Many steroids are well known to be capable of solvation, and various specific states of solvation are often associated with individual crystallographic forms. In the present work, X-ray diffraction studies indicated that the two steroids showing trace retention of ETO were submitted to sterilization in unsolvated crystal forms, whereas the two steroids showing appreciable ETO retention were sterilized in solvated (principally hydrated) forms. In both the latter cases, however, the samples were incompletely solvated and possessed additional capacity equivalent to at least 0.5 of a monosolvate. The residual ETO content of these samples, amounting to less than 0.1 of a monosolvate, thus could be accommodated readily on accessible solvation sites. Mixed monosolvate formation has been observed previously by the author for compounds crystallized from mixed solvents and is not an isolated phenomenon.

Mechanism of Glycol Retention.—Glycol is a secondary residue, resulting from the hydrolysis of ETO. The presence of ETO in the head space over samples stored in closed containers indicates that it is sufficiently mobile to react with water in the vapor phase (26) as well as within or on the samples.

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Interactions of Xanthine Derivatives with Bovine Serum Albumin II

Spectrophotometric Studies

By DAVID E. GUTTMAN and ANTONI E. GADZALA*

The spectral characteristics in aqueous solution of a number of xanthine derivatives were found to be influenced by the presence of bovine and human serum albumin. The observed effects were assumed to be due to the binding of the xanthines by the protein. Changes in spectra were thus used as the basis for an experimental method for determining the extent and nature of the small molecule-protein interaction. The effects of temperature, pH, and protein modification on binding behavior were studied. Comparative studies on a number of structurally different xanthines were conducted. The results indicate that a rather specific orientation of the protein molecule was required for optimal interaction. Expansion or other configurational changes induced by pH or temperature effects resulted in decreased binding. The binding site appears to contain a grouping which can hydrogen-bond with the xanthine. The participation of other types of interaction forces are suggested also.

A PREVIOUS REPORT (1) communicated the results of a study in which the binding of various xanthines by bovine serum albumin (BSA) was investigated by an equilibrium dialysis technique. By utilizing this experimental method, it was possible to demonstrate that a number of xanthines were bound reversibly by BSA, that the binding was pH dependent, and that structural modification of the small molecule had a pronounced influence on the degree and nature of the binding. It was the purpose of this investigation to restudy the previously investigated systems by a spectrophotometric

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method with the hope that this would provide a more sensitive means of detection and thus might yield additional information about the interactions.

Spectrophotometry can be used to detect binding if interaction is manifested by changes in the spectral characteristics of the small molecule. This method of detection, therefore, necessitates participation in the interaction of a chromophoric system. Disturbances in the system can result in bathochromic or hypsochromic shifts in spectra and/or hypochromic or hyperchromic effects. Although the method has limitations and, in some cases, can fail to detect interaction, it has been successfully utilized, for example, by Klotz (2) to investigate the protein binding of certain dyes and by Westphal (3), who studied the binding of a number of ketosteroids by human serum albumin (HSA). As evidenced by a number of reports, spectral changes also can be valuable in suggesting the nature of the forces responsible for interaction. As will be seen, the spectrophotometric technique used in this study yielded results which in some respects were qualitatively similar to those obtained from dialysis experiments and in addition did make apparent certain occurrences not observed with the dialytic technique.

EXPERIMENTAL

Materials.—The proteins used were BSA, fraction V (lots 32878, 33574, 35278), and HSA, fraction V (lot 33479) (purchased from Calbiochem Laboratories). Reagent grade mono-, di-, and trisodium phosphate and phosphoric acid were used to prepare buffer solutions. The xanthine derivatives were either synthesized by standard procedures or obtained from commercial sources.

Procedure.—Spectrophotometric examinations of xanthine-containing solutions in the absence and presence of protein were made on either a Cary model 15 recording spectrophotometer or a Beckman model DU spectrophotometer equipped with a dual thermal spacer assembly. The Cary was used primarily to obtain complete spectra of xanthines in the absence and presence of protein, while the Beckman was utilized in making absorbance measurements at single wavelengths. For each solution that contained xanthine and protein, a corresponding solution containing an equivalent concentration of protein was prepared. The latter solution was used as a reference blank in the spectrophotometric examination of the sample and served to blank out absorbance contributions from the protein. When possible, the concentration of xanthine was chosen to yield an absorbance reading below 0.800. When higher concentrations were required, such as with weakly interacting systems, silica spacers were used to decrease the light path length. Phosphate buffers were used to control pH, and all solutions were maintained at an ionic strength of 0.16. Calculation of protein molarity was based on an assumed molecular weight of 69,000.

RESULTS

Comparison of Spectra Obtained in the Presence and Absence of Protein.—Three characteristically different effects were observed when the spectra of various xanthines, which were obtained in the presence and absence of protein, were compared. Nonionizable compounds studied which included caffeine, 8-methylcaffeine, 8-methoxycaffeine, 8-ethoxycaffeine, 8-propoxycaffeine, 8-amoxycaffeine, and theophylline at lower pH values, were characterized by an effect, illustrated in Fig. 1, which represents the caffeine–BSA system. Here, the presence of protein resulted in a hypochromic effect. Absorbance values throughout the wavelength range of the spectrum were lower in the presence of protein than in protein-free solutions for all of these compounds. In all cases, the degree of hypochromism increased as the concentration of protein was increased. The presence of protein had no apparent effect, however, on the position of the wavelength of maximum absorption.

The 8-nitrotheophylline system yielded somewhat different results, represented by Fig. 2. Here, the presence of protein resulted in a blue shift. As the protein concentration was increased, absorbance at the 386 mμ peak decreased, the peak was shifted to slightly lower wavelengths, and absorbances at wavelengths below 355 mμ increased. Similar results were obtained with HSA.

An unusual effect was observed with 8-chlorotheophylline and is shown in Fig. 3. Here, the presence of protein resulted in an apparent shift of the spectrum to longer wavelengths with a concomitant hyperchromism at the wavelength of the new peak.

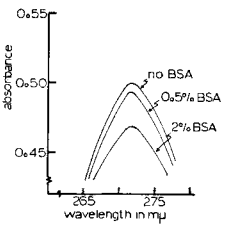

Fig. 1.—The influence of BSA on the spectrum of caffeine at a pH of 6.85.

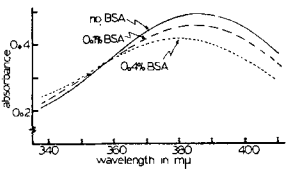

Fig. 2.—The influence of BSA on the spectrum of 8-nitrotheophylline at a pH of 6.85.

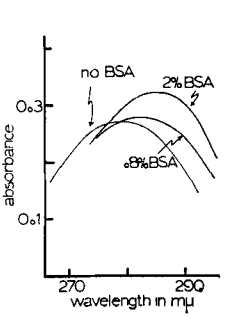

Fig. 3.—The influence of BSA on the spectrum of 8-chlorotheophylline at a pH of 6.85.

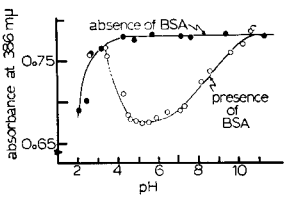

Fig. 4.—The influence of pH on the absorbances of solutions of 8-nitrotheophylline in the absence and presence of BSA.

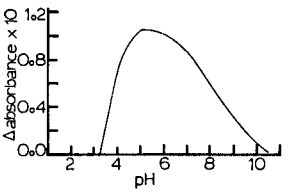

Fig. 5.—The influence of pH on the binding of 8-nitrotheophylline by BSA, reflected by changes in absorbance.

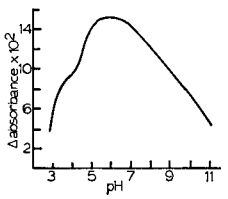

Fig. 6.—The influence of pH on the binding of 8-chlorotheophylline by BSA, reflected by changes in absorbance.

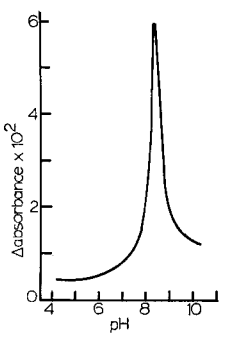

Fig. 7.—The influence of pH on the binding of theophylline by BSA, reflected by changes in absorbance.

The spectrum of theobromine was not altered by the presence of protein and confirmed a previous observation (1) that theobromine was weakly bound by BSA.

Influence of pH on the Interaction.—For each compound studied, absorbance determinations as a function of pH were made on two corresponding solutions which were equimolar with respect to the xanthine. One solution contained protein; the other did not. Typical results representing the 8-nitrotheophilline–BSA system are shown in Fig. 4. Here, absorbance as a function of pH is shown for the xanthine alone by the top curve and that for xanthine plus protein by the bottom curve. The top curve reflects pH dependent charges in absorbance which resulted from ionization of the compound (pKa 2.07), while the bottom curve reflected changes which occurred due to both ionization and interaction with the protein. At any pH value, the difference in absorbance between the two solutions resulted from the hypochromic effect which occurred on interaction with the protein. The magnitude of the difference is a qualitative measure of the extent of binding. Differences were interpolated from the two curves and are plotted as a function of pH in Fig. 5 to yield the pH profile of the interaction. It is seen that essentially no interaction occurred below a pH of 3.3. With increases in pH above this point, the extent of binding increased until a maximum was achieved at a pH of approximately 5.2. With subsequent increases in pH, the extent of binding decreased until complete abolition of interaction occurred at pH values of 10.5 and above. The 8-nitrotheophylline–HSA system yielded a pH profile almost identical to that obtained with BSA. Profiles for 8-chlorotheophylline, theophylline, caffeine, and a number of caffeine derivatives were obtained in a similar manner. All are qualitatively similar and are shown in Figs. 6–8. It is interesting to observe that, in contrast to the other compounds significant binding of 8-chlorotheophylline was observed at pH values above 10.5. The curve for theophylline indicates that strong binding of this compound occurred only when significant concentrations of the ionized form were present. The pH profiles for caffeine, 8-methylcaffeine, 8-methoxycaffeine, 8-ethoxycaffeine, and 8-propoxycaffeine were obtained by using an identical concentration of 6.0×10^{-4} M in each case. Because of this and the fact that all other experimental variables were the same, the pH profiles provide a means for qualitative comparisons of the binding affinities of the five compounds. It may be seen that the 8-alkoxy compounds are all bound to approximately the same extent, which is somewhat greater than that for the 8-methylcaffeine and considerably greater than that exhibited by caffeine. Solubility considerations necessitated using a less concentrated solution of the 8-amoxy compound; thus, its pH profile is lower than the others and cannot be compared to them.

Strength of Binding at pH 6.85.—Semiquantitative estimations of the strength of binding can be made in some cases from spectrophotometric data. The approach here requires the experimental determination of the absorbance or absorptivity of the small molecule in the bound state. This was achieved experimentally in the present study for 8-nitrotheophylline and 8-methoxycaffeine by the method described by Westphal (3). Absorbance values of solutions equimolar with respect to xanthine, but containing varying concentrations of protein, were determined in the usual manner. The apparent molar absorptivity for the xanthine was calculated for each solution and was plotted as a function of the ratio of xanthine molarity to protein molarity. Such a plot is shown in Fig. 9 for 8-nitrotheophylline. Extrapolation to zero yields an estimate of the molar absorptivity of a small molecule at infinitely high concentrations of protein where all xanthine would be expected to be in the bound form. The fraction of xanthine bound (f_b) in any solution was calculated from

$$f_b = \frac{a_f - a_x}{a_f - a_b}$$

where

- a_f = molar absorptivity of xanthine in the absence of protein,
- a_x = apparent molar absorptivity of xanthine at the protein concentration under consideration, and
- a_b = molar absorptivity of the bound xanthine as determined by extrapolation.

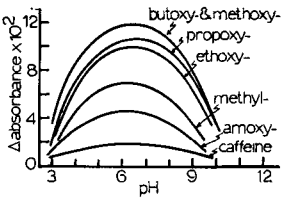

Fig. 8.—The influence of pH on the binding of caffeine and 8-substituted caffeine derivatives, reflected by changes in absorbance.

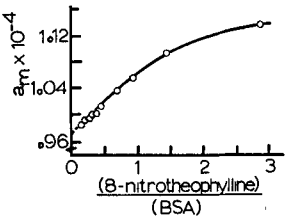

Fig. 9.—The influence of BSA concentration on the molar absorptivity of 8-nitrotheophylline at 386 mμ.

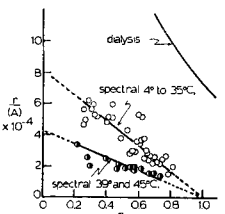

Fig. 10.—A plot showing the binding of 8-nitrotheophylline by BSA at a pH of 6.85.

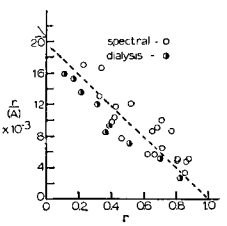

Fig. 11.—A plot showing the binding of 8-methoxycaffeine by BSA at a pH of 6.85.

The fraction of free xanthine (f_f) in a solution was calculated from

$$f_f = \frac{a_x - a_b}{a_f - a_b}$$

In this way it was possible to calculate for each protein concentration studied the number of moles of xanthine bound, the moles of xanthine bound per mole of protein (r), and the concentration of free xanthine in the solution (A). These values permitted construction of the conventional r *versus* $r/(A)$ plots which have been used frequently to estimate the number of sites available for binding on the protein and the association constant for the interaction (4). These studies were made at a pH of 6.85 in order to compare results to those obtained at the same pH in the previously reported dialysis study. Typical plots are shown in Figs. 10 and 11 for 8-nitrotheophylline and 8-methoxycaffeine. Results obtained by Eichman *et al.* (1) in their dialytic study are presented in these figures for comparative purposes. It is seen that the binding behavior of 8-nitrotheophylline, indicated by spectrophotometric examination, is quite different than that detected by dialytic methods. The spectral data indicated interaction with a single site on the protein with an association constant of approximately 8×10^4. The results obtained from the dialysis studies showed that binding was considerably more extensive and that more than one site on the protein were involved. In contrast, the binding behavior of 8-methoxycaffeine indicated by the spectral study was consistent, within experimental error, with that observed dialytically.

Quantitative treatments of the binding behaviors of caffeine and theophylline could not be made because of their relatively weak binding tendencies. The results obtained with 8-chlorotheophylline were completely inconsistent with those obtained by dialysis.

Influence of Temperature.—The binding of 8-nitrotheophylline by BSA (fraction V) was studied at 10 different temperatures ranging from 4.0° to 45°. The results are summarized in Fig. 10. The interaction appeared to be independent of temperature in the range of 4.0° to 35°. Binding at the two highest temperatures studied, 39° and 45°, was less than that found at the lower temperatures but appeared to be the same at the two temperatures.

Additional Studies.—BSA was acetylated by the method of Tabachnick and Sobotka (5). The modified protein did not bind 8-nitrotheophylline or other xanthines detectably.

EDTA was incorporated into protein–small molecule systems to test for the possibility of metal-ion mediated interactions. No difference in binding behavior was observed between systems which contained EDTA and those which did not.

DISCUSSION

Spectrophotometric examination of the interaction between xanthines and BSA yielded results in general agreement with those obtained by utilizing equilibrium dialysis as the investigational tool. Thus, both techniques showed that 8-nitrotheophyllinate interacted most strongly with the protein, followed by the 8-alkoxycaffeines, 8-methylcaffeine, and caffeine. The association constant for 8-methoxycaffeine was found to be approximately the same in both studies. Both techniques detected the lack of significant binding of theobromine, and both demonstrated a similar pH dependency for the binding of theophylline. That a single site on the protein was responsible for the binding of nonionizable xanthines, like 8-methoxycaffeine, was suggested similarly by both studies.

The nature of the binding of 8-nitrotheophyllinate, assessed by spectral studies, was somewhat different from that obtained from dialysis measurements. The spectrophotometric data showed binding to a single site, while dialysis indicated the involvement of two or more sites with a higher strength of binding. This discrepancy can be explained by assuming that the compound can be bound, as indicated by dialysis, to more than one site but that interaction with only one of them involved the chromophoric system. This seems reasonable in view of the fact that a single site appears to be responsible for the binding of nonionizable xanthines.

Results from the dialysis studies (1) did not make apparent the complete pH profile for the interaction and the fact that the influence of pH was similar for all of the xanthines. The more sensitive and convenient spectral method did permit a more comprehensive investigation of this effect. The in-

complete pH profile obtained by dialysis experimentation motivated the suggestion that a protonated amino group, possibly the ϵ amino group of a lysine residue, functioned as a binding site and that neutralization or acetylation of this site was responsible for inhibition of binding. The more definitive study permitted by spectral methods indicates that the nature of the binding site and the effect of pH on the interaction was more complex. Variation in pH above and below a value corresponding to approximately the isoelectric point of the protein resulted in decreased binding and complete inhibition at both high and low pH extremes. The pH profile on the acid side of the maximum parallels the pH-dependent N to F transition described by Foster (6). This transition is an expansion of the protein, which is electrostatic in nature and occurs due to protonation of carboxylate groupings. Similar expansions have been reported on the basic side of the isoelectric point (7, 8). It would appear that the site responsible for the binding of xanthines is contained by the compact N form of the protein and that expansion of the protein or other changes in its configuration resulted in the loss of the binding site. The extent of xanthine binding appears to be a sensitive indicator of such configurational changes. The interrelationships between conformational alterations of the albumin molecule and its ability to bind small molecules and ions has been reviewed extensively and discussed by Foster (6).

The lack of a temperature dependency in the temperature range of 4.0° to 35° indicates that the binding of 8-nitrotheophyllate, in common with many other anions, was due to a large increase in entropy resulting from the association. The decrease in binding observed at the two higher temperatures might reflect a temperature induced expansion of the macromolecule. Similarly, acetylation could have blocked binding, not necessarily by blocking a specific grouping, but by precluding a configuration necessary for interaction.

It was surprising to observe three different types of spectral effects in spite of the fact that all of the xanthines were quite similar in chemical structure. The hypochromism obtained when albumin was incorporated into solutions of nonionizable xanthines was quite similar to that caused by replacing or partially replacing water from aqueous solutions of the xanthines by solvents of lower dielectric constant, such as dioxane or ethanol. By analogy, this might mean that there was a difference in polarity between the microenvironment of the small molecule in the bound state and that in the free state. The shift of the spectrum of 8-nitrotheophylline to lower wavelengths caused by BSA is similar to that reported by Carsten and Eisen (9) in their study of the binding of 2,4-dinitrophenol to BSA. They suggested hydrogen-bond formation with a grouping on the protein as a possible explanation. Such an explanation is reasonable for this interaction as well. Evidence in support of this is that both protonation of 8-nitrotheophylline and changing from a nonhydrogen bonding solvent, such as dioxane to water or alcohol, result in a similar shift of the spectrum. It is pertinent that the anionic forms of xanthines like the theophyllinates are thought to exist through resonance stabilization, with the negative charge localized in the vicinity of the oxygen on C_6 of the molecule. The unusual shift observed in the 8-chlorotheophylline study as well as its pH profile, which was somewhat different from the others, and the fact that results from the spectral determination of the strength of binding were so different from those found with dialysis, motivates the suggestion that this compound underwent a nonreversible reaction of some type with the protein. Additional studies are being conducted currently to test this possibility.

The evidence obtained permits some speculation about the nature of the xanthine-albumin interaction. It appears that a rather specific orientation of the protein molecule is required for optimal interaction. Expansion of the macromolecule or other types of configurational changes result in loss of the binding site. This site probably contains a grouping which functions as a hydrogen donor that can undergo hydrogen-bond formation with the xanthines. Indications are that the microenvironment of the bound small molecule is less polar than water. The results suggest that it is the oxygen on C_6 of the xanthine nucleus that can hydrogen bond with the protein. This is supported by the spectral shift observed with 8-nitrotheophylline, by the fact that the anionic 8-nitrotheophylline interacts more strongly than the nonionizable xanthines, and by the fact that the ionized form of theophylline is more strongly bound than the nonionized form. That theobromine, which differs from the other xanthines by the absence of a methyl group at N_1 did not interact detectably also suggests the involvement of a part of the xanthine molecule in the immediate vicinity of N_1. The weaker binding of caffeine relative to 8-substituted caffeines shows that the interaction is sensitive to and strengthened by the presence of nonpolar substituents in the 8 position. Hydrophobic bonding between nonpolar groupings on the protein in the area of the binding site and the 8-substituent is, therefore, indicated. The stronger binding of 8-alkoxycaffeines, compared to 8-methylcaffeine, is suggestive of a degree of size specificity for optimal interaction.

Perhaps the most interesting observation made in the course of this study was the sensitivity of the binding behavior to pH dependent changes in the protein structure. The binding behavior of xanthines might provide a useful approach to obtaining additional information about such transitions.

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