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COMPARATIVE STUDY OF THE BINDING OF COUMARIN ANTICOAGULANTS AND SERUM ALBUMINS*

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Abstract—The interaction between coumarin anticoagulants and congeners and albumins from different species was studied using the technique of equilibrium dialysis. Coumarin was bound slightly, the addition of a hydroxyl group to either the 4- or 7-position resulted in a marked increase in binding, especially at low concentrations of ligand. The addition of an α-acetonylbenzyl side chain to the 4-hydroxycoumarin molecule, which is in both warfarin and acenocoumarin, caused a further increase in binding. The compounds containing two 4-hydroxycoumarin ring systems, Dicumarol and ethyl biscoumacetate, showed the greatest degree of binding. At low concentrations the binding of both of these was similar, however, at high concentrations Dicumarol continued to bind while the binding of ethyl biscoumacetate fell off sharply. A Scatchard plot of the data for the interaction of the coumarin with human serum albumin suggested that more than one binding site was involved in the binding process. Bishydroxycoumarin was bound extensively to all of the serum albumins studied, but only slightly to ovalbumin. A comparison of the amino acid compositions of each protein revealed striking similarities between the serum albumins, and marked differences in comparison with ovalbumin.

THE COUMARIN anticoagulants have been used for a number of years for prophylactic purposes in thromboembolism. Many of these agents are bound to serum proteins, especially serum albumin. This binding affects their pharmacologic and pharmacokinetic properties. The agents which have been studied, generally, have been bound to a high extent which, in part, contributes to their long duration of action.

Other drugs may be bound to the same sites as the anticoagulants which may complicate therapy. For example, in a recent epidemiological study conducted by Hansten,[1] it was reported that drug interactions with the coumarin anticoagulants may be responsible for numerous bleeding episodes. Some of these bleeding episodes were attributed to the displacement of bound anticoagulant. For example, Aggeler *et al.*[2] found the anticoagulant effect of warfarin was potentiated by pyrazolone derivatives. Equilibrium dialysis studies showed that the pyralozones displaced warfarin bound to serum albumin. The effect of such a displacement *in vivo* would be to permit the amount of anticoagulant in the liver to become elevated, thus accounting for an enhanced effect. Other drugs have been reported to displace bound anticoagulant, e.g. clofibrate, diphenylhydantoin[1] and tolbutamide.[3]

The purpose of the present study was to obtain further information concerning the structural features of the coumarins which favor their combination with the albumins, as well as to compare the binding of these substances by albumins from different species.

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MATERIALS AND METHODS

Albumins

The albumins used in this study were obtained from several sources. Bovine serum albumin (BSA), rabbit serum albumin (RSA) and human serum albumin (HSA) were four times recrystallized preparations obtained from the Nutritional Biochemicals Corp. Twice crystallized ovalbumin was obtained from the Worthington Biochemicals Corp. Further purification of the crystalline albumins was not deemed necessary. Ovine (sheep) serum albumin (OSA) was obtained from Calbiochem Corp. This fraction was reported to contain impurities[4] some of which were removed by treatment with Norit.[5] Amino acid analysis of the albumins was performed on a Bio-Cal BC 200 amino acid auto-analyzer. The protein solutions used in the dialysis studies were made at a concentration of 4 mg/ml in 0·01 M tris pH 7·4.

Drugs and chemicals

Tris (Hydroxymethyl) Aminomethane (Fisher Scientific Co.) was used as a buffer throughout this study at a concentration of 0·01 M and at pH 7·4, unless otherwise stated.

The drugs were obtained from various sources. Bishydroxycoumarin (Dicumarol) and coumarin from the Nutritional Biochemicals Corp., 4-Hydroxycoumarin from the Aldrich Chemical Co., and warfarin sodium from Endo Products, Inc., Ethyl biscoumacetate (Tromexan) and acenocoumarin (Sintrom) from Geigy Pharmaceuticals.

The drug solutions were prepared to provide a concentration of 500 μmoles/l. in 0·01 M tris pH 7·4 and diluted as necessary. Since warfarin sodium and coumarin were soluble in tris alone, they required no prior treatment. However, bishydroxycoumarin, 4-hydroxycoumarin, and acenocoumarin are not readily soluble in water; therefore each compound was dissolved in a small volume of 0·01 N NaOH after which each was diluted to volume with tris buffer. Ethyl biscoumacetate was dissolved in small volume of acetone and brought to volume with 0·01 M tris buffer.

Equilibrium dialysis

Equilibrium dialysis was used to study the binding of the coumarins by the albumins. Suitable portions of Visking casings (16 mm dia.) were soaked for 24 hr or more in 0·01 M tris pH 7·4 prior to use, in order to leach out absorbing materials which might interfere. Five or 10-ml aliquots of albumin were dialyzed against about 2 vol. of medium containing the coumarin congener studied, in triplicate. The tubes were capped and shaken for 24 hr at about 27° after which the amount of free anticoagulant was measured and subtracted from the initial amount added to obtain the amount bound. Appropriate controls were carried out to determine whether the albumins penetrated the dialysis casing or whether the casing contributed any ultraviolet absorbing materials. Pilot experiments with bishydroxycoumarin showed that equilibration occurred in about 6 hr under the experimental conditions employed. To insure that equilibration had occurred, experiments were carried out for 24 hr. Electrophoretic analysis revealed that albumins exposed to these experimental conditions migrated the same as samples which had not been shaken, which suggested that denaturation had not taken place as a result of the treatment. The concentration of anticoagulant was determined at appropriate wavelengths (ranging from 300 to 320 nm).

Analysis of data

The data were analyzed by two procedures: (1) by means of a multiple site plot in which r, μmoles of drug bound per μmole of protein, was plotted as a function of (A) unbound drug concentration and (2) by the procedure of Scatchard.[6]

RESULTS AND DISCUSSION

The dialysis studies were carried out using albumin concentrations of 4·0 mg/ml and generally using six concentrations of anticoagulant which at the start of the experiment ranged from 0·025 to 0·500 μmoles/ml in the dialysis media. In Fig. 1 is a summary of the results of the studies of the interaction between BSA, a series of

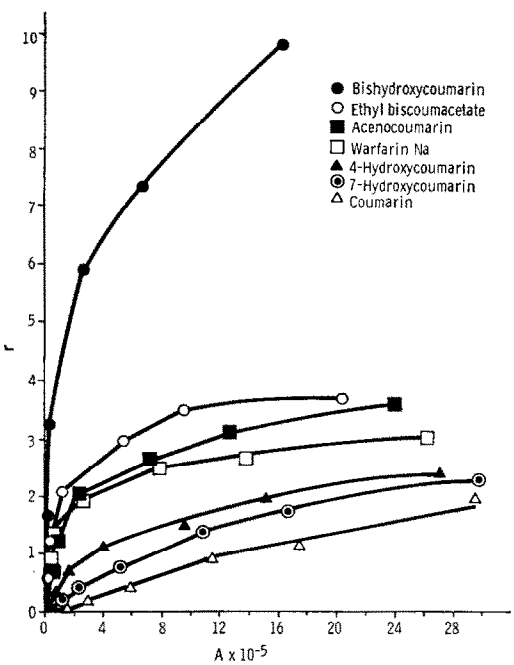

FIG. 1. A comparison of the binding of different coumarin congeners by BSA. Six different amounts of each compound were used, while the amount of protein was held constant. The samples were dialyzed 24 hr and analyzed. Each point is the mean of three determinations.

anticoagulants and some related compounds. As illustrated, coumarin was bound to the slightest extent while bishydroxycoumarin was bound to the greatest extent. The other compounds were intermediate in the degree of binding. In the case of coumarin, the stoichiometry was about 2 moles/mole of protein. While with bishydroxycoumarin more than 9 moles were bound per mole of protein. Due to the limited solubility of Dicumarol in aqueous media at neutral pH, it was not feasible to attain saturation under the experimental conditions employed.

Coumarin was bound, and the presence of a hydroxyl group at either the 4- or the 7- position of the coumarin molecule resulted in a marked increase in the binding, especially at low concentrations. The presence of the hydroxyl group may have provided a negatively charged group on the coumarin molecule which could interact

with a cationic center on the protein. In comparing coumarin with the 4- and 7-hydroxy derivatives, two to three times as much was bound at the highest concentration studied. It is interesting to note that the minimum requirements for anticoagulant activity are an intact 4-hydroxycoumarin molecule with the 3-position containing a hydrogen atom.[7]

The addition of an α-acetonylbenzyl side chain to the 4-hydroxycoumarin molecule which is present in both warfarin and acenocoumarin increased the binding further. BSA, as well as the other serum albumins, bound both of these drugs to about the same extent (3–4 moles/mole of protein).

Compounds containing two 4-hydroxycoumarin ring systems such as ethyl biscoumacetate and bishydroxycoumarin, showed an even greater degree of binding by bovine serum albumin than the previous compounds. In the study with bishydroxycoumarin, as greater amounts of drug were used, greater amounts were bound. At the highest drug concentration employed, approximately 9–10 molecules of drug were bound. Similar findings were observed in studies with the other serum albumins. At low drug concentrations, the binding of both compounds were similar. This may be due to the possibility that the initial binding of the drugs might involve the two 4-hydroxyl groups with the cationic centers on the surface of the serum albumin molecule. As the concentration of the drug increased, the binding of ethyl biscoumacetrate fell off sharply while the extent of binding of bishydroxycoumarin increased. The leveling off of the binding of ethyl biscoumacetate by BSA suggested that saturation of some of the binding sites on the albumin molecule had occurred which was not the case with bishydroxycoumarin. It should be pointed out that in the use of these two anticoagulants, ethyl biscoumacetate was reported to have an onset of action of about 12 hr, while bishydroxycoumarin had a latency period of 24–48 hr.[8] Some of the differences in onset between these two drugs may be due to differences in binding. The addition of the esteratic substituent to the carbon bridge separating the two 4-hydroxycoumarin moieties as in ethyl biscoumacetate may result in an increase in the polarity of the compound. This would tend to diminish the hydrophobic interactions of the non-polar parts of the molecule with the aliphatic side chains on the serum albumin molecule. It is also possible that the esteratic substituent might provide some steric hindrance in an interaction with certain sites. Chignell reported that the bishydroxycoumarin complex produced an extrinsic cotton effect while the other anticoagulants did not.[9] The addition of a carbethoxy group resulted in a loss of optical activity which suggested that steric factors are important.

Similar binding studies were carried out with the human serum albumins. In this case, the data were also analyzed using the procedure of Scatchard.[6] Assuming that the law of mass-action applies, the equation may be written as follows:

$$\frac{r}{(A)} = K_a (n\text{-}r),$$

where n is the total number of equivalent and independent sites per molecule of protein that are available for binding; K_a is the summation of the intrinsic association constants; r, the binding ratio, the moles of drug bound per mole of protein; and (A) is the equilibrium concentration of free drug. The results of the study with HSA are summarized in Fig. 2. The curvilinear relationships suggested that more than one type of binding sites on the albumin molecule were involved.

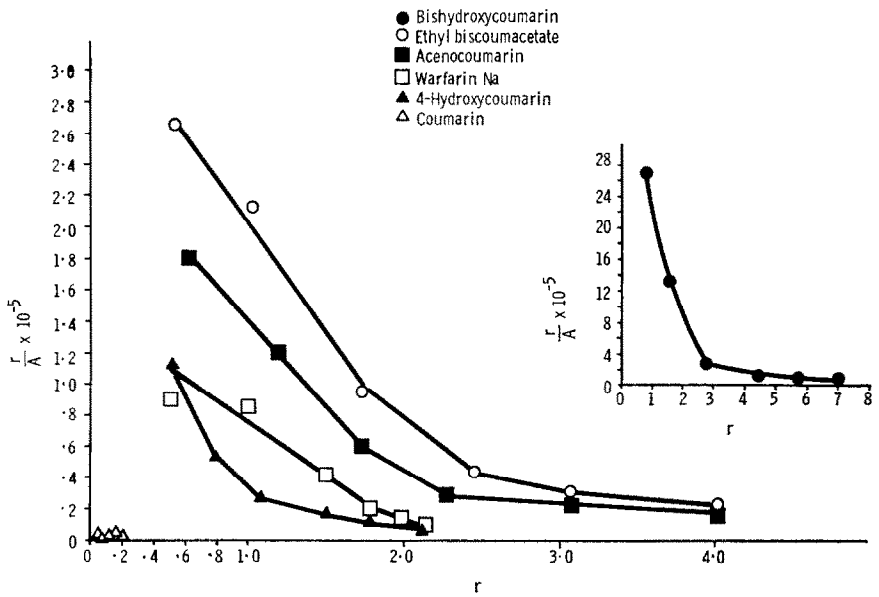

FIG. 2. Scatchard plot of the binding of coumarin congeners by HSA. Six different amounts of each compound were used, while the amount of protein was held constant. The samples were dialyzed for 24 hr and analyzed. Each point is the mean of three determinations.

The simplest interpretation is there may be two classes of binding sites with two different affinities. It may be speculated that at low concentrations of drug there are a limited number of binding sites on the serum albumin molecule which upon being occupied results in conformational changes which exposes additional binding sites. Inaccessibility of the latter binding sites could be due to the possibility that they were buried in the interior of the protein (hydrophobic in nature) or they may have been on the surface of the protein but positioned inappropriately with respect to auxiliary binding sites in the anticoagulant. Such a possibility is supported by the optical rotatory dispersion studies of Markus and Karush,[10,11] who observed changes in the configuration of the albumin molecule during the binding of azo dyes and detergents.

A comparison of the binding of bishydroxycoumarin by bovine, ovine, rabbit, human serum albumin and ovalbumin was made (Fig. 3). Ovalbumin was included because it was an albumin outside the class of the serum albumins. The binding studies revealed that bishydroxycoumarin was bound extensively to all of the serum albumins studied. Although there are scattered reports of the amino acids present in some of these proteins, the methods used varied. For this reason all of the proteins studied were analyzed for amino acid composition by the same procedure (Table 1). Differences in amino acid composition possibly could help explain the differences in the affinity observed.

In considering the amino acids involved in binding, several amino acids have been suggested as being important in the binding of anions. Cationic centers on the protein molecule are intimately involved in the binding process of other substances.[12] These centers consist of basic amino acids such as histidine, lysine and arginine. Relatively large amounts of the basic amino acids were found in all of the serum albumins,

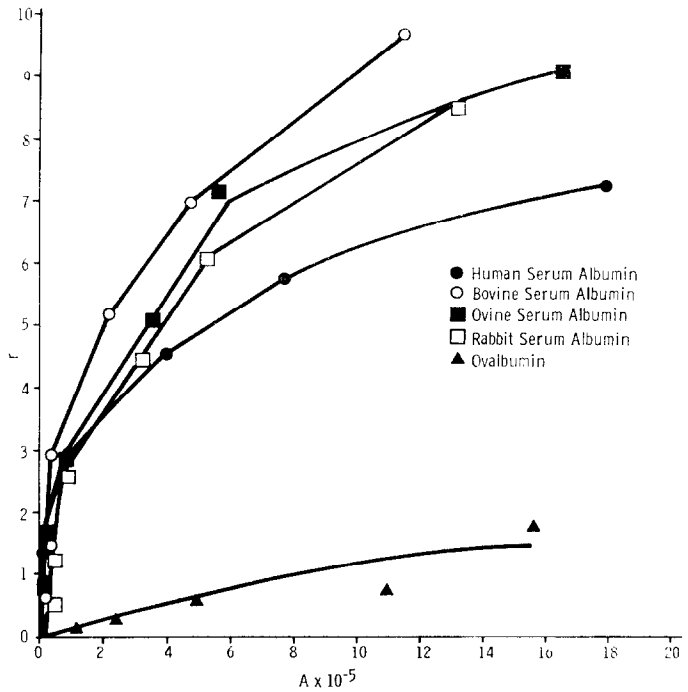

FIG. 3. A comparison of the binding of bishydroxycoumarin and different albumins. Six different amounts of drug were used; the amount of protein was held constant. The samples were dialyzed 24 hr and analyzed. Each point is the mean of three determinations.

especially lysine, as compared with ovalbumin. That more was involved in the binding process, than just the interaction with lysine residues, was demonstrated by Wosilait[13] who reported that polylysine bound Dicumarol only slightly.

Other amino acids may also be involved in binding. For example, some aliphatic amino acids have also been suggested to have a high affinity for the non-polar portion of the anionic molecules.[14] These amino acids contain non-polar side chains such as the isopropyl group of valine, the secondary and iso-butyl groups of the leucines and the benzyl group of phenylalanine. Both the serum albumins and ovalbumin contained comparable amounts of the aliphatic amino acids. It is possible these groups may interact with the hydrophobic moieties of the anticoagulants.

Hydrophobic bonding was found to be accompanied by a positive entropy change because of the increased freedom of the water molecules which were in the neighborhood of the non-polar groups before association.[15] O'Reilly reported an entropy change of +11·2 entropy units for the interaction between warfarin sodium and human serum albumin.[16] On the basis of microcalorimetric studies between warfarin and HSA, O'Reilly[17] provided additional evidence for the involvement of hydrophobic bonds in the formation of the complex. Hydrophobic bonding may involve the benzene rings.

Stahman reported that the pK of bishydroxycoumarin was 7·2.[18] Thus, at pH 7·4 more than half of the material will exist in a negatively ionized form and the remainder in the enol form. Since hydrophobic forces diminish inversely proportional to the

TABLE 1. COMPARISON OF THE AMINO ACID COMPOSITION OF ALBUMINS

Amino acid	Human serum albumin	Bovine serum albumin	Rabbit serum albumin	Ovine serum albumin	Ovalbumin
			(Residues/mole of protein)		
Glycine	11–12	16	19–20	20–21	19–20
Alanine	65–66	47	56	50	36–37
Valine	41	36	37	39	33
Leucine	66	65–66	66	63–64	33
Isoleucine	8	13	15	14–15	23
Serine	17	20–21	19	20–21	32
Threonine	26–27	32–33	24–25	31	14
Tyrosine	18	19–20	23	19–20	12
Aspartic	54	56	57	60	32
Glutamic	92	89–90	76	78	55
Histidine	15	17	21	18	2
Arginine	23–24	23	22–23	24	15–16
Lysine	67–68	72	69	64	20–21
Phenylalanine	32	26–27	24–25	28	20
Tryptophan			Not determined		
Half-cystine	32–33	33	42	37	3–4
Methionine	6	4–5	2	6	16
Proline	18	21	24	22	13
Ammonia*	41	37–38	37	43	54
Total number of residues	591–597	589–596	596–600	593–598	378–383

Molecular weight of serum albumins was taken as 69,000.
Molecular weight of ovalbumin was 45,000–12,000 (CHO moiety) = 43,800.
* Not included in the total.

seventh power of the distance the hydrophobic forces will diminish to a relatively small value at relatively short distances. On the other hand, electrostatic forces diminish as the reciprocal of the square of the distance, thus will have a somewhat greater influence at a greater distance from the surface of the albumin molecule than the hydrophobic forces. Hydrogen bonds probably lie in between the above. In view of these differences, it seems possible that the earliest forces involved in the interaction between these drugs and serum albumin could involve cationic centers on the surface of the serum albumin molecule in an electrostatic interaction with a negative charge on the anticoagulant. This possibility was supported by an experiment in which the dialysis was carried out at pH 9·5, at which pH there was a decrease in binding (unpublished observations). At pH 9·5 there would be a lesser extent of ionization of lysine and a greater degree of ionization of bishydroxycoumarin. At present it would appear that there are multiple factors involved in the binding process.

Further studies are in progress to clarify and elucidate the sites on serum albumin which are capable of interacting with the anticoagulants.

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