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Induced Conformational Changes in Novobiocin

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SUMMARY

The techniques of ultraviolet absorption spectroscopy and circular dichroism have been utilized to demonstrate in the substituted coumarin antibiotic, novobiocin sodium, a conformational change which could be induced by making the solution acidic. By comparing the ultraviolet spectra of novobiocin with those of coumarin and warfarin, a less complex substituted coumarin, the region of interest in the spectrum has been assigned to transitions within the coumarin ring. Upon acidification a large bathochromic shift was observed only for novobiocin, and has been attributed to increased conjugation as a result of an alignment of the benzamido group attached to position 3 of the coumarin skeleton of the drug. Also upon acidification, an inversion in sign of the circular dichroic band has been noted and has been attributed to a movement of the perturbing benzyl group to a negatively rotating quadrant in accordance with the octant rule. The entirely different circular dichroic spectra of the human albumin–novobiocin complex and the bovine albumin–novobiocin complex may be explained in terms of different conformational changes induced in the novobiocin molecule upon being bound to the two proteins.

INTRODUCTION

Circular dichroism and optical rotatory dispersion have been used as conformationally specific techniques to chronicle the interaction of proteins with ligands (1, 2). The present report resulted from initial observations during a study of the binding of the antibiotic novobiocin sodium to the two most widely used albumins, human and bovine. In using circular dichroic spectra to gain further insight into the binding process, vast differences were noted between the spectra of the two complexes. Subsequent studies, employing ultraviolet absorption and circular dichroism of the drug in solution, revealed unusual solvent-induced spectral changes. By observing the behavior of certain model compounds of similar basic structure, we reasoned that novobiocin sodium may undergo a conformational change as the composition of the solvent is varied from neutral to acid pH values below 3. The power of using circular dichroic measurements for the purpose of demonstrating conformational changes in small molecules has been shown by Wellman, Briggs, and Djerassi (3) for temperature perturbations of the structure of certain cyclohexanones.

Novobiocin is a dibasic acid isolated from *Streptomyces* bacteria (4) and is generally active against Gram-positive and some Gram-negative bacteria (5). Its formal chemical

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name is 7-[4-(carbamoyloxy)-tetrahydro-3-hydroxy-5-methoxy-6,6-dimethylpyran-2-yloxy]-4-hydroxy-3-[4-hydroxy-3-(3-methyl-2-butenyl)benzamido]-8-methylcoumarin. The structure of the drug was determined by Hoeksema *et al.* (6), and the monosodium salt appears in Fig. 1. The noviose sugar is joined by an α-glycosidic linkage with position 7 of the coumarin ring and is in the 1C conformation (7). Little else is known about the absolute conformation of the molecule. The diacid form is insoluble in water, but the monosodium compound which is obtained at physiological pH is extremely soluble. Dilute solutions at pH 2 are stable at 24°, but a half-life of about 60 days was observed at pH 7–10 (4). The drug is known to bind avidly to serum proteins (8).

Novobiocin sodium is an asymmetrical compound which exhibits a nonzero circular dichroic spectrum, the major bands of which correspond to its ultraviolet absorption maxima. By far the majority of the studies on ligand-macromolecular interaction using circular dichroism have thus far utilized inherently symmetrical lignads which, when bound to the macromolecule, exhibit extrinsic Cotton effects (1, 2). As evidenced by the circular dichroic spectrum of the drug-albumin complex in the region above 285 nm, novobiocin, being inherently asymmetrical, reveals a surprising change in its inherent asymmetry when bound to some albumin proteins. The binding process must perturb the asymmetry of the drug in a unique manner, either extrinsically, by imparting the asymmetry of the protein to the already asymmetrical drug, or intrinsically, by actually changing the conformation of the bound novobiocin. By demonstrating that novobiocin can undergo a solvent-induced conformational change, the present study lends credence to the intrinisc mechanism.

MATERIALS AND METHODS

Novobiocin sodium, lot ZD-692, was generously supplied by Dr. G. B. Whitfield of the Upjohn Company. It exhibited an absorption maximum at 304 nm with a molar absorptivity of 2.10×10^4 M^{-1} cm^{-1}. The absorptivity of the drug has been reported to be some 2% higher than this value (9). The ultraviolet absorption spectrum of the drug in 70% ethanol was found to be identical with that previously published (4). Coumarin was obtained from Aldrich Chemical Company, and warfarin sodium was bought as the single injection unit (75 mg) of Coumadin from Endo Laboratories.

Crystalline bovine serum albumin was lot 18 from Pentex Corporation, and crystalline human serum albumin was lot control No. 08029T from Grand Island Biological Comany. The albumins were dissolved initially to a concentration of 5% in 0.5 M NaCl, dialyzed against 200 times their volume of 0.5 M NaCl at 4° for 18 hr, and then twice against 200 times their volume of 0.1 M NaCl at 4° for 12 hr. Finally the albumins were dialyzed in the cold against distilled deionized water at a 200-fold volume excess for 72 hr with changes in dialysate every 12 hr. The albumins were then lyophilized to dryness and stored in a desiccator in the cold.

Further purification of the bovine albumin was achieved by defatting it according to the method of Chen (10). This defatted albumin was then used in all analyses reported here. In 0.1 M sodium phosphate buffer, pH 7.4, the bovine albumin had an $E_{1\,cm}^{1\%}$ at 279 nm of 6.60 while the human serum albumin had a $E_{1\,cm}^{1\%}$ at 279 nm of 5.29. All other chemicals and solvents were reagent grade.

Ultrafiltration was carried out by a modification of Toribara's method (11). Since novobiocin sodium was found to be retarded significantly by the dialysis membranes (Nojax 23, Union Carbide), the modification permitted a reproducible, small, and constant membrane surface area to be exposed to the drug-albumin-buffer solution ultrafiltered. Further details of the apparatus have been presented elsewhere (12). The concentration of the drug in the ultrafiltrate was measured by its absorbance at 304 nm in a Beckman DU-2 spectrophotometer either in a 10-mm microcell or a 1-mm standard quartz cell. For the bovine albumin the results obtained with the ultrafiltration method agreed within 2% with those from an equilibrium dialysis study.

Continuous scan ultraviolet absorption spectra were taken at room temperature (23–25°) on a Beckman DK recording spectrophotometer, while point measurements were made on a Gilford 220 absorbance attach-

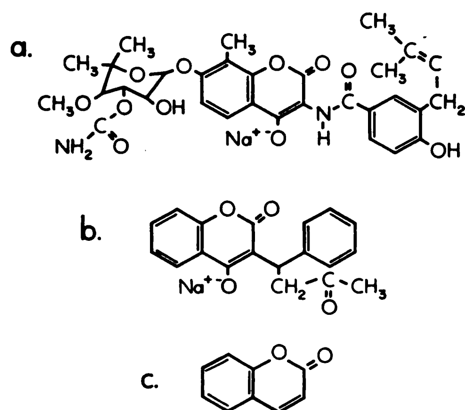

FIG. 1. *Structural formulae of novobiocin sodium (a), warfarin sodium (b), and coumarin (c)*

ment to a Beckman DU monochromator. Circular dichroic measurements were made on the 6002 attachment to a Cary 60 spectropolarimeter. Circular dichroic spectra were taken either at 29°, the thermostated temperature of the machine, or at 38°, using a jacketed 5-mm cell. At all times care was taken to keep absorbance values below 1.2. By analogy with molecular rotation, molecular ellipticity has been calculated using the formula

$$[\theta] = \text{molecular ellipticity} = \frac{\theta_{obs}\, 10^2}{lc}$$

where θ_{obs} is the observed ellipticity in degrees at wavelength λ, l is the path length in centimeters, and c is the molar concentration of the solute in question. $[\theta]$ has dimensions of degrees-cm^2 per decimole.

RESULTS

In Fig. 1 are shown the structures of (a) novobiocin, a complex substituted hydroxycoumarin (b) warfarin sodium, of lesser complexity, and (c) coumarin. In Fig. 2 are shown the ultraviolet absorption spectra of the three compounds at the physiological pH of 7.4 and at an acid pH near unity. Above 220 nm exactly the same spectra were obtained for coumarin at the two pH values. The spectrum of novobiocin at neutral pH reveals two prominent long-wavelength transitions: a peak at 304 nm with a molar absorptivity of 2.10 × 10^4 M^{-1} cm^{-1} and a shoulder at 289 nm having a molar absorptivity of 1.8 × 10^4 M^{-1} cm^{-1}. In the lower-wavelength region several transitions are visible, including prominent ones at 255 and 238 nm. In an organic molecule as complex as novobiocin sodium, it is difficult to assign these transitions to a particular chromophoric group of the molecule, but one can obtain a reasonably accurate estimate of their origins by studying simpler molecules of similar basic structure. This has been done for the higher-wavelength transitions.

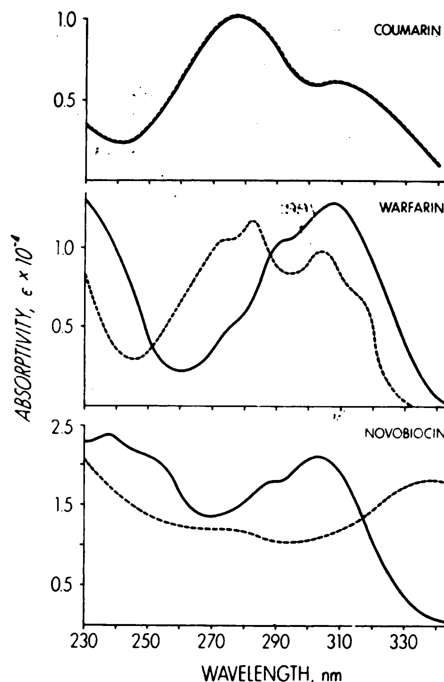

FIG. 2. *Ultraviolet absorption spectra of coumarin, warfarin, and novobiocin in aqueous phosphate buffer, pH 7.4* (——), *and aqueous acid pH 1* (- - -)
All spectra were recorded at room temperature, 23–25°.

Consideration of the coumarin ring system alone in novobiocin shows that it is a 4-hydroxy-8-methylcoumarin. The molecule coumarin, shown in Fig. 1c, is devoid of either substituent and thus is a simple model of the core of novobiocin. In the utralviolet absorption spectrum of coumarin shown in Fig. 2 there are two strong high-wavelength transitions: one at 277 nm and an intense shoulder at 308 nm. It is interesting that in apolar solvents such as cyclohexane and heptane (not shown in this paper) we observed that the lower-wavelength peak seen at 277 nm in

aqueous medium reveals fine structure and undergoes a hypsochromic shift whereas the shoulder seen at 308 nm in aqueous medium undergoes a bathochromic shift. Even in this case, in which the two transitions become well separated, no additional transitions were observed between them. Specifically, we observed no shoulder or peak in the ultraviolet absorption spectrum around 290 nm.

A second substituted coumarin drug is warfarin sodium, whose structure is shown in Fig. 1b. This bompound is a 4-hydroxycoumarin with a benzene ring attached to it through a secondary methyl linkage. Thus there is no possibility that the π-electron system of the coumarin ring could be conjugated with that of the benzene ring. Since further π-electron delocalization is not possible, there should be no pronounced shift of the higher transitions compared with those seen for the molecule coumarin (13). In Fig. 2 the ultraviolet absorption spectrum of warfarin sodium at pH 7.4 confirms this fact. There is a peak at 308 nm and a shoulder at 293 nm, along with a less intense shoulder around 276 nm.

It has already been mentioned that upon acidification of the solutions the spectrum of coumarin does not change above 220 nm. In the cases of warfarin and novobiocin some change might be expected by the conversion of the sodium salt to the protonated forms of the respective compounds. The acid warfarin spectrum shows additional fine structure throughout the ultraviolet region. In particular, no new peak appeared in the longer-wavelength region. The acid novobiocin curve, in contrast, seems to have lost much of its structure, but of special interest is the appearance of a strong peak at the longer wavelength of 340 nm. When a neutral solution was acidified and then neutralized to pH 7.4, exactly the same ultraviolet absorption spectrum was obtained at that given by the original solution. This indicates that acidification produced no irreversible change and that no degradation of the novobiocin had occurred.

Since coumarin and warfarin do not exhibit nonzero circular dichroic spectra in the region of interest, studies involving pH and solvent perturbation using circular dichroic measurements have been confined to novobiocin.

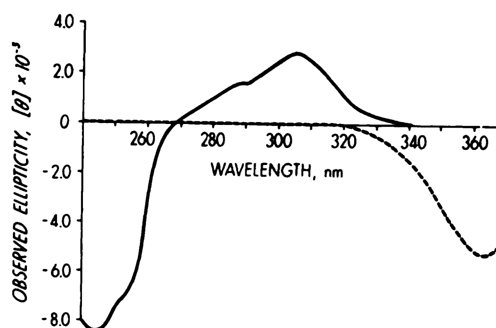

FIG. 3. *Circular dichroic spectra of novobiocin in aqueous phosphate buffer, pH 7.4 (——), and aqueous acid, pH 1 (- - -), at 29°*

The circular dichroic spectra of novobiocin sodium in aqueous 0.1 M sodium phosphate buffer, pH 7.4, at 29° and of the acid form at pH 1 are shown in Fig. 3. At pH 7.4 the two long-wavelength transitions observed in the ultraviolet absorption spectrum of the drug at 304 and 289 nm exhibit positive ellipticity. The molecular ellipticity of the 304 nm transition under these conditions is 7.61×10^3 deg-cm^2/dmole. Below 265 nm the drug begins a negative rotation, with a negative peak occurring at 245 nm and another at 236 nm, followed by a continuous decrease in rotation below 228 nm. It is this large negative rotation which undoubtedly accounts for the negative molecular rotation of the drug measured at the sodium *D*-line (4). At the acid pH the long-wavelength ellipticity has inverted in sign and the peak appears to be at 365 nm. The ellipticity observed at lower wavelengths at pH 7.4 has disappeared.

Since the acid form of novobiocin is not very soluble in aqueous media, the spectra obtained here were from a dilute solution of the sodium salt converted to the acid pH by the addition of concentrated hydrochloric acid just before measurement. The solution may have been supersaturated. The acid form is soluble in nonpolar solvents such as dichloroethane, and the circular dichroic spectrum of such a solution is shown in Fig. 4. Here both high-wavelength transitions observed in the ultraviolet absorption spectrum of the drug in this solvent at 340 and 305 nm have negative ellipticity. The highest-wavelength transition in Fig. 4 appears

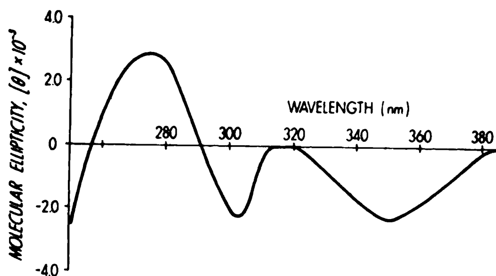

FIG. 4. *Circular dichroic spectrum of novobiocin in 1,2-dichloroethane at 29°*

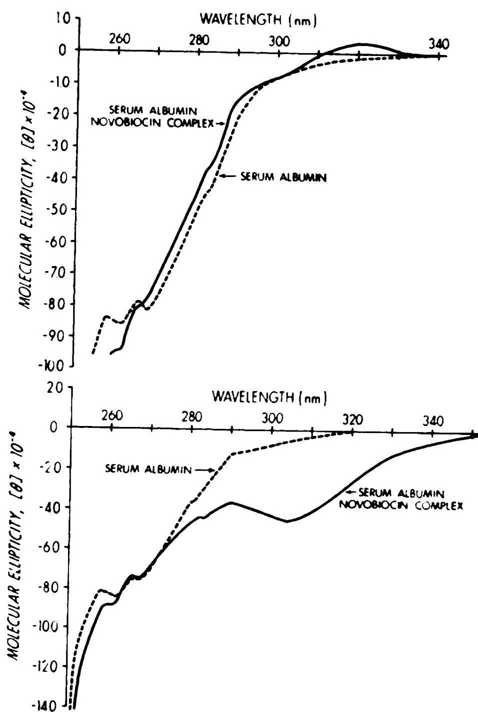

FIG. 5. *Circular dichroic spectra of two serum albumins and their respective novobiocin complexes at 38° in 0.1 M phosphate buffer, pH 7.4*

Upper curve: bovine serum albumin (- - -) and its complex with novobiocin (——) at a mole ratio of drug to albumin of 1.3. The concentration of bovine albumin in both cases was 35 μM; the total novobiocin concentration in the complex was 60 μM. The concentration of drug bound to albumin, determined by ultrafiltration at 38°, was 45.5 μM. Lower curve: human serum albumin (- - -) and its complex with novobiocin (——) at a mole ratio of 1.4. The concentration of human albumin in both cases was 36 μM; the total novobiocin concentration in the complex was 60 μM. The concentration of drug bound to albumin, determined by ultrafiltration at 38°, was 50.5 μM.

at 350 nm, and a second, of equal magnitude ($-2.0 \pm 0.3 \times 10^3$ deg-cm²/dmole), is observed at 305 nm. A positive peak at 274 nm is also seen, for which no comparable transition can be found in the ultraviolet absorption spectrum, although there is a weak trransition in the absorption spectrum in 1,2-dichloroethane at 261 nm.

The upper portion of Fig. 5 shows the circular dichroic spectra of bovine serum albumin and the bovine serum albumin–novobiocin complex at 38°. In both cases the albumin concentration was 35 μM (assuming a molecular weight of 69,000). The total drug concentration was 60 μM, and the concentration bound (determined by ultrafiltration) was 45.5 μM, giving a mole ratio of bound drug to albumin of 1.3. The bovine serum albumin curve is featureless until the inflection noted at 282 nm, followed by the appearance of two peaks which have ben attributed to disulfide bond transitions in human albumin (14). The complex shows an initial positive rotation at 319–320 nm, followed by a drop to the position of the free albumin curve from 304 to 299 nm. Because of the positive rotation of the drug and possible participation of the 282 nm inflection from albumin, the complex then becomes more positive and remains so until 265 nm. Below 265 nm the complex becomes more negative than the free albumin, probably because at this point the circular dichroic spectrum of the drug begins a negative rotation, which is apparently little changed in complexing with bovine serum albumin.

The lower portion of Fig. 5 shows the circular dichroic spectra of free human serum albumin and its novobiocin complex at a mole ratio of 1.4 at 38°. Human albumin in both traces was at a concentration of 36 μM (assuming a molecular weight of 67,000), while the total (free plus bound) novobiocin was 60 μM. The albumin spectrum agrees well with that published by Legrand and Viennet (14) and by Chignell (2). As with all other albumins tested, the human albumin also reveals an inflection at 282 nm. The human serum albumin–novobiocin complex reveals an inversion and enhancement by some 3.5

times of the 304 nm transition of the drug upon being bound. There is no shift in wavelength in this case, as in the positive rotation noted in the bovine albumin complex. After the negative rotation at 304 nm, the spectrum of the complex begins to move positively until it forms a peak around 290 nm, then continues a negative rotation until it coalesces with the free albumin spectrum from 272 to nearly 263 nm. Below 263 nm the complex reveals more negative rotation than free albumin, again probably because of the negative rotation of the bound drug below this point.

DISCUSSION

The ultraviolet absorption spectrum of the molecule coumarin revealed a relatively intense transition at 308 nm as well as one at 277 nm. Likewise, the substituted coumarin drug warfarin sodium has its highest-wavelength, very intense transition at 308 nm, with a shoulder at 289 nm. It can therefore be concluded that in warfarin sodium the transition seen at 308 nm arises from an electronic transition on the coumarin ring system. It is possible, though unproved, that the shoulder found at 289 nm may be a result of the 4-hydroxy substituent on the coumarin ring or, just as likely, is absorption due to the benzene ring moiety of warfarin. The absorption spectrum of 4-hydroxycoumarin in acid solution is at least qualitatively identical with that of warfarin sodium in acid medium, leading one to suspect the former hypothesis. Even though these transitions on both molecules undergo a bathochromic shift on being transferred to solvents of lower dielectric constant, their intensity makes it unreasonable to assume that they represent an $n \rightarrow \pi^*$ transition (15). At best the transition at 308 nm may be caused by a mixture of $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions.

In view of the ultraviolet absorption spectra of coumarin and warfarin sodium, a cursory look at the structure of novobiocin sodium indicates that the highest-wavelength transition should be somewhere above 308 nm, since the π-electron delocalization of the courmarin ring could extend over the amide bond to the benzyl moiety (13). However, as the absorption spectrum reveals, this is not the case. The highest-wavelength transition appears at 304 nm, actually 4 nm lower than that of coumarin. It must be tentatively concluded that the benzyl moiety is not conjugated with the courmarin portion of the molecule. Indeed, construction of the molecular model of novobiocin using Corey-Pauling-Koltun space-filling models illustrates that only with extreme steric strain can the two aromatic systems lie on the same plane. From the model it appears more likely that the amide bond is nonplanar Therefore the spectral results in aqueous medium at pH 7.4 suggest that there is no π-electron delocalization across the amide bond, a condition requiring the two aromatic systems to be perpendicular to each other.

Upon changing the pH of an aqueous solution from neutral to acid a large bathochromic shift from 304 to 340 nm occurs. Simple protonation of the 4-hydroxy substituent cannot account for this change, because warfarin and 4-hydroxycoumarin show no such extreme bathochromic shift. Rather, it may be assumed that novobiocin sodium undergoes a conformational change prmitting the benzyl moiety to come into partial conjugation with the coumarin ring. The circular dichroic spectral study of novobiocin sodium should confirm the suspicion of a conformational change, and indeed the spectra shown in Fig. 3 are markedly different for the two pH values. In the acid solution the sign of the ellipticity has inverted to negative, and the maximum appears at 365 nm, as compared to 340 nm in the ultraviolet absorption spectra. The circular dichroic spectrum of the acid form of novobiocin in dichloroethane shows the same inversion in sign of the long-wavelength transition, but the maximum appears at 345 nm. In addition, the dichloroethane solution also shows nonzero ellipticity in regions of the spectrum where the aqueous acid solution shows zero ellipticity. The absorption spectrum of novobiocin in dichloroethane is practically identical with that of the drug in aqueous solution. A 70% ethanol solution of the acid form gave substantially the same circular dichroic spectrum as the dichloroethane solution. These data suggest that the dielectric nature of the

solvent system also influences the conformation of the molcule. Hoeksema *et al.* (4) reported the optical rotation at the sodium *D*-line of the acid form to be −63.0 degrees (*c*, 1% in ethanol) and to vary with pH. The optical rotations of the monosodium salt, which gave a pH of 7.5 in a 1% aqueous solution, were −38 degrees (*c*, 2.5% in 95% ethanol) and −33 degrees (*c*, 2.5% in water). The data of Hoeksema *et al.* show changes in optical rotation with both pH and dielectric constant, which we interpret in terms of conformational changes. Although the exact nature of the presumed conformational changes cannot be determined solely from absorption and circular dichroic spectral studies, it may be reasonable to assume that the conformational change involves a rotation of the amide bond (both C═O and NH—CO bonds) of novobiocin sodium. This rotation must then involve a change in a region critical to the sign and magnitude of the ellipticity of a transition within the coumarin skeleton, since ultraviolet absorption studies have indicated that the region of interest (290–310 nm) arises from absorption by the coumarin ring. This possibility has been discussed more fully elsewhere (12), but obviously is only one of several mechanisms which can account for the observed phenomena.

When novobiocin sodium is bound to the serum albumins, dramatic changes in the circular dichroic spectrum of the drug are observed. The perturbations involve spectral transitions both from the drug and, presumably, from albumins at 282 nm. The two most commonly used albumins, human and bovine, give considerably different novobiocin complex circular dichroic spectra at physiological pH. The region of the spectrum of primary interest here involves that in which the molecular ellipticity of novobiocin in aqueous medium is positive. The spectrum obtained when the drug is complexed by bovine albumin is altered somewhat, but the ellipticities remain positive. Human albumin has an entirely different effect, causing not only an inversion in the sign of the ellipticities but also a 3.5-fold increase in intensity at the maximum, with no shift in its position. This type of inversion on binding has been reported at least once before, for the binding of flavin adenine dinucleotide to D-amino acid oxidase from pig kidney (16), but has not, to our knowledge, been found before for a drug bound to one of the serum albumins. This inversion of the FAD Cotton effect was interpreted as indicative of a change in conformation of the FAD upon binding to the enzyme. The changes reported here in novobiocin binding, particularly to human serum albumin, could be interpreted in a similar manner, especially since it has been argued that novobiocin undergoes simple solvent and pH-dependent conformational changes.

The inversion in sign of the ellipticity of novobiocin, with no bathochromic shift when bound to human albumin, contrasts with the observations made upon acidification of a neutral solution of novobiocin alone. Whereas changes in ultraviolet absorption and the circular dichroic spectra may both be interpreted as conformational changes, their causes are somewhat different. Extensive studies (13) of the influence of substituents on the location of an electronic transition indicated that a bathochromic shift occurs on introducing conjugation into the molecule. Therefore the bathochromic shift observed upon acidification of a novobiocin solution may be interpreted as a conformation change which introduces more conjugation into the molecule. In the case of optical rotation, Moffit *et al.* (17) have explained the influence of perturbing groups on the sign of the rotation by the octant rule, which states simply that the product of the $x \cdot y \cdot z$ coordinates of the group determines the sign. Thus a molecule may undergo a conformational change which manifests itself by changes in the ultraviolet absorption spectrum or in the sign and shape of the circular dichroic spectrum, or in both types of spectra simultaneously.

The reaction of novobiocin with human albumin may cause a conformational change in the drug which does not introduce further conjugation, but does permit the perturbing benzyl group to move into a negative quadrant. Bovine albumin, in complexing with novobiocin, may not move the benzyl

moiety into a negative quadrant but may, in contrast, introduce conjugation as indicated by the bathochromic shift, probably through rotation of the amide group. On the other hand, the actual conformation of the drug may be little changed when complexed with bovine albumin. The bathochromic shift may simply be a manifestation of the fact that the drug is now in an environment having a polarity different from water. Acidification of the neutral solution of novobiocin alone may then be interpreted as producing a protonated form which assumes a conformation with further conjugation as well as a movement of the benzyl group into a negative quadrant. The possibility always exists, of course, that the changes seen here in the circular dichroic spectra of the complexes are extrinsic in origin, i.e., that they arise not from a change in conformation of the bound drug but rather as a result of association with a unique asymmetrical center on each protein in question. Resolution of this problem would be difficult indeed; yet the fact that the drug readily undergoes a solvent-dependent conformational change argues for the intrinsic mechanism. The explanation given here for the observed phenomena is thus consistent with established rules. Chignell (18) studied the binding of 4-hydroxycoumarin and two of its substitution drugs (warfarin and acenocoumarin, which is warfarin with a nitro group on the benzyl ring) with human albumin. He reported no extrinsic Cotton effects for these cases.

More extensive investigations have been performed on the human and bovine albumin–novobiocin complexes. These will be reported subsequently.

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