drugs known to form strong complexes with HSA, only *p*-acetanilides and acetylsalicylic acid failed to give extrinsic Cotton effects with HSA at the experimental conditions used. The structural requirement for binding of the drugs to HSA and the possibilities for studying the binding sites of HSA are discussed. In some cases (e.g. the benzdiazepine and dibenzazepine derivatives) a plane ring system with high electron density seems to be an essential factor for strong binding capacity.

ONE OF the most important factors for the understanding of the pharmacokinetic properties of a drug is knowledge about its binding properties to serum proteins. The binding constant is of fundamental importance for the concentration of free drug, which in turn determines the pharmacodynamic activity of the drug and the rate of inactivation and elimination.[1] For the binding in plasma of both endogenous and exogenous substances[2] serum albumin quantitatively is of greatest significance in most cases. Moreover, many pharmacological interactions observed can be explained by a competition between different substances for the same binding site of albumin.[3,4]

Many different methods have been used for studying protein–drug binding, e.g. equilibrium dialysis, gelfiltration and NMR.[5] However, circular dichroism (CD) studies seem to be an attractive alternative which hitherto has been in limited use.[6,7] Drugs often contain chromophores absorbing ultraviolet light in the wavelength region over 250 nm. When these chromophores are bound to proteins in an asymmetric environment, one can expect them to give rise to so-called extrinsic Cotton effects,[8] which can be used for determinations of, for instance, the concentration of drug–protein complexes, binding constants and the number of ligands bound.[9] Moreover, in favourable cases, CD-studies can also give information about binding-site characteristics.[9]

The purpose of the present work is to show the usefulness of the CD-technique for

the study of the binding to human serum albumin (HSA) of certain drugs, namely some analgesics, sedatives and antidepressive agents.

MATERIALS AND METHODS

Human serum albumin (HSA), obtained by ethanol fractionation and lyophilization, was received as a gift from KABI, Stockholm. Of all the commercially available samples this preparation contains the highest percentage of the monomeric form.[10] In order to eliminate any remaining adsorbed impurities, it was treated with activated charcoal at pH 3·0 according to Chen.[11] The albumin monomer was thereafter isolated by repeated gelfiltration on Sephadex G-200 and G-100 in 0·1 M KCl. The purity of the monomeric fractions was controlled by electrophoresis on 7% polyacrylamide gels at pH 8·3. The albumin monomer was stored at −20° until use. No dimerization could be detected after storage for more than 4 months. The concentration of HSA was determined from the optical density at 280 nm ($E_{1\,cm}^{1\%} = 5{\cdot}80$).[12] The spectra were recorded from 400 nm in order to correct for the light scattering effect according to Beaven.[13]

Drugs. Officinal drugs used fulfilled the requirements of the Nordic Pharmacopoeia. All other drugs were obtained as gifts from the different manufacturers and were used without further purification. Absorption spectra were recorded on an automatic Cary 15 double beam spectrophotometer at 25°.

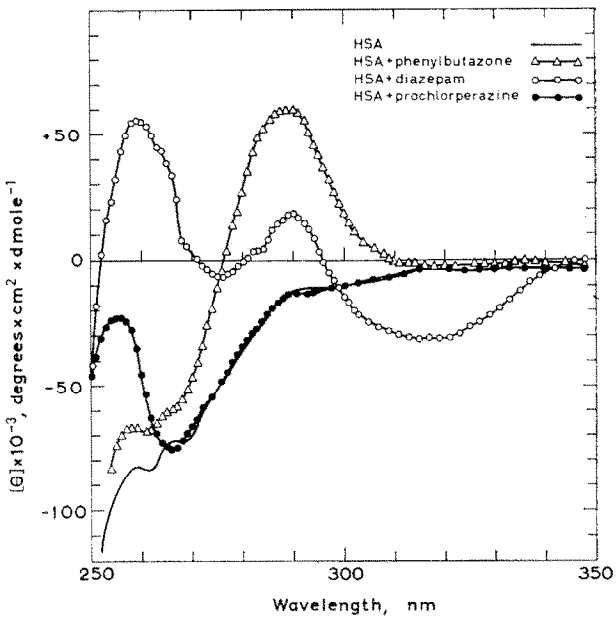
Circular dichroism measurements. These were made on a JASCO J-20 spectropolarimeter, Japan Spectroscopic Co., Tokyo, at 25–27°. The spectra were recorded in the range of 350–250 nm. Cylindrical cells with 10 mm pathlength were used. In some cases, the strong absorption of some drugs prevented scanning in the lowest wavelength region under the conditions used. The instrument was calibrated with D-10-camphorsulphonic acid and checked daily with a built-in test signal system. Before scanning each sample was filtered through a Millipore filter (0·22 μ). The result is expressed as molar ellipticity, $[\theta]$, in deg. $\times$ cm^2 $\times$ dmole^{-1}, calculated with reference to the HSA concentration using a mol. wt of 69,000.

Experimental procedure. To test the ability of the different drugs to produce new Cotton effects with HSA, each drug was dissolved in either 0·1 M KCl, or ethanol, or in a mixture of 0·1 M KCl–ethanol (1:1 or 4:1). The concentration of the drugs was 2·6 mM. The freshly prepared drug solution (100 or 200 μl) was added to 3·0 ml of a solution of HSA (1·2 mg/ml of 0·1 M KCl) corresponding to a 5- or 10-fold molar excess of drug, respectively. All solutions used were adjusted to pH 7·4 with 6 M sodium hydroxide.

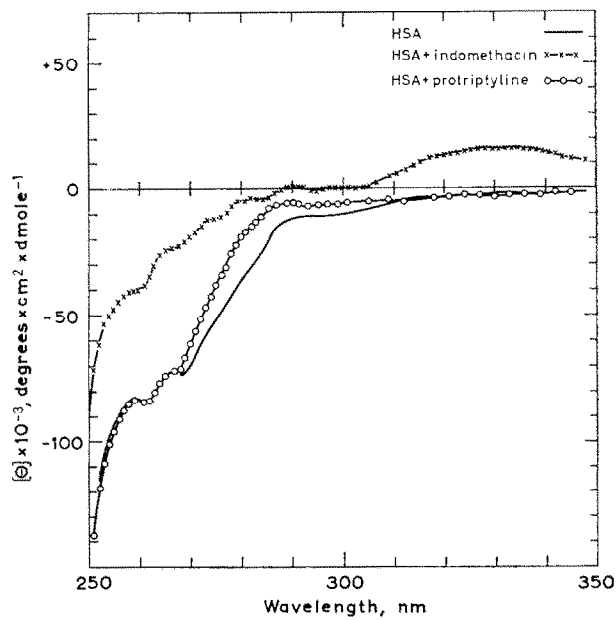
The ellipticity of the drugs alone was tested in 10 mm cells at the same concentration as above. No drug except levomepromazine showed any Cotton effect in the wavelength region studied. The ellipticity of levomepromazine ($[\theta]^{max}_{264\,nm}$ and $[\theta]^{max}_{315\,nm} = -2{\cdot}2 \times 10^3$ and $-1{\cdot}2 \times 10^3$ deg. $\times$ cm^2 $\times$ dmole^{-1}, respectively) was corrected for during the study of the HSA-complex.

RESULTS

HSA monomer produces a negative CD-spectrum below about 320 nm down to very low wavelengths (about 201 nm). At pH 7·4 characteristic negative maxima are obtained at 268 and 262 nm (see Figs. 1a and b) probably originating from phenylalanine



(a)



(b)

FIG. 1(a). Circular dichroism spectra of human serum albumin (HSA) (———), of HSA in the presence of a 5-fold molar excess of diazepam (○——○), in a 5-fold molar excess of phenylbutazone (△——△) and in a 10-fold molar excess (●——●) of prochlorperazine. (b) Circular dichroism spectra of human serum albumin (HSA) (———), of HSA in the presence of a 5-fold molar excess of indomethacin (×——×) and a 10-fold molar excess of protriptyline (○——○). The concentration of HSA was 1·1 mg/ml in 0·1 M KCl, pH 7·4 and the temperature 25–27°. Molar ellipticity, [θ], was calculated with respect to the HSA concentration.

side chains.[14] For the study of the complex binding of smaller compounds to proteins, the wavelength region above 250 nm is of particular interest, as the contribution of the proteins themselves to the ellipticity (i.e. the intrinsic Cotton effects) in this area generally is comparatively small, which means that any changes of the spectrum can be detected more easily. Figs. 1a and b show some examples of changed spectra obtained when different types of drugs are bound to HSA monomer.

In the present study, 52 different drugs were tested for their ability to change the CD spectrum of HSA. The drugs are arranged in 6 different groups according to their pharmacologically active chemical structures and are presented with chemical formulas in Table 1. The recommended international non-proprietary names of the drugs are used, when such names are available. The characteristic features of the results are also indicated in the Table. A positive result means that a drug gives a spectrum with HSA which deviates by more than 1×10^{-3} degrees from the spectrum of HSA at the experimental conditions used. In cases where maxima of the difference spectra are obtained within the wavelength region studied, the sign and location of the maxima

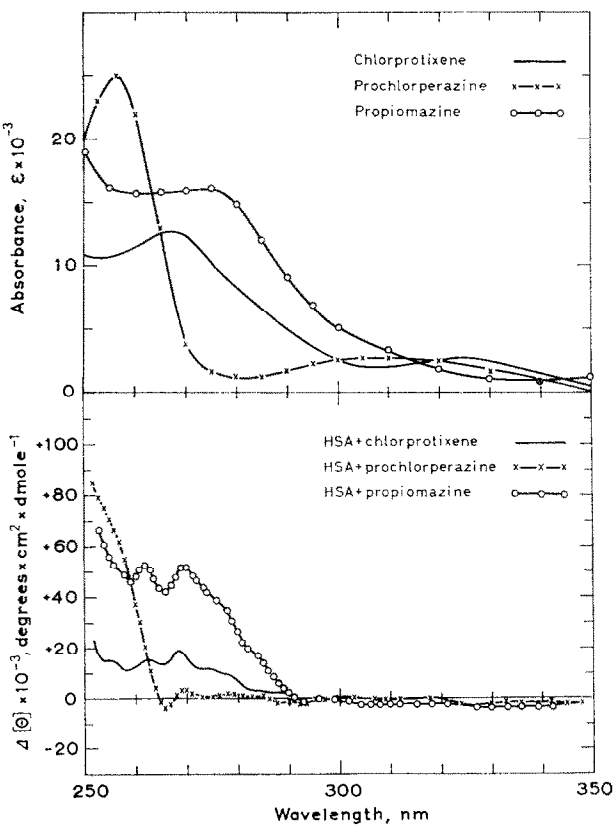


FIG. 2. Absorption spectra (upper part) of chlorprotixene (———), prochlorperazine (×——×) and propiomazine (○——○) in 0·1 M KCl and difference CD spectra (lower part) of the HSA-complexes with the respective drugs, obtained after subtraction of the CD spectrum of HSA alone. The concentration of HSA was 1·12 mg/ml in 0·1 M KCl and the drugs were present in a 10-fold molar excess. The measuring conditions were the same as in Fig. 1.

are noted in the table. In some cases, however, the scanning of the HSA-complexes was discontinued before a difference maximum was reached because of a too high light absorption of the sample at the standard conditions used.

Ten different phenothiazines—together with chlorprotixene and prothipendyl having similar structure—have been collected in group 1, Table 1. All the drugs tested gave rise to changed CD spectra when bound to the HSA monomer. Some examples are given in Fig. 2. It was initially noted that only small differences could be observed when a 5 molar excess of drug was used, consequently, the higher concentration of the drugs corresponding to a 10-fold molar excess was subsequently used.

In group 2, Table 1, eight different tricyclic antidepressive agents are collected. The common feature of those drugs giving positive results (Fig. 3) is the central unsaturated ring. No positive results, not even with a 10-fold molar excess are obtained from the 10, 11-dihydrogenated structures.

Figure 4 summarizes the results obtained with the benzdiazepine drugs in group 3. All the substances tested gave positive results. All the chloro derivatives exhibited very characteristic difference spectra, while nitrazepam, containing a nitro group instead of the chloro group, gave a quite different spectrum. The chloro-compounds showed a

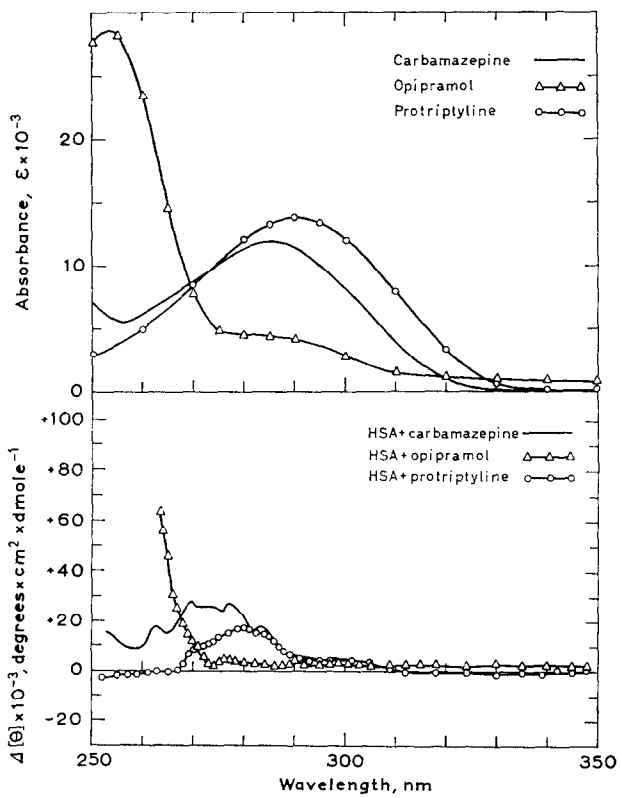


FIG. 3. Absorption spectra (upper part) of carbamazepine (———), opipramol (△——△) and protriptyline (○——○) in 0·1 M KCl and difference CD spectra (lower part) of the HSA complexes with the respective drugs, obtained after subtraction of the CD spectrum of HSA alone. The concentration of HSA was 1·12 mg/ml in 0·1 M KCl and the drugs were present in a 10-fold molar excess. The measuring conditions were the same as in Fig. 1.

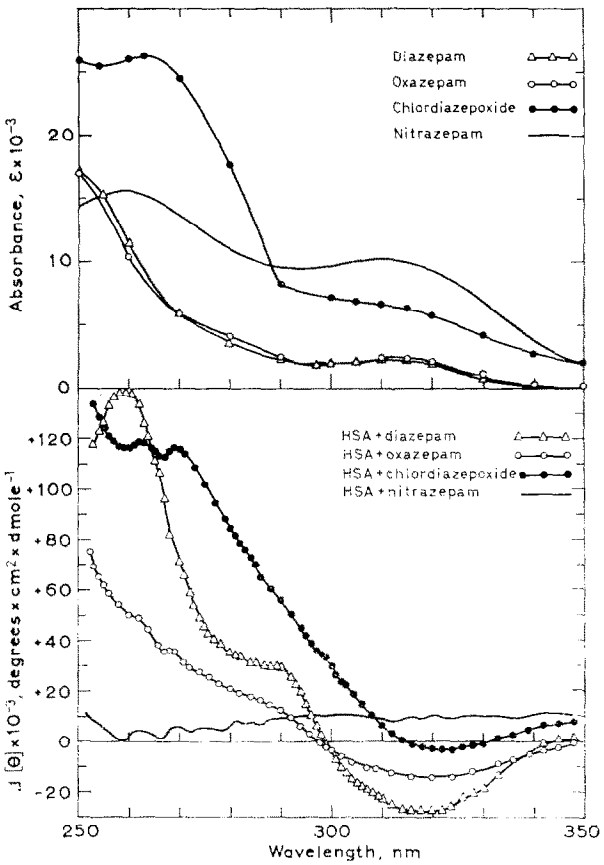


FIG. 4. Absorption spectra (upper part) of diazepam (△——△), oxazepam (○——○), chlordiazepoxide (●——●) and nitrazepam (———) in 0·1 M KCl and difference CD spectra (lower part) of the HSA-complexes with the respective drugs, obtained after subtraction of the CD spectrum of HSA alone. The concentration of HSA was 1·16 mg/ml in 0·1 M KCl and the drugs were present in a 5-fold molar excess. The measuring conditions were the same as in Fig. 1.

typical negative maximum at about 320 nm and positive maxima in the lower wavelength region.

The pyrazolones and pyrazolidines are classified in group 4. The drugs belonging to the former group gave negative results, while the drugs in the latter group showed significant difference spectra, depicted in Fig. 5. The results from oxiphenbutazone and phenylbutazone are consistent with those of Chignell[15] and Rosen.[7] The concentration dependence of the sign of the Cotton effects given by the oxiphenbutazone–HSA complexes noted by Rosen[7] was also confirmed.

Group 5, Table 1, contains the seven barbiturates studied, all of which gave negative results at a 10-fold molar excess over the HSA monomer.

Group 6 consists of the remaining drugs studied. Indomethacin and salicylic acid were the only ones of this group giving new Cotton effects with HSA. Figure 6 shows a comparison between the CD-difference spectra and the absorption spectra of the drugs in question.

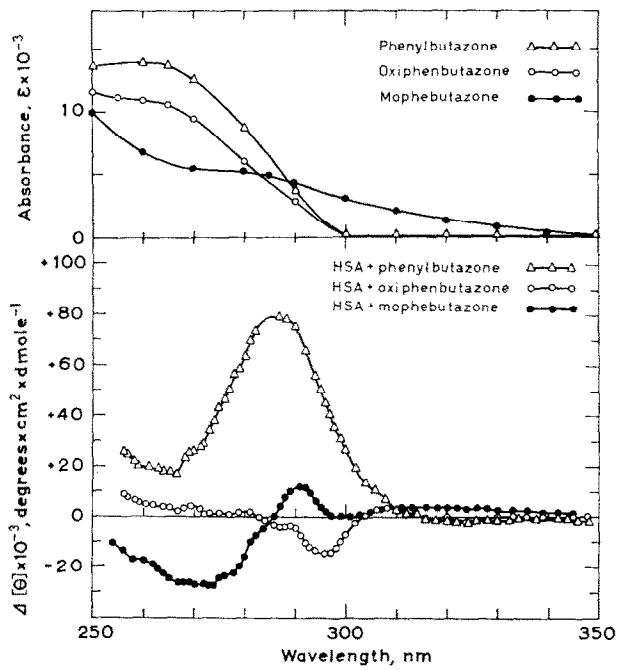


FIG. 5. Absorption spectra (upper part) of phenylbutazone (△——△), oxiphenbutazone (○——○) and mophebutazone (●——●) in 0·1 M KCl and difference CD spectra (lower part) of the HSA complexes with the respective drugs, obtained after subtraction of the CD spectrum of HSA alone The concentration of HSA was 1·16 mg/ml in 0·1 M KCl and the drugs were present in a 5-fold molar excess. The measuring conditions were the same as in Fig. 1.

DISCUSSION

Of the 52 drugs tested, 24 gave new significant Cotton effects with HSA monomer at pH 7·4 and 25° in 0·1 M KCl when a 5- or 10-fold molar excess of drug over HSA was used. This means that the drugs in question had formed complexes with the albumin molecule in such a way as to cause new extrinsic Cotton effects and/or to modify the intrinsic Cotton effects of HSA. "Extrinsic" Cotton effects arise when a chromophore of the drug is bound in an asymmetric conformation on the albumin surface and the changes of the "intrinsic" Cotton effects are due to a changed conformation of chromophoric amino-acid side chains in the albumin molecule.

However, additional requirements must be fulfilled. The complex constant must be high enough and/or the rotational strength strong enough to give significant deviations from the CD spectrum of HSA. With an albumin concentration of about 1·2 mg/ml ($1{\cdot}7 \times 10^{-5}$ M), and assuming that 25 per cent of all binding sites are covered in a 1 : 1 complex, one can estimate the minimum complex constant to be about 4×10^3 M^{-1}, if the drug is present in a 5-fold molar excess.

As to the change of ellipticity, this has to be larger than 23×10^3 deg. $\times$ cm^2 $\times$ $dmole^{-1}$, if the recorded difference (with a 1 cm cuvette) is larger than 1 cm (that is 1×10^{-3} deg. with the most sensitive settings of the spectropolarimeter) with the same assumptions as above. If the complex constant is higher than assumed, a larger part of the binding sites will be covered, which means that the Cotton effect in such a

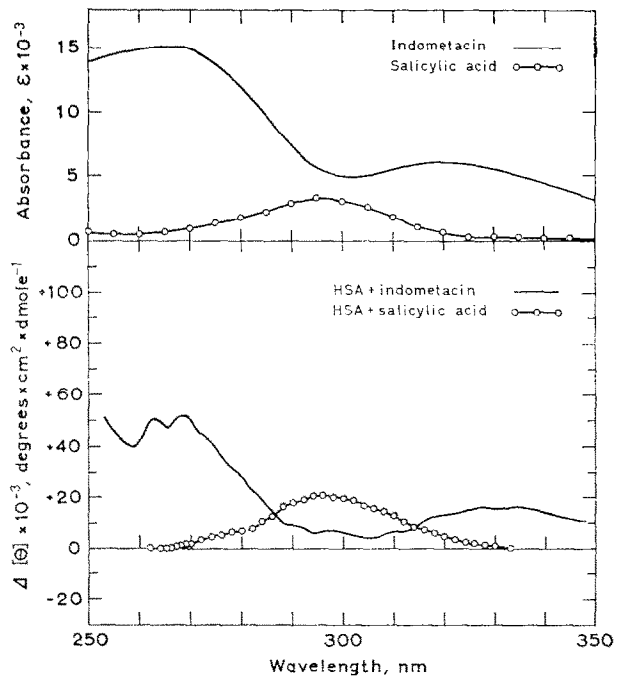


FIG. 6. Absorption spectra (upper part) of indomethacin (———) and salicylic acid (○——○) in 0·1 M KCl and difference CD spectra (lower part) of the HSA-complex with the drugs, obtained after subtraction of the CD spectrum of HSA alone. The concentration of HSA was 1·16 mg/ml in 0·1 M KCl and the drugs were present in a 5-fold molar excess. The measuring conditions were the same as in Fig. 1.

case can be weaker without changing the minimum deflection on the recorder and vice versa.

The phenothiazines classified in group 1 seem to give complexes with HSA which are just within the limit detectable by the method described. From Table 1 and Fig. 2 is seen that a 10-fold molar excess of drug has to be used in some cases. This can mean that the pharmacological importance of the protein binding is limited. The phenothiazines have two benzene rings, but the π-electron systems of these rings are not conjugated which may be the reason for the relatively weak binding, as other ring systems discussed below with larger planar π-electron clouds give strong Cotton effects. The similarities between the absorption and the difference spectra (Fig. 2) indicate that the strongest effects seen are due to extrinsic Cotton effects originating in the protein complex. However, a thorough investigation of the difference spectra around 260–270 nm shows in several cases small S-shaped irregularities which are sometimes (e.g. with propiomazin) superimposed on other larger Cotton effects. These changes occur in the wavelength region where the phenyl side chains of phenylalanine show CD maxima.[14] It is also well known (e.g. ref. 6, 16) that the absorption of a chromophore can be "red-shifted" or "blue-shifted" if it is transferred to a more hydrophobic or hydrophilic medium, respectively. The changes seen in the difference spectra can thus be an indication that phenyl groups from HSA take part in the complex binding of

phenothiazines, forming a hydrophobic bond due to $\pi - \pi$-electron interaction. More detailed quantitative studies are needed on this point before any definite conclusions can be drawn.

Group 2 contains tricyclic compounds consisting of two benzene rings separated by a seven-membered ring. It is noteworthy that only those forming a conjugated double-bond system gave positive results. Thus, carbamazepine, opipramol and protriptyline gave significant new extrinsic Cotton effects, while dibenzepine and others, which are 10,11-dihydrogenated compounds, gave no new Cotton effects. This suggests that an extended π-electron system, which gives a planar configuration, is of great importance for the manifestation of the new extrinsic Cotton effects observed. Space-filling models of the conjugated and the dihydrogenated ring systems are shown in Fig. 7, which clearly reveals that the two benzene rings of the dihydrogenated derivatives are not situated in the same plane. Further quantitative studies should give a more conclusive answer to this problem.

The benzdiazepines comprising group 3 also have a large planar π-electron system. The chlorinated benzdiazepine derivatives all gave characteristic difference spectra, which coincided well with the absorption spectra of the drugs themselves. Thus it is evident that the chromophores of the drugs are bound in an asymmetric conformation on the HSA surface. In nitrazepam, the nitro group is substituted for the chloro group, which modification changes the binding characteristics appreciably. The magnitude of the extrinsic Cotton effects is less significant, which might be due to weaker binding. The nitro group is more electron attracting than the chloro atom and therefore decreases the electron density of the π-electron ring system with a concomitant decreased tendency to form hydrophobic bonds. Moreover, the bulky nitro group may hinder contact with the binding site of HSA.

Phenylbutazone and oxiphenbutazone are known to yield extrinsic Cotton effects with HSA and give complexes with primary constants exceeding 10^5 M^{-1},[15,17] The present study gave the same qualitative results and the same concentration-dependent changes of the ellipticity of the HSA-oxiphenbutazone complex as noted by Rosen.[7] Mophebutazone also showed positive results indicating that two phenyl rings are not a necessary requirement for binding to HSA. However, despite large structural similarities, the pyrazolone derivatives did not change the CD spectrum of HSA.

As to the barbiturates, the ring should be planar but does not constitute a conjugated π-electron system. The planar structure has been confirmed in X-ray diffraction studies of some barbiturates in complex with 8-bromo-9-ethyl-adenine.[18] The barbiturates studied also contain three carbonyl oxygens imparting to the compounds slightly acidic properties which reduce the tendency of the ring to form hydrophobic bonds. On the contrary, some indications suggest that they bind to plasma proteins by electrostatic interactions. Thus the pH dependence points to the pKa of the barbiturates being an important factor[19,20] and interactions observed with acidic drugs, (e.g. sulphonamides)[19] indicate that ionic bonds can be involved in the formation of protein–barbiturate complexes. However, available information show that the complexes are relatively weak. Goldbaum and Smith[20] have noted that the binding by bovine serum albumin is dependent on the length of the side chains of the drugs. Among the drugs studied by us, they found that at most only 37 per cent of the drug (pentobarbital) was bound at drug and protein concentrations which exceeded by about 10 times those prevailing in this study. Moreover, thiopental, which seems to be

one of the barbiturates with the highest tendency to form protein complexes, has a k_1 of $1{\cdot}2 \times 10^4$ M^{-1} at 7° and pH 7·42[21] and Brånstad *et al.*[19] found phenobarbital and pentobarbital to have $k_1 < 3 \times 10^3$ at 25° and pH 7. It is therefore highly probable that the barbiturates studied do not form complexes with HSA which are stable enough to be detected under the experimental conditions used in the present work.

Group 6 contains several chemically different drugs, many of which have acidic properties. The protein binding characteristics of these drugs are mainly unknown. However, the primary binding constants of acetylsalicylic acid and salicylic acid for the binding to bovine serum albumin have been estimated to be 3·5 and $4{\cdot}0 \times 10^4$ M^{-1}, respectively, at pH 5·4 and 4°.[22] Also, the constants for paracetamol and phenacetin are known, being 1·7 and $2{\cdot}0 \times 10^4$ M^{-1} at pH 7·2 and 19°.[23] If it is assumed that the binding constants with HSA are of the same magnitude, the constants are high enough to yield appreciable amounts of complexes, which should be detected at the experimental conditions used here, provided that the drugs can modify the CD spectrum of HSA. Except salicylic acid the drugs, however, did not give any new Cotton effects, but interaction studies with diazepam and mophebutazone have shown that acetylsalicylic acid does compete for the binding site on HSA and therefore is indeed bound.* Our CD studies thus indicate that acetylsalicylic acid and the *p*-acetanilides are not bound to HSA in such a way as to impart to the chromophores significant rotational strength.

Indomethacin is the second drug of group 6 which gives Cotton effects with HSA. This result is contradictory to the findings of Chignell,[17] who did not find any. Indomethacin has two planar ring systems, the indole and *p*-chlorophenyl groups. It is probable that the indole ring system is involved in the complex formation, as tryptophan gives a complex (with $k_1 = 8{\cdot}1 \times 10^4$ at 10°) detectable with the CD technique[24] and as other indole compounds are bound as well.[25]

The present work has clearly shown the usefulness of CD measurements for the study of drug–protein binding. Most of the drugs known to be bound to HSA with $k > 4 \times 10^3$ M^{-1}, which is the limiting value used in the above calculation, have given rise to new Cotton effects. The only exceptions hitherto noted are acetylsalicylic acid and the *p*-acetanilides studied, which do not give any extrinsic Cotton effects when they are bound to HSA. The reason for this can be that the chromophore is not fixed in the complex or that the rotational strength of the chromophore is too weak to produce a measureable change of the HSA ellipticity. It is thus not possible to definitely conclude that the absence of new Cotton effects mean that a drug does not form a complex with the protein studied. Anyhow, the experimental conditions used in this work are chosen in such a way that all drug–protein complexes of pharmacokinetic significance giving Cotton effects are detected. Martin[1] showed that only complexes having $k > 10^4$ are strong enough to appreciably affect the drug distribution *in vivo* at equilibrium. Assuming a total body water volume of 42 l. and a plasma volume of 3 l., he showed that of a drug (total amount in body 1·5 mmoles) bound to albumin in a 1 : 1 ratio with $k = 10^4$ only about 20 per cent in the plasma is indeed free, but about 78 per cent is free in the whole body. This means that pharmacodynamic and pharmacokinetic properties, which are related to the concentration of free drug in the intracellular and interstitial fluid, are affected only marginally by plasma protein binding, if the association constant is 10^4 or smaller. In the present study all complexes with $k > 4 \times 10^3$ giving reasonably strong Cotton effects were detected.

* I. Sjöholm and, A. Grahnén, unpublished work.

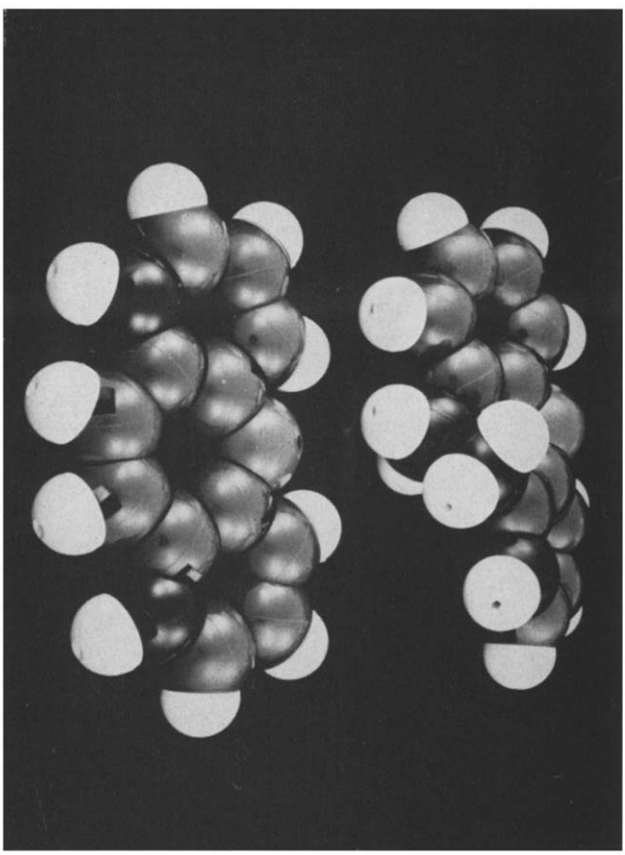


FIG. 7. Space-filling models of dibenz(b,f) azepine (left) and 10,11-dihydro-dibenz (b,f) azepine (right).

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The present work has dealt only with qualitative aspects, but the CD technique has also a great potential value as a more general method for quantitative studies on protein binding. Rosen,[7] Ikeda and Hamaguchi[9] and Sjöholm and Grahnén[24] have, for instance, used CD measurements for quantitative binding studies on physiologically and biochemically important compounds. A drawback in this respect is, however, that only reasonably strong complexes can be studied. With the instruments available today, the limiting value of the association constant seems to be approximately 10^3, which can vary somewhat depending on the rotational strength of the complex and the optical properties of the compounds under study. This is due to the fact that the concentrations of the proteins and ligands cannot be increased very much over the level used in this work, as too much of the light will then be absorbed by the sample with a concomitant too large increase in the photomultiplier voltage. It should, however, be pointed out in this context that only complexes with $k > 10^4$ (for 1:1 complexes) have pharmacological, pharmacokinetic or physiological significance, as is discussed above. On the other hand, it can certainly be of interest to investigate also weaker complexes, e.g. in studies on the structural requirement for binding to macromolecules of a certain group of compounds.

In comparison with other methods used for drug-protein studies, the CD measurements are not affected by such side effects as non-specific adsorption to dialysis membranes or gelfiltration materials or influenced by Donnan equilibrium phenomena. Another advantage is the information about the binding site and the participating structure of the drug, which can be obtained from the intrinsic and extrinsic Cotton effects, respectively. This information can be of great value in future studies on drug interactions which depend on the competition of two or more drugs for the same binding site. The application of the CD technique to the study of drug–protein complexes thus seems to be a valuable alternative to other methods hitherto used.

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