Studies on Protein Binding I

Interaction of *para*-Hydroxybenzoic Acid Esters with Bovine Serum Albumin

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The binding of methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid (parabens) by bovine serum albumin (BSA) was investigated by an equilibrium dialysis procedure. All the compounds were found to interact with BSA. The association constants were ascertained from the dialysis data, and free energy change, ΔF^0, enthalpy change, ΔH^0, and entropy change, ΔS^0, of the interaction were computed from the data at two temperatures. The possible mechanism of the interaction is discussed. The spectrophotometric study of the interaction revealed hypochromic effects in the absorption spectra of all the parabens in the presence of BSA. The *in vitro* antifungal activity of methyl- and propylparaben was found to be related to the concentration of free or unbound drug.

INTERACTION OF NUMEROUS small molecules with serum proteins has been extensively reviewed by Goldstein and most of the drugs studied have been found to bind primarily to the albumin fraction of plasma proteins (1). Although a majority of the earlier reports were of a qualitative nature, attempts have been made to quantify the data with an ultimate aim of elucidating the nature of equilibria and forces involved in the drug-macromolecular interaction (2, 3). With the advancement of the theoretical knowledge of these interactions, considerable research has been conducted on the binding of salicylates (4, 5), sulfonamides (6–8), penicillins (9), xanthines (10), steroids (11), and other therapeutic agents with serum albumins.

In their extensive reviews Goldstein (1) and Brodie and Hogben (12) have discussed the importance of drug–protein association in the transport, distribution, and elimination of medicinal agents. In addition, it is generally recognized that the specificity of pharmacologic agents lies in the structure of complexes which they form with macromolecular systems. The investigation of the factors involved in such interactions may shed some light on the understanding of drug action.

Interactions of some benzoic acid derivatives (5) with serum albumins have been investigated. The degree of ionization and the relative size of the anion of the interacting molecule influenced the protein binding, and Boyer *et al.* (13) concluded that an anion with a fairly large nonpolar group imparted an increase in its binding capacity for BSA. Both textbook and reference works (14–16) indicate a significant loss of biological activity of some antifungal agents in the presence of protein materials and Alto *et al.* observed that the antimicrobial activity of *p*-hydroxybenzoates was diminished in the presence of protein (17). No reports could be found of experimentation on the direct correlation of binding data with the *in vitro* antimicrobial activity of *p*-hydroxybenzoates. The purpose of this work was to evaluate quantitatively the binding of a series of *p*-hydroxybenzoic acid esters by bovine serum albumin (BSA) and to show that the *in vitro* antifungal activity of methyl- and propylparaben was primarily a function of free or unbound drug.

EXPERIMENTAL

Materials—Bovine serum albumin, fraction V (BSA) purchased from the Armour Pharmaceutical Company (lots B24011 and D25901), was used throughout this study. The albumin[1] content of lots B24011 and D25901 were 97 and 95%, respectively. Both lots of albumin exhibited similar binding tendencies for methyl *p*-hydroxybenzoate. The moisture content of protein was determined and appropriate corrections were made in weighing it; a molecular weight of 69,000 was assumed. Reagent grade monobasic potassium phosphate and dibasic potassium phosphate were employed to prepare the buffer solutions. Reagent grade potassium chloride was used to obtain an equilibrium ionic strength of 0.16 where necessary. The *p*-hydroxy-

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[1] The analytical results were kindly supplied by Dr. T. Douglas of the Armour Pharmaceutical Company, Kankakee, Ill.

benzoic acid esters (parabens) and the synthetic culture medium were the same as described in a previous paper (18). All paraben and protein solutions were prepared immediately prior to use.

Dialysis Studies—The general approach, technique, and treatment of data of this study have been described previously (19, 20). Dialysis cells were the same as those employed by Patel and Foss (20) with the exception that seamless cellulose membranes (Fisher Scientific Company) were employed in the present work. The membranes were placed in distilled water and heated to 90° for 1 hr., and they were washed with distilled water several times in order to remove any contaminating substances in the cellophane (7). The membranes were permeable to *p*-hydroxybenzoates and impermeable to BSA. Binding data of an extensively dialyzed sample of the BSA yielded results similar to those from an undialyzed commercial material. Thus undialyzed BSA was used in the entire investigation. Binding of parabens by the membranes and the dialysis cells was found to be insignificant. Equilibrium was established at the end of 16 hr. by agitation on a rotational shaker in an Aminco constant-temperature laboratory bath (American Instrument Co. Inc.).

The dialysis studies were performed on 0.1 m*M* BSA, with the exception that in the case of propylparaben–BSA interaction, an additional 0.25 m*M* BSA was included. All parabens were studied at pH 7.4 and their initial concentrations ranged from 5.15×10^{-4} *M* to 1.32×10^{-2} *M*. Binding data were also collected for the methyl- and propylparaben–BSA systems at pH 6.6 for the purpose of approximating the pH of the synthetic culture medium (18).

Spectrophotometric Studies—Spectrophotometric examination of paraben solutions in the absence and presence of BSA was made at room temperature on a Hilger Ultrascan recording spectrophotometer, model H999. The concentration of paraben used was 6.4×10^{-5} *M*. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell.

Analysis After Equilibration—At the end of equilibrium time 1- or 2-ml. aliquots were removed from both sides of the membrane and were diluted with 0.1 *N* HCl to suppress the dissociation of parabens. The concentrations of parabens were determined using a Beckman DU spectrophotometer at a wavelength of their maximum absorbance, 256 mμ. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell and it was found that at very high dilutions employed for the analysis of parabens, there was no effect on the absorbance of parabens in the presence of BSA. Any absorption contribution by the dialyzable components in BSA was assumed to be negligible in lieu of the high dilutions employed in the analysis. The pH values of the solutions were recorded with a Beckman zeromatic pH meter, model 76, after each dialysis experiment, with no appreciable change noted.

Microbiological Studies—Observations were made on the influence of BSA on the antifungal activity of methyl- and propylparaben for *Aspergillus niger*.[2] The culture medium and methods as described previously (18, 21) were employed with the following exception in sterilization procedure. In order to avoid denaturation of protein at the high autoclave temperature, the solutions were sterilized by Millipore filtration using a hypodermic syringe, Swinney adapter, and Millipore filter.[3] Inhibitory concentrations for methyl- and propylparaben, in the presence and absence of BSA, were obtained through visual observation of samples each day for a period of 2 weeks.

RESULTS

Treatment of Data—The mathematical analysis of binding data from equilibrium dialysis studies has been described and discussed by Klotz (2, 22) and Scatchard (23). If the intrinsic association constants for all polymer sites are identical, the data can be plotted according to the equation of Scatchard (23),

$$\frac{r}{(A)} = nk - rk$$

where r = moles of bound drug/mole of protein, (A) = free drug concentration, n = number of binding sites available on each protein molecule, and k = association constant. Plotting $r/(A)$ *versus* r will give a straight line with slope = $-k$, and ordinate and abscissa intercepts of nk and n, respectively. By the method of least squares the quantities nk and k were calculated using an IBM 360/67 computer. From the value of k, the thermodynamic functions were calculated according to the following equations (2, 24):

$$\Delta F^0 = -RT \ln k$$

where ΔF^0 = the free energy change, R = gas constant, and T = absolute temperature.

$$\ln \frac{k_2}{k_1} = \frac{\Delta H^0}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where ΔH^0 = the enthalpy change and k_1 and k_2 are binding constants at temperatures T_1 and T_2.

$$\Delta F^0 = \Delta H^0 - T\Delta S^0$$

where ΔS^0 = the entropy change.

Dialytic Data of the Paraben-Protein Interaction—The data for the interaction of methyl-, ethyl-, propyl-, and butylparaben at pH 7.4 and at two temperatures are plotted in Figs. 1–4. Within the concentration ranges of the drugs and the protein, a linear relationship of $r/(A)$ *versus* r, as described by Scatchard's equation, was noted. The k and n values were obtained by linear extrapolation to the ordinate and abscissa axes.

Absorption Spectra in the Presence and Absence of Protein—The absorption spectra of certain molecules are altered in the presence of proteins and this spectral change is indicative of an interaction between two compounds. This change usually consists of a decrease in the absorption coefficient. The pronounced hypochromic effects of methyl-, ethyl-, propyl-, and butylparaben in the presence of increasing concentrations of BSA are depicted in Figs. 5–8. The method has been employed by var-

[2] UAMH No. 1456, kindly supplied by Miss Marjorie Dineen, of the Provincial Laboratory of Public Health, Edmonton, Canada.

[3] Mixed esters of cellulose, marketed as MF-Millipore, Type HA, Millipore Ltd., Montreal, Canada.

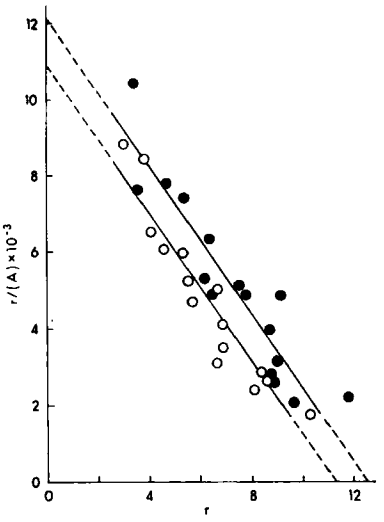

Fig. 1—Binding of methylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 10°.

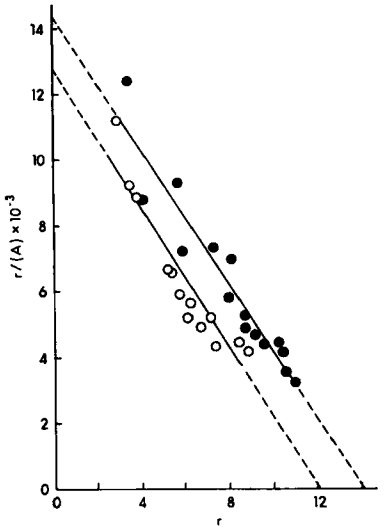

Fig. 2—Binding of ethylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

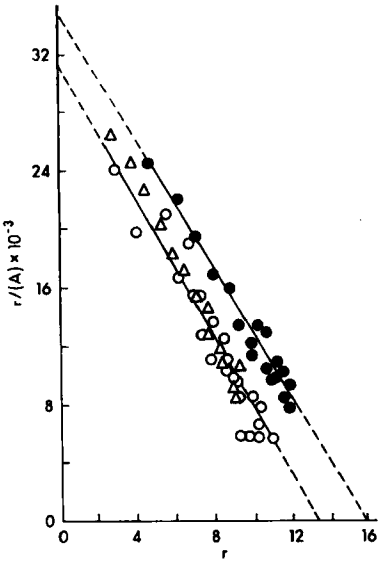

Fig. 3—Binding of propylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°; △, data collected employing 0.25 mmole BSA, pH 7.4, at 30°; all other data of Figs. 1–4 were obtained using 0.1 mM BSA.

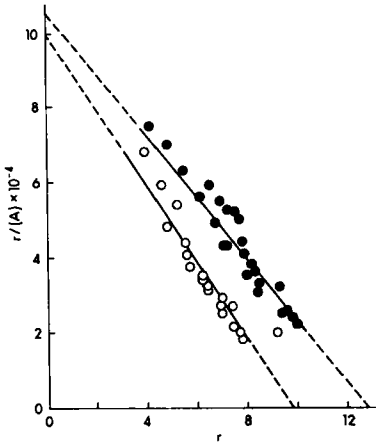

Fig. 4—Binding of butylparaben by BSA, pH 7.4. Key: ○, 30°; ●, 20°.

ious investigators (25–27) to study the binding of some drugs by proteins.

Inactivation by BSA Related to Binding—It was shown in earlier studies that the ratio, R, of total to free preservative for a given concentration of polysorbate 80 and cetomacrogol 1,000 was fairly constant (18–21). If the antifungal activity of parabens can be assumed to be due to the free or unbound form, the R value may be used to predict the minimum inhibitory concentration of antifungal agent in the presence of a definite quantity of BSA by a procedure described previously (18, 21). It is recognized that in the case of BSA the R value would not be constant over a wide range of concentrations of the parabens. However, within a narrow range of concentrations, especially around the minimum inhibitory concentration of the drug, it can be assumed to be reasonably constant, as outlined below. The R values, as obtained employing 0.1 mM BSA solution, at pH 6.6 and 30°, were 1.19 (range 1.16–1.23 with standard deviation of 0.026) and 1.75 (range 1.60–1.95 with standard deviation of 0.12) for methyl- and propylparaben, respectively. The total paraben concentrations on the protein side at the end of the dialysis experiment were 0.062–0.110% and 0.030–0.05% in the cases of methyl- and propylparaben, respectively. The results of the *in vitro* correlation of binding data with the antifungal activity of methyl- and propylparaben for *Aspergillus niger* in the presence of 0.1 mM BSA are summarized in Table I. It is evident from these data that there is a good correlation between the predicted minimum inhibitory concentration and the experimental antifungal concentration for both the parabens.

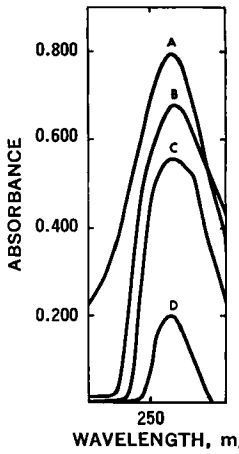

Fig. 5—Absorption spectra of methylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

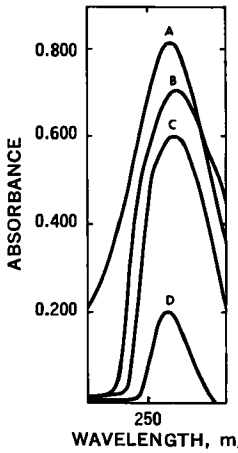

Fig. 6—Absorption spectra of ethylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

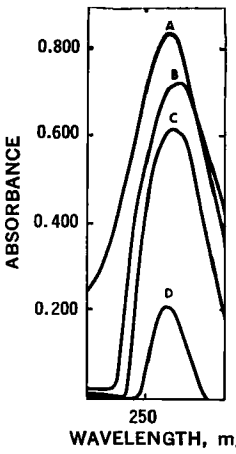

Fig. 7—Absorption spectra of propylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

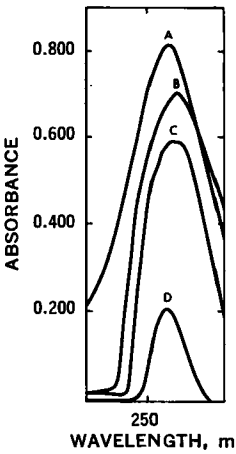

Fig. 8—Absorption spectra of butylparaben at pH 7.4. Key: A, buffer; B, 0.025 mM BSA; C, 0.050 mM BSA; D, 0.10 mM BSA.

DISCUSSION

The ability of *p*-hydroxybenzoic acid esters to interact with several macromolecules has been well documented (18, 19, 28, 29). Based upon this observation, it was expected that BSA might provide binding sites for the parabens. From Figs. 1–4, it is evident that all the parabens interacted with BSA. The values for the association constants, k, and number of binding sites, n, together with the thermodynamic parameters of the interaction, are summarized in Table II. From this table it is noted that the values of n are not integers. This might be either due to the experimental error in dialytic studies as explained by Klotz (30) or due to the view that protein might not be molecularly homogeneous with respect to binding properties as theorized by Karush (31).

The exact mechanism of binding of *p*-hydroxybenzoates by BSA cannot be elucidated from this study, since the results were obtained primarily from the binding of methyl-, ethyl-, propyl-, and butylparaben. However, certain tentative conclusions may be drawn. A comparison of association constants for various parabens at a definite temperature (Table II) reveals that the binding increases from methyl- to ethyl- to propyl- to butylparaben. Due to the limited solubility of hexyl- and heptylparaben it was not possible to evaluate the association constants for these compounds by dialysis studies. An increase in the solubility of these compounds in aqueous BSA solution at pH 7.4 over their water solubility at the same pH indicated that hexyl- and heptylparaben would be bound to a greater extent than butylparaben (32). About 40% of the total amino acids in the protein are nonpolar residues (33) and it is quite possible that the association between these residues and the nonpolar moieties of the paraben molecule (aromatic ring and hydrophobic chain of ester) may contribute to the overall interaction. An examination of the data of Table II shows that the magnitude of binding increased with an increase in temperature, demonstrating that the binding process was endothermic (unfavorable positive enthalpy). More significantly, the binding was accompanied by an increase in entropy term which is typically a thermodynamic behavior in common

TABLE I—CORRELATION OF PROTEIN BINDING DATA WITH *In Vitro* ANTIFUNGAL ACTIVITY OF PARABENS, OBSERVED FOR TWO WEEKS

Concn., BSA	Minimum Inhibitory Concn. Methylparaben, g./100 ml. Pred.[a]	Expt.[b]	Propylparaben, g./100 ml. Pred.	Expt.
0		0.060		0.020
0.1 m*M*	0.071	0.074	0.035	0.035

[a] Predicted from the binding data. [b] Experimental values.

TABLE II—ASSOCIATION CONSTANTS AND THERMODYNAMIC PARAMETERS OF PARABEN–PROTEIN INTERACTION IN AQUEOUS SOLUTION AT pH 7.4

Paraben	Temp.	Binding Constant (k), l./mole × 10^{-3}	nk × 10^{-3}	No. of Sites, n	Free Energy Change (ΔF^0), kcal./mole	Enthalpy Change (ΔH^0), kcal./mole	Entropy Change, (ΔS^0) e.u.
Methyl-	30°	0.987	10.9	11.2	−4.15	0.0845	14.0
	10°	0.968	12.1	12.5	−3.87		14.0
Ethyl-	30°	1.05	12.8	12.2	−4.19	0.529	15.6
	20°	1.02	14.4	14.1	−4.04		15.6
Propyl-	30°	2.33	31.3	13.4	−4.67	0.876	18.3
	20°	2.22	34.9	15.7	−4.49		18.3
Butyl-	30°	10.1	99.4	9.9	−5.56	4.00	31.5
	20°	8.11	105	12.9	−5.24		31.5

with that described by Schachman (34) for hydrophobic bonding. The positive entropy change for this type of interaction has been ascribed by Kauzman (35) and others (36, 37) as resulting from the energy change accompanying the disruption of the "iceberg" structure of water molecules around hydrocarbon groups in aqueous solution (hydrophobic bonding). The large difference in the values of ΔH_0 and ΔS_0 of propyl- and butylparaben was probably due in part to the experimental error which resulted from a significant scatter in the experimental points in the case of butylparaben (Fig. 4), especially at 30°.

In Figs. 5–8, the degree of hypochromism increases with an increase in concentration of protein. The decrease in the absorption has been interpreted by Klotz (25) as an effect due to the formation of drug–protein complex, if the complexation involves a chromophor system of drug. Although spectrophotometric study can often yield information as to the magnitude of the interaction as well as to the nature of forces responsible for the interaction, and such study is in progress, this part of the work was limited to the qualitative nature of the interaction.

The results of correlation of binding data with the inhibitory concentration of methyl- and propylparaben for *Aspergillus niger* in the presence of 0.69% BSA are in good agreement. This part of the study shows that the protein-bound paraben is devoid of *in vitro* antifungal activity.

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Keyphrases

Protein binding
Bovine serum albumin interaction—*p*-hydroxybenzoic acid esters (parabens)
Dialysis—bovine serum albumin-parabens
Parabens, antimicrobial activity—binding effect
IR spectrophotometry—analysis