Stability of GDA-3,16 *versus* Solvent Composition—Some data accumulated to date on short-term stability of GDA-3,16 in some solvents are given in Table II. These observations were obtained during the course of experiments to devise efficient reaction media for the conversion of GDA-3,16 to either Unknown 1 or GMA-16.

SUMMARY

GDA-3,16 degrades rapidly in water at its natural pH (about 9.5). In addition to the expected ester hydrolysis to germine monoacetate and germine, rapid conversion of GDA-3,16 to another as yet unidentified GDA occurred. Degradation of GDA-3,16 solution was accompanied by a drop in pH, reflecting the ester hydrolysis. A kinetic model describing these changes was proposed. TLC was used to monitor the chemical changes of GDA-3,16.

Overall stability of GDA-3,16 increases with decreasing pH ($t_{1/2}$ <1 hr. at pH 9.5, $t_{90\%}$ ~ 1 hr. at pH 7.2) and reaches a maximum near pH 4.6 (no instability detected after 24 hr.). Directions are given for preparing an aqueous GDA-3,16 solution stable for several days at room temperature.

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Interaction of Bishydroxycoumarin with Human Serum Albumin

M. J. CHO*, A. G. MITCHELL, and M. PERNAROWSKI†

Abstract □ The binding of bishydroxycoumarin to human serum albumin was studied by means of spectrophotometry, solubility analysis, and equilibrium dialysis. The data were interpreted on the basis of the theory of multiple equilibria. The human serum albumin–bishydroxycoumarin association is exothermic and occurs spontaneously under the experimental conditions. The α,β-unsaturated lactone structure in bishydroxycoumarin is involved in the complexation. A heterogeneity of binding sites on human serum albumin was observed. The average number of the first-type binding sites is approximately three. The corresponding intrinsic association constant is equal to 1.7×10^5 l./mole at 40° and 3.5×10^5 l./mole at 20°. The binding sites are believed to be hydrophobic regions, probably located in the interior of the human serum albumin molecule whose van der Waals' contour approximates and thus, in a sense is selective for, the bishydroxycoumarin molecule. The selectivity of the binding sites was supported by the large contribution of $\Delta H°$ to $\Delta G°$. The main binding energy is derived from nonionic sources.

Keyphrases □ Bishydroxycoumarin–human serum albumin—interaction □ Serum albumin, human—bishydroxycoumarin binding □ Solubility analysis—human serum albumin □ Equilibrium dialysis—analysis □ UV spectrophotometry—analysis

The binding of bishydroxycoumarin to human serum albumin was first reported about 20 years ago (1). However, the mechanism of interaction has not been extensively studied. The present work was designed to evaluate the mechanism.

EXPERIMENTAL

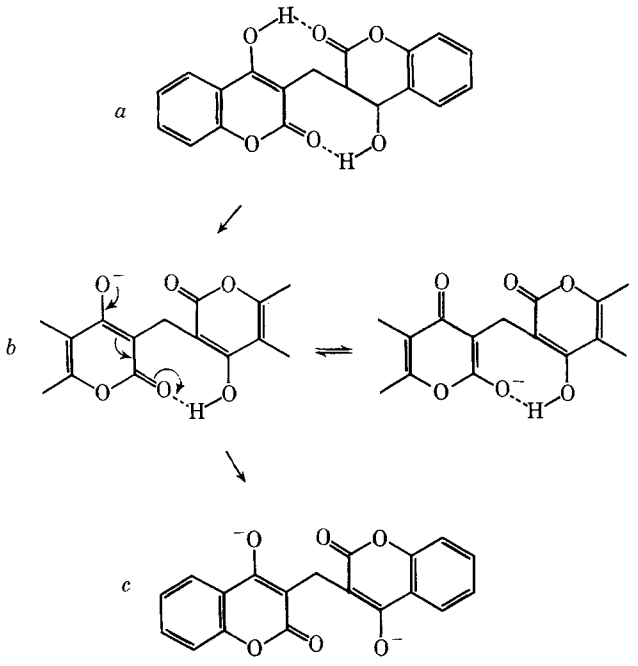
Materials—Bishydroxycoumarin USP[1] and human serum albumin Fraction V[2] were used in this study. Tris(hydroxymethyl)aminomethane was obtained commercially. Cellophane dialysis tubing[3] was cut to the size of the dialysis cell, washed thoroughly with distilled water, and stored in the refrigerator.

Buffer System—The effect of pH and ionic strength on the apparent solubility of bishydroxycoumarin was investigated (2). Solubility increased rapidly with increase in pH from 7.6 to 8.0 at a constant ionic strength of 0.2. This is the pH range where bishydroxycoumarin starts to undergo the second ionization. It is believed that completely ionized bishydroxycoumarin has a strong ion–dipole interaction with water molecules, which results in a marked increase in solubility.

The solubility also increased as the ionic strength, provided by chloride ion, was increased at a constant pH of 7.2. Frank and Wen (3) claim that halide ions, except fluoride, are "breakers" of water structure. The number of "unbound" water molecules is thus greater in the presence of chloride ion than in the ordinary liquid water, and this will provide more cavities for the solution of hydrocarbon (4, 5).

In the biologically significant pH range, there is little choice of buffer system. Phosphate buffer interferes with the protein binding of small molecules (6). Tris(hydroxymethyl)aminomethane has intramolecular hydrogen bonds (7) and, hence, is expected to be relatively inert. Chloride ion may interfere with the human serum albumin–bishydroxycoumarin interaction. However, its competitive

[1] Abbott Laboratories, Montreal, Quebec, Canada.
[2] Pentex Inc., Kankakee, Ill.
[3] Fisher Scientific Co., No. 8-667-2, 4.41-cm. (1.735-in.) flat width.



Scheme I—Chemical structure of bishydroxycoumarin showing two intramolecular hydrogen bonds (a), *resonance after the first ionization* (b), *and completely ionized bishydroxycoumarin* (c)

interaction was neglected since the intrinsic affinity of chloride ion for human serum albumin (8) would be far less than that observed for human serum albumin–bishydroxycoumarin.

On the basis of the preliminary experiments, a tris(hydroxymethyl)aminomethane–hydrochloric acid buffer system was selected having a pH of 7.4 and an ionic strength of 0.15. The pKa of tris(hydroxymethyl)aminomethane is 8.075 (9), and the buffer capacity of the system was 0.021. The ionic strength is sufficiently high to prevent the Donnan effect (10) in dialysis experiments.

Solubility Analysis—A series of solutions containing various amounts of human serum albumin was prepared in tris(hydroxymethyl)aminomethane buffer. Excess bishydroxycoumarin was added to 25.0 ml. of each solution in a 30-ml. centrifuge tube. The tubes were sealed and tumbled in a water bath at 20 ± 0.1°. After 40 hr., they were centrifuged for 20 min. at 2500 r.p.m. The temperature increase after centrifugation was always less than 1.0°. The bishydroxycoumarin content of the supernatant was determined spectrophotometrically.

Equilibrium Dialysis—Dialysis cells similar to those described by Patel and Foss (12) were used, and the procedures of Yang and Foster (13) and Pollansch and Briggs (14) were followed to cover a wide range of free drug concentration. The volume ratio of human serum albumin to human serum albumin-free compartments was varied by adding an extra spacer on either side of the membrane.

The cells were tumbled at 30 r.p.m. in a constant-temperature bath until equilibrium was reached (approximately 40 hr.). The human serum albumin-free compartment was analyzed for free bishydroxycoumarin. Experiments were carried out at 20 and 40° using 0.1, 0.2, and 0.4% human serum albumin.

Spectrophotometric Analysis—Bishydroxycoumarin in samples from the solubility and dialysis experiments was analyzed spectrophotometrically[4] at 304 nm. using tris(hydroxymethyl)aminomethane buffer as a blank. Corrections were made, where appropriate, for both human serum albumin content and depression of absorbance due to complex formation. The percent depression of the absorbance readings in the presence of excess human serum albumin was used to estimate the molar absorptivity value of bound bishydroxycoumarin. The absorptivity ratio of bound to free drug was 0.899 ± 0.012. The correction procedure and the calculation of binding parameters from the spectrophotometric data will be described in a subsequent paper (11).

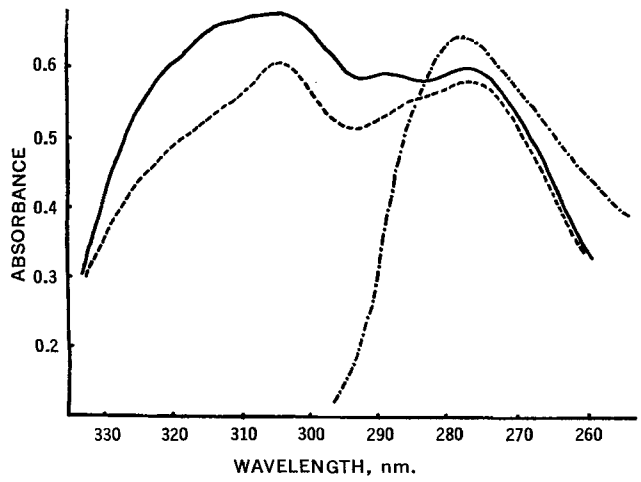


Figure 1—*Absorption spectra for 0.1% human serum albumin* (···) *and for bishydroxycoumarin in the presence* (- - -) *and absence* (——) *of human serum albumin at pH 7.4.*

RESULTS AND DISCUSSION

Apparent pKa Values of Bishydroxycoumarin—Recently, Hutchinson and Tomlinson (15) proposed a hydrogen-bonded structure for bishydroxycoumarin (Scheme I*a*). Two ionizable hydrogen atoms are involved in the internal hydrogen bonds. As shown in Scheme I*b*, the negative charge on the oxygen atom produced by the first ionization is expected to make the oxygen atom in the carbonyl group more negative by an electronic shift through the conjugated chain. This increase in electronegativity apparently increases its ability to form a hydrogen bond with the remaining hydrogen atom. The strengthened hydrogen bond will, therefore, make it more difficult for the second hydrogen atom to ionize. Only one ionization constant has been reported in the literature (16, 17).

Bishydroxycoumarin is very insoluble in acidic solution (0.5 mg./l. or 1.5×10^{-6} mole/l.), and the basic titrimetric approach in aqueous media cannot be utilized to estimate the pKa value. By use of precision spectrophotometry (18), the apparent pKa value was determined in dimethylformamide (DMF)–water mixtures (2). Extrapolation to 0% DMF resulted in pKa values of 4.4 and 8.0 for the first and second ionization constants, respectively. At pH 7.4, therefore, 80% bishydroxycoumarin exists as the monoionized species and 20% bishydroxycoumarin exists as the diionized species.

Spectrophotometric Analysis—As shown in Fig. 1, the spectral characteristics of bishydroxycoumarin are changed by human serum albumin. It is most likely that the functional group producing the absorption peak at 304 nm., which is depressed in the presence of human serum albumin, is involved in the complexation process. The same absorption peak is shifted to a lower wavelength as bishydroxycoumarin undergoes the second ionization (2).[5] The predominating chromophore on bishydroxycoumarin is probably the α,β-unsaturated lactone structure, which shows an electronic shift as the —OH group in the β-carbon ionizes (Scheme I*b*). The changes in electronic configuration which accompany the ionization process are believed to cause the shift of the absorption peak.

An attempt was made to evaluate the spectral changes due to complexation in a quantitative way following the method of Klotz (19). Agreement between binding data calculated from the spectrophotometric and equilibrium dialysis methods was poor. However, the dialysis method indicates a heterogeneity of binding sites on human serum albumin. Hence, it is likely that the estimated molar absorptivity value of bound bishydroxycoumarin is not a constant but varies according to the proportion of bishydroxycoumarin on each type of site.

Solubility Analysis—Many investigators, particularly Higuchi and Connors (20), have used phase-solubility analysis to study a wide variety of molecular interactions. Under favorable conditions, this method yields quantitative information about complexation. The method deals with a single free drug concentration and, there-

[4] Beckman DU-2 spectrophotometer.

[5] It was not possible to determine the changes in absorption spectrum associated with the first ionization of bishydroxycoumarin because of the extremely low solubility of bishydroxycoumarin in acidic solutions.

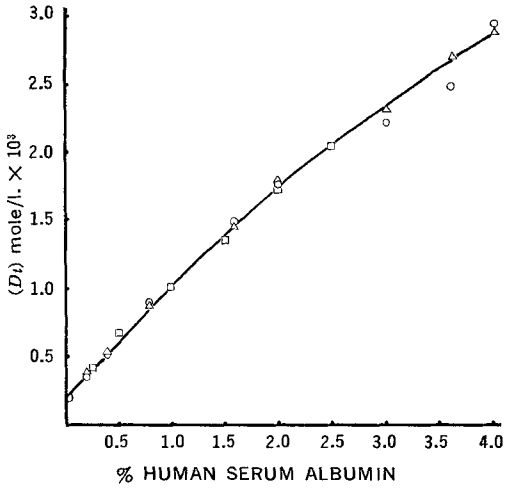


Figure 2—*Effect of human serum albumin on the apparent solubility of bishydroxycoumarin at 20° in tris(hydroxymethyl)aminomethane buffer. Different symbols represent separate experiments.*

fore, gives only one point on any binding curve based on the theory of multiple equilibria. However, this method is important since it provides information on binding at a high free drug concentration.

The effect of human serum albumin on the apparent solubility of bishydroxycoumarin is illustrated in Fig. 2. The solubility increases linearly with human serum albumin up to a concentration of 1.0%. Over this concentration range, the r value in Eq. 1 (the slope of the line) is a constant.

Equilibrium Dialysis—On the basis of preliminary experiments, a correction factor of 4% of the total bishydroxycoumarin[6] was used to correct for losses due to binding to the membrane and possibly to the Plexiglas of the dialysis cell.

Human serum albumin has a net negative charge of 18 at pH 7.4 (22). The ionic strength of the tris(hydroxymethyl)aminomethane buffer was 0.15, and the highest human serum albumin concentration used was 0.4% (5.8×10^{-5} mole/l.). Under these conditions, the distribution ratio of univalent anions across the membrane is equal to 1.0035 (10). It is possible, therefore, to neglect the abnormal distribution of bishydroxycoumarin anion due to the Donnan effect.

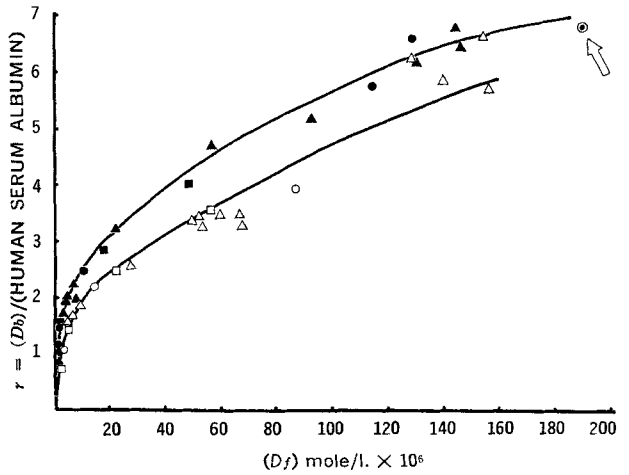


Figure 3—*Langmuir-type plot for human serum albumin–bishydroxycoumarin interaction in tris(hydroxymethyl)aminomethane buffer at 20° (closed symbols) and 40° (open symbols). Human serum albumin concentration: 0.1%, △; 0.2%, ○; and 0.4%, □. Data for solubility method indicated by an arrow.*

[6] Meyer and Guttman (21) suggested that corrections should be based on the amount of free drug only. Neither approach is strictly correct, and the membrane should be treated as a separate binding site. It is believed that the procedure described in this article will have a smaller error for a system in which most drug molecules exist in the bound form. The approach of Meyer and Guttman will have a smaller error for a system in which the extent of binding is low.

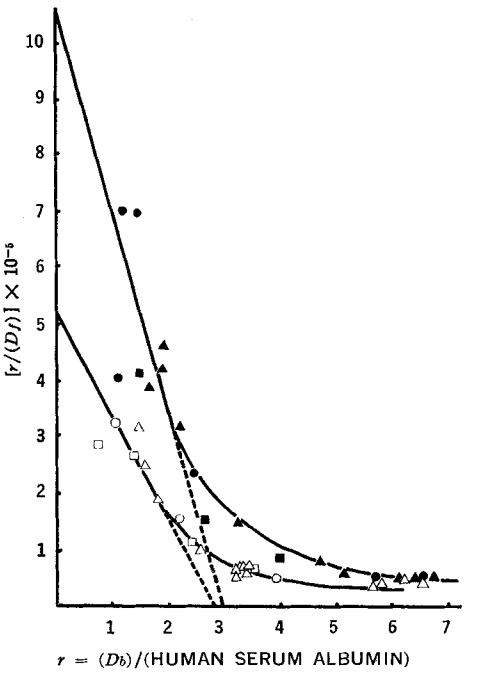


Figure 4—*Scatchard plot for human serum albumin–bishydroxycoumarin interaction in tris(hydroxymethyl)aminomethane buffer at 20 and 40°. Symbols as in Fig. 3.*

Binding data obtained using equilibrium dialysis are shown in Figs. 3 and 4.

Theory of Multiple Equilibria—The principles and concepts fundamental to an understanding of protein binding are found in *References 23–28.*

The molar ratio of bound drug to total protein, r, is a measure of binding. The value of r is expressed in terms of the intrinsic association constant, k, the maximum number of binding sites on a protein molecule, n, and the concentration of free drug (D_f).

$$r = \frac{nk(D_f)}{1 + k(D_f)} \qquad \text{(Eq. 1)}$$

The intrinsic association constant, k, is defined as the equilibrium constant for the association reaction between 1 mole of free drug and 1 mole of unoccupied binding site to form 1 mole of complex.

Equation 1 is similar to the equation derived by Langmuir (29) to describe a certain type of adsorption isotherm. Although they are similar, it is not always correct to assume that binding and adsorption are identical processes. The equations are similar because both have been derived from the law of mass action.

The so-called Scatchard equation (25) is derived from Eq. 1:

$$r/(D_f) = kn - kr \qquad \text{(Eq. 2)}$$

A plot of $r/(D_f)$ *versus* r is a straight line, where the intercept on the abscissa yields n and the intercept on the ordinate is equal to kn. Equation 1 is valid only if there is one type of binding site between which no electrostatic interaction exists. Most binding systems fail to obey these conditions.

Interpretation of Binding Data—The simplest way to treat the binding data is with a Langmuir-type plot of r value *versus* (D_f). Equation 1 is a segment of a rectangular hyperbola passing through the origin. If (D_f) in Eq. 1 becomes infinite, the r value approaches n as a limit:

$$\lim_{(D_f) \to \infty} r = n \qquad \text{(Eq. 3)}$$

and at $r = n/2$:

$$(D_f) = 1/k \qquad \text{(Eq. 4)}$$

These two equations show the importance of a wide concentration range of free drug in any binding study.

Figure 3 shows that, at low concentrations, bishydroxycoumarin is more easily bound to human serum albumin than at high concentrations. Only the data obtained from the solubility method show saturation of the binding sites. Quantitative information derived from Eqs. 3 and 4 is valid only if there is a single type of independent binding site. Therefore, binding parameters were not derived from this plot.

The binding data are replotted on the basis of the Scatchard equation (Eq. 2) in Fig. 4. The sharp bend near the abscissa indicates either that more than one type of binding site is present in the human serum albumin molecule or that there is a significant electrostatic interaction between the binding sites. The "screening" effect of high ionic strength in the buffer system rules out the latter possibility. Therefore, using the procedure of Rosenthal (28), each curve was resolved into two straight lines, each of which represents a different type of binding site. The values of n of the first type of binding site (3.0 at 20° and 2.8 at 40°) were rounded off to the nearest whole integer, 3. The intercepts on the ordinate, nk, are 10.5×10^5 and 5.1×10^6, which yield k values of 3.5×10^5 and 1.7×10^5 l./mole at 20 and 40°, respectively. The second type of binding site will not be considered because the intrinsic affinity of the bishydroxycoumarin molecule for these sites is much smaller than for the first type.

Thermodynamic Analysis and Mechanism of Interaction—Assuming that there is no significant temperature dependence of enthalpy change within the temperature range in which the interaction was carried out, it is possible to estimate the standard enthalpy change, $\Delta H°$, for the association of 1 mole of bishydroxycoumarin with 1 mole of the binding site from Eq. 5:

$$\log \frac{k \text{ at } 20°}{k \text{ at } 40°} = \frac{-\Delta H°}{2.303R}\left(\frac{1}{293} - \frac{1}{313}\right) \qquad \text{(Eq. 5)}$$

The standard free energy, $\Delta G°$, for complex formation is estimated from

$$\Delta G° = -RT \ln k \qquad \text{(Eq. 6)}$$

and the entropy change, $\Delta S°$, is obtained by substituting $\Delta H°$ and $\Delta G°$ into the Gibbs–Helmholtz equation:

$$\left(\frac{\partial \Delta G°}{\partial T}\right)_p = -\Delta S° \frac{\Delta G° - \Delta H°}{T} \qquad \text{(Eq. 7)}$$

Thermodynamic parameters for the association reaction are reported in Table I. The decrease in binding strength of human serum albumin for bishydroxycoumarin with increasing temperature is characteristic of an exothermic reaction and has been reported for many protein interactions (*Reference 30* gives some examples). The negative sign for $\Delta G°$ means that the binding process is spontaneous. The $\Delta S°$ is positive, in agreement with observations for other albumin–anion interactions (31).

A small temperature dependence of binding strength is a characteristic of an interaction between oppositely charged species (32). If the nature of the human serum albumin–bishydroxycoumarin interaction was largely electrostatic, *i.e.*, if the ionic part of the bishydroxycoumarin molecule combined with the cationic parts of the human serum albumin molecule, the main source of the $-\Delta G°$ value would be a large contribution of $+\Delta S°$ term with little contribution from the $\Delta H°$ factor. Thermodynamic changes for the human serum albumin–bishydroxycoumarin interaction indicate a large contribution to the $-\Delta G°$ value by the $-\Delta H°$ factor. It is very unlikely, therefore, that the association is ionic in nature. A similar interpretation for the interaction between human serum albumin and warfarin has been suggested by O'Reilly and Kowitz (30).[7]

In the absence of information about attendant conformational changes, most interpretations of binding processes make the assumption that preexisting binding sites are involved. On the other hand, Karush (33) postulated that serum albumin possesses "configurational adaptability" for a variety of small molecules and that binding sites might form during the binding process. Using this hypothesis, Karush (34) interpreted the differences in thermodynamic parameters observed in nonspecific albumin–anion and specific antibody–hapten interactions.

Table I—Thermodynamic Data for Human Serum Albumin–Bishydroxycoumarin Interaction

Temperature	$k \times 10^{-5}$ l./mole	$-\Delta H°$ kcal./mole	$\Delta G°$	$\Delta S°$, e.u.
20°	3.5	−6.58	−7.43	+2.90
40°	1.7	−6.58	−7.49	+2.91

The positive $\Delta S°$ associated with many reactions involving proteins is usually attributed to disorientation and unfolding of the protein molecule. This does not appear to be a satisfactory explanation for the human serum albumin–bishydroxycoumarin interaction, because the enthalpy changes observed are very negative. A process of unfolding presumably requires the breaking or bending of several bonds and should result in an endothermic reaction of appreciable magnitude (35). It is postulated, therefore, that the human serum albumin molecule has some kind of preexisting sites for the bishydroxycoumarin molecule.

The binding sites are probably hydrophobic regions whose van der Waals' contour is closely complementary to, and thus in a sense is selective for, the bishydroxycoumarin molecule. The selectivity is explained by a large contribution of enthalpy to the free energy (approximately 88% at both 20 and 40°). In this respect, the human serum albumin–bishydroxycoumarin interaction is in between the human serum albumin–warfarin interaction, in which the $\Delta H°$ contribution to $\Delta G°$ is approximately 50% (30), and specific interactions between antibody and hapten, in which the free energy term is due almost entirely to the enthalpy term (34). However, the human serum albumin–bishydroxycoumarin interaction is presumably quite different from antibody–hapten interactions because the site on human serum albumin is not completely rigid and the "key to lock" concept cannot be applied. It is suggested that hydrophobic interaction acts as the driving force for binding and that the sites are located in the interior of the protein molecule. The binding site cannot resist the disruptive tendencies either of thermal energy or of intramolecular electrostatic repulsion to which the human serum albumin molecule is subject at extreme values of pH. Hence, as Nagashimi *et al.* (17) observed, the human serum albumin–bishydroxycoumarin interaction is much less at pH 3.0–3.5 where a configurational change of the protein from a "compact form" to an "expandable form" takes place. Displacement of bishydroxycoumarin by another drug molecule from the protein (36) may arise in a similar way. It can be postulated that van der Waals' contour is disturbed by the third molecule resulting in a drastic decrease in binding affinity to the protein.

The human serum albumin–bishydroxycoumarin interaction involves the transfer of a hydrophobic molecule from an aqueous environment to a region with a lower dielectric constant. After binding occurs, a hypothetical hole previously occupied by the bishydroxycoumarin molecule remains and will be filled with an equal volume of hydrogen-bonded water molecules (34). The number of hydrogen bonds formed in this way will exceed the number of hydrogen bonds that the free bishydroxycoumarin molecule previously formed with its neighboring water molecules. It is believed from the intramolecularly hydrogen-bonded structure of bishydroxycoumarin (Scheme I*a*) that the number of hydrogen bonds between water and bishydroxycoumarin molecules is practically nil. On the basis of this explanation, complex formation will be exothermic and associated with a substantial decrease in the enthalpy of the system.

The "iceberg" concept of water structure (37) assumes that hydrocarbon groups, such as those present both in human serum albumin and bishydroxycoumarin molecules, are surrounded in aqueous solution with one or more layers of water molecules which are more highly ordered than the molecules in ordinary liquid water. The number of "structured" water molecules around the bishydroxycoumarin–human serum albumin complex will be less than those around the two unbound entities. Consequently, heat will be required (endothermic) for the fusion of the "icebergs." The exothermic nature of the binding, caused by an increase in the number of hydrogen bonds accompanying complexation, is believed to exceed the endothermic melting of the "icebergs," resulting in net negative $\Delta H°$ (Table I).

[7] In this paper, there is an error in the calculation of r (21). However, this error should not affect the thermodynamic data.

The melting of "icebergs" around bishydroxycoumarin and human serum albumin molecules will result in an increase in randomness (*i.e.*, positive $\Delta S°$) (4, 5, 38–40). The bishydroxycoumarin molecule will lose rotational and translational degrees of freedom after binding to yield a higher ordering (*i.e.*, negative $\Delta S°$). Formation of a bonded water cluster at the hypothetical hole left by the bishydroxycoumarin molecule will give an ordering effect (41). The disordering effect due to the melting of "icebergs" probably exceeds the ordering effects, resulting in the net entropy change of +2.9 (Table I). In more specific interactions, the net entropy change will be smaller than the value reported here and may become negative because of a significant loss of degrees of freedom.

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