Table III—α- and β-Tribenosides (Nanograms per Milliliter) in the Plasma of a Human Subject Given 800 mg of Tribenoside Orally for 7 Days

Anomer	Time after Administration								
	0 hr	1 hr	2 hr	3 hr	4 hr	6 hr	8 hr	Day 2	Day 8
α-Tribenoside	ND[a]	41	53	20	10	6	6	11	14
β-Tribenoside	ND	94	92	51	36	24	24	52	47

[a] ND = not detected.

A 2-μl portion of the heptane solution was injected into the gas chromatograph by the solvent flush technique.

The α- and β-tribenoside content was calculated from the ratio of the peak areas by reference to a calibration curve prepared from a series of dried methanolic α- and β-tribenoside solutions to which plasma was added to yield concentrations between 2 and 150 ng/ml.

Human Subject—A healthy male subject, who had not received any drug for 8 days prior to the experiment and did not take any other drug during the study, received two 400-mg tribenoside capsules before breakfast for 7 days.

Blood samples were collected on the 1st day before and 1, 2, 3, 4, 6, and 8 hr after drug administration. Blood was collected on Days 2 and 3, just before the morning drug administration.

RESULTS AND DISCUSSION

Reaction Time—The derivatization was applied to 10, 25, and 500 ng of α- and β-tribenosides, and the reaction time was varied from 1 to 30 min. Derivative formation was evaluated by peak areas; derivatization was immediate, and the yield of the α- and β-tribenosides was the same over 1–30 min. For convenience and safety, the suggested derivatization time is 10 min.

Sensitivity and Precision—Tables I and II show that when the concentrations of α- and β-tribenosides reached the lower limits, the coefficient of variation increased to ~10. These lower concentrations (10 and 5 ng/ml for α- and β-tribenosides, respectively) may be taken as the assay sensitivity limits; lower concentrations could be detected but could not be determined accurately.

Plasma Interferences—Figures 1*a* and 1*b* show the chromatograms of a blank human plasma extract and the same plasma spiked with 100 ng of α- and β-tribenosides and 150 ng of β-clobenoside, respectively. No interference of the normal plasma components was noted.

Specificity—The four main metabolites (all a mixture of the corresponding α- and β-anomers) were derivatized under the same conditions as tribenoside. These metabolites were ethyl 3,5-di-*O*-benzyl-D-glucofuranoside, ethyl 3,6-di-*O*-benzyl-D-glucofuranoside, ethyl 5,6-di-*O*-benzyl-D-glucofuranoside, and 3,5,6-tri-*O*-benzyl-D-glucofuranoside. Their derivatives were not recorded when chromatographed under the same conditions as tribenoside at concentrations of ≤1 mg/ml.

Application—The technique was used to study the elimination of α- and β-tribenosides after daily oral administration to humans. The plasma concentrations of α- and β-tribenosides are given in Table III. The determinations were done in duplicate.

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ACKNOWLEDGMENTS

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Effect of Polyols on Interaction of Paraben Preservatives with Polysorbate 80

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Abstract □ A quantitative study of the interaction of the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid with polysorbate 80 in the presence and absence of two polyols (propylene glycol and glycerol), which were potential competitors, was performed. The results indicate that neither competitor displaced significant amounts of the parabens from their binding sites on polysorbate 80. The previously observed synergistic antimicrobial effects of these polyols appear to be due to a mechanism other than the displacement of the parabens from their micellar binding sites.

Keyphrases □ Polyols—effect of propylene glycol and glycerol on interaction of parabens with polysorbate 80 □ Propylene glycol—effect on interaction of parabens with polysorbate 80 □ Glycerol—effect on interaction of parabens with polysorbate 80 □ Parabens—interaction with polysorbate 80, effect of propylene glycol and glycerol □ Preservatives—parabens, interaction with polysorbate 80, effect of propylene glycol and glycerol □ Polysorbate 80—interaction with paraben preservatives, effect of propylene glycol and glycerol

Several reports described the ability of nonionic surfactants (including polysorbates) to interfere with the activity of phenolic preservatives such as the parabens (1–4). This inactivation is believed to be due to the formation of a complex between the preservative molecules and the surfactant micelle, based on the assumption that the preservative antimicrobial activity is primarily due to the unbound form (3).

Previous work indicated that polyols such as propylene glycol or glycerol could function as preservatives or synergists to preservatives (5–7). Since these studies were designed to determine only whether preservative activity was enhanced by polyol addition, a systematic study was needed to evaluate the mechanism underlying the increased preservative activity associated with the polyols. Thus, a model system was developed to evaluate the ability

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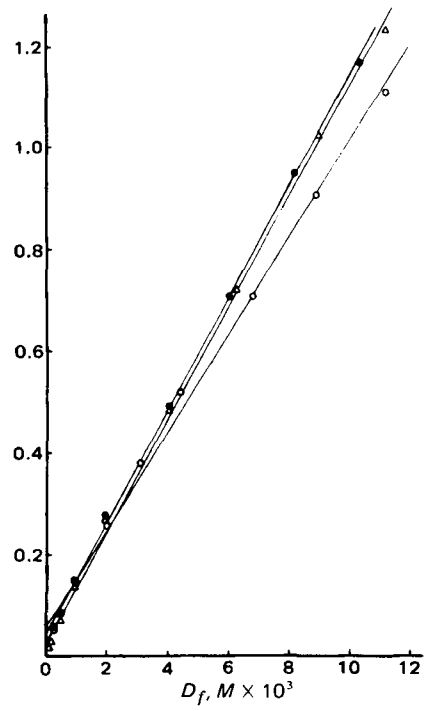

Figure 1—*Influence of polyol on methylparaben binding to polysorbate 80 at 23–25°. Key: ○, methylparaben alone; ●, methylparaben plus propylene glycol (10:1); and △, methylparaben plus glycerol (5:1). All data points represent the average of duplicate determinations.*

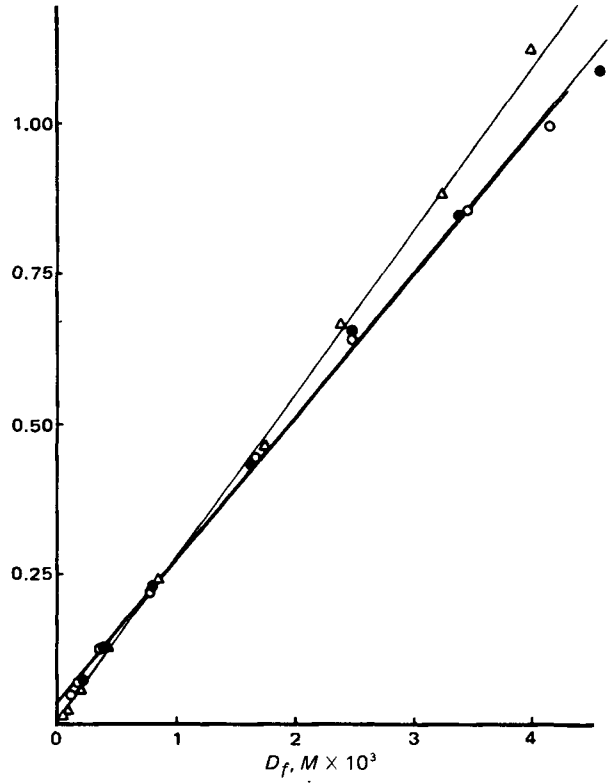

Figure 2—*Influence of polyol on ethylparaben binding to polysorbate 80 at 23–25° Key: ○, ethylparaben alone; ●, ethylparaben plus propylene glycol (10:1); and △, ethylparaben plus glycerol (5:1). All data points represent the average of duplicate determinations.*

of various polyols to inhibit the complexation of several paraben preservatives with polysorbate 80.

In initial efforts (8), sorbitol was the polyol chosen for study because of its compatibility with ingredients common to pharmaceutical formulations. Since sorbitol proved to be an ineffective competitive complexing agent, presumably because of its relatively high polarity, less polar polyols such as propylene glycol and glycerol were selected; these agents had previously been reported to act as synergists to preservatives (6, 7).

EXPERIMENTAL

Reagents—The following were used: methylparaben[1] (I), mp 130°; ethylparaben[2] (II), mp 117°; propylparaben[3] (III), mp 96–97°; butylparaben[4] (IV), mp 70°; polysorbate 80[5]; propylene glycol (reagent grade)[6]; and glycerol[7] (spectrophotometric grade).

The parabens were recrystallized from distilled water (9, 10) and dried in a desiccator for at least 48 hr prior to use. Polysorbate 80, propylene glycol, and glycerol were used without further purification. All solutions were prepared with double-distilled water from an all-glass apparatus.

Procedures—The dialysis procedures and equipment were similar to those described previously (8). Commercially available[8] acrylic plastic dialysis cells were employed. Nylon[9] membranes, previously washed for 1 hr in distilled water and heated to 80° to remove contaminants, were satisfactory. These membranes were previously shown to be impermeable to the polysorbate yet permeable to the parabens, thereby allowing equilibration of the latter (2, 11).

After the cells were assembled, 5 ml of distilled water was added to one cell compartment (the aqueous compartment) and 5 ml of a mixture of varying volume ratios of preservative in 10% (w/v) polysorbate 80 and polysorbate 80 (10% w/v) was added to the other cell compartment (the surfactant compartment). This procedure was employed to vary the amount of preservative present in the surfactant compartment while maintaining the polysorbate concentration constant at 10% (w/v). The cells were then placed on a shaker bath[10] and agitated at 120 oscillations/min for 20 hr at room temperature (23–25°) to achieve equilibrium.

Samples of preservative were removed from both sides of the membrane and assayed spectrophotometrically at 255 nm (2, 12) against blanks prepared identically, except for the omission of the parabens. This procedure was essential to compensate for any polysorbate absorption. Polysorbate 80 solutions stored for more than 4 weeks sometimes gave evidence of microbial growth. To avoid this problem, fresh polysorbate 80-containing solutions were prepared every 3 weeks.

In developing the spectrophotometric assay, it was noted that the absorption spectra of the parabens were pH dependent. As the pH was increased, the peak at 255 nm gradually decreased in intensity and a peak, centered at about 296 nm (13–15), increased in intensity. This second peak was likely due to the ionization of the weakly acidic phenolic proton of the parabens, which have pKa values of 8.4–8.5 (14). The simultaneous existence of the two paraben forms (*i.e.*, ionized and unionized) was undesirable in assaying the solutions accurately and in studying the binding of only the more active unionized form (16, 17), which reportedly binds more avidly to polysorbate 80 than the ionic form (18).

In addition, the parabens are reported to be relatively unstable at pH values above 6 (18, 19). Since the absorption spectra of the parabens should be pH independent at pH 6 or below (where they would be essentially 100% unionized), a study was performed to determine whether the paraben binding profile in a system buffered to pH 5.5 (0.1 *M* acetate) would differ significantly from that observed with an aqueous unbuffered system, as employed by Patel and Kostenbauder (2). The results of this comparison indicated that the use of unbuffered aqueous systems provides binding data that are equivalent to data obtained using a pH 5.5 system. Thus, in subsequent experiments, unbuffered aqueous systems were employed. Measurements at the start and completion of several

[1] Lot L49260, Ruger Chemical Co., Irvington, NJ 10533.
[2] Eastman Organic Chemicals, Rochester, NY 14650.
[3] Lot C708922, Ruger Chemical Co., Irvington, NJ 10533.
[4] Lot 750543, Calbiochem, La Jolla, CA 92037.
[5] Lot 1546, ICI Americas Inc., Atlas Chemicals Division, Wilmington, DE 19899.
[6] Mallinckrodt Chemical Works, St. Louis, MO 63160.
[7] Aldrich Chemical Co., Milwaukee, WI 53233.
[8] Model 289, Bel-Art Products, Pequannock, NJ 07440.
[9] Capran 77C, 0.6-mil, Allied Chemical Corp., Morristown, NJ 07960.
[10] Model 50, GCA/Precision Scientific, Chicago, IL 60647.

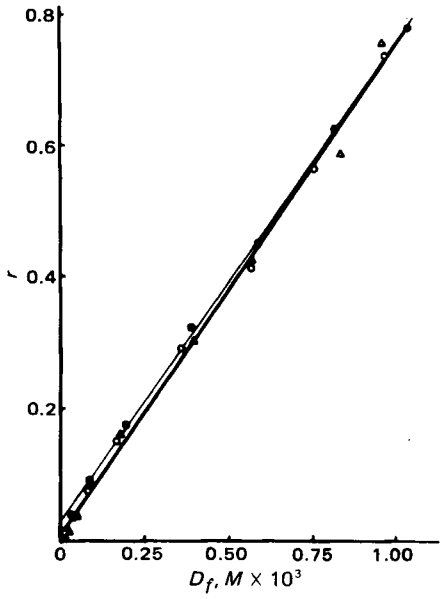

Figure 3—*Influence of polyol on propylparaben binding to polysorbate 80 at 23–25°. Key: ○, propylparaben alone; ●, propylparaben plus propylene glycol (10:1); and △, propylparaben plus glyercol (5:1). All data points represent the average of duplicate determinations.*

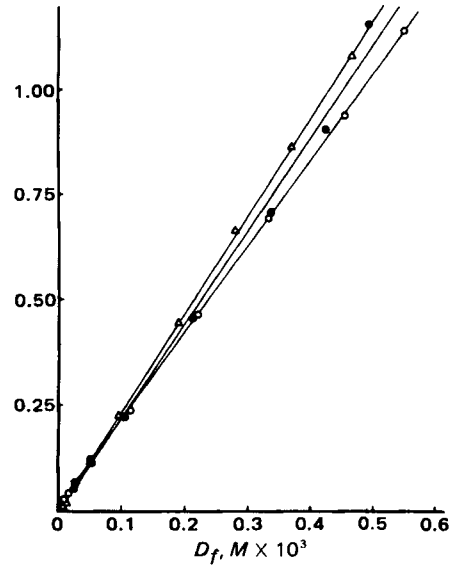

Figure 4—*Influence of polyol on butylparaben binding to polysorbate 80 at 23–25°. Key: ○, butylparaben alone; ●, butylparaben plus propylene glycol (10:1); and △, butylparaben plus glycerol (5:1). All data points represent the average of duplicate determinations.*

experimental runs indicated that the pH remained relatively constant (pH ≤ 6).

Propylene glycol and glycerol were added as possible competitors to the preservative in 10% (w/v) polysorbate 80 solution such that, in any aliquot portion, the molar ratio of polyol to paraben was constant at either 10:1 (propylene glycol) or 5:1 (glycerol). The assays were identical to those in the experiments involving no competitor since neither polyol interfered with the preservative assay at the dilutions necessary to assay for the parabens.

RESULTS AND DISCUSSION

The degree of interaction of each paraben with polysorbate 80 was determined by assaying spectrophotometrically the surfactant compartment of the dialysis cell to determine the total paraben concentration, D_t, and then assaying the aqueous compartment to determine the free (unbound) paraben concentration, D_f. The bound paraben concentration, D_b, was calculated as the difference between these two values.

To obtain information about the binding mechanism, the following mass action treatment was employed (20). It is applicable to binding phenomena in undersaturated systems such as those utilized here.

For a macromolecule with m classes of independent and equivalent binding sites, with each class i having n_i sites with an intrinsic association (binding) constant K_i, the following expression may be written:

$$r = \frac{[D_b]}{[M_t]} = \sum_{i=1}^{m} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \qquad \text{(Eq. 1)}$$

where r is the number of moles of paraben bound per mole of surfactant; $[D_b]$ and $[D_f]$ are the concentrations of bound and free (unbound) paraben, respectively; and $[M_t]$ is the concentration of macromolecule (surfactant). Theoretically, $[M_t]$ should be the micelle concentration, n the number of binding sites per micelle, and K the association constant for the reaction with the micelles since the binding interaction reportedly occurs between the preservative and the surfactant micelles rather than the monomer surfactant molecules (11, 21). From a practical standpoint, however, it is more convenient to express K, n, and M_t in terms of the surfactant concentration since the micellar molecular weight is not known precisely. Since the critical micelle concentration of polysorbate 80 is very low (21, 22), the monomer concentration may be neglected (11). In analyzing the data, the molecular weight of polysorbate 80 was assumed to be 1470[11].

A Scatchard (23) plot results when $r/[D_f]$ *versus* r is plotted. By rearranging Eq. 1, it can be observed that this type of plot should be linear with a slope of $-K$ and ordinate and abscissa intercepts of nK and n, respectively, when only one class of binding sites is present.

This simplest of binding models is referred to as the "two-parameter model" (24) since only n and K need to be determined to describe the binding adequately. However, as noted previously (9, 11, 25), Scatchard plots frequently exhibit curvature indicative of more than one class of binding sites. To describe these more complex binding processes, Eq. 1 may be rewritten as:

$$r = \sum_{i=1}^{m-1} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} + n_m K_m [D_f] \qquad \text{(Eq. 2)}$$

when one association constant becomes very small. This can occur when the second class of binding sites is not saturable within the experimentally investigated range of ligand concentrations. In such instances, $[D_t] \ll 1/K_2$, where $[D_t]$ = the total drug (ligand) concentration employed. The second term on the right-hand side of Eq. 2 is, therefore, indicative of a class of binding sites that possesses an "infinite" (nonsaturable) binding capacity but "zero" affinity, thereby producing a horizontal asymptote on the Scatchard plot. This binding process can be described by a three-parameter model, which corresponds to a value of $m = 2$ in Eq. 2, and may be written as:

$$\frac{r}{[D_f]} = \frac{n_1 K_1}{1 + K_1 [D_f]} + C \qquad \text{(Eq. 3)}$$

where $C = n_2 K_2$. Often these two different classes of binding sites are designated as "specific" (subscript 1) and "nonspecific" (subscript 2) binding (26).

As illustrated by Winkler and Hubner (26), it is often necessary to use other plots in conjunction with the Scatchard plot to ascertain the binding models correctly. For example, if it is assumed that the smallest unbound ligand (preservative) concentration in the experiment is large relative to $1/K_1$, Eq. 3 can be rewritten as:

$$r = n_1 + C[D_f] \qquad \text{(Eq. 4)}$$

Equation 4 indicates that a plot of r *versus* $[D_f]$ should produce a straight line of slope C and an ordinate intercept of n_1.

To determine the applicability of Eq. 4 in describing the binding, the equation was fitted to the data plotted in Figs. 1–4. These plots illustrate the interaction between 10% (w/v) polysorbate 80 and I–IV, alone and in the presence of propylene glycol or glycerol. The best-fit binding parameters for the interaction of the various parabens with polysorbate 80 (Table I) were calculated by least-squares linear regression.

The linearity of these plots, as indicated by the high coefficients of determination for the linear regressions, is good evidence of the adherence of the binding data to Eq. 4. The values of C listed in Table I may be interpreted as being indicative of a weak, nonspecific (nonsaturable) type of binding analogous to a partitioning of the preservative between the micellar and aqueous phases (17, 21). In a Scatchard plot, they would

[11] Dr. Paul Becher, ICI Americas Inc., Wilmington, DE 19897, personal communication.

Table I—Binding Parameters for the Interaction of Parabens with Polysorbate 80 (at 23–25°) in the Presence and Absence of Polyols

Preservative	Competitor[a]	Coefficient of Determination (r^2)	n_1	C	Partition Coefficient[b]
Methylparaben	None	0.9944	0.0565 ± 0.0101[c]	95.5 ± 1.80	91.2
	Propylene glycol (10:1)	0.9991	0.0424 ± 0.0049	109.6 ± 0.90	
	Glycerol (5:1)	0.9973	0.0263 ± 0.0078	109.4 ± 1.43	
Ethylparaben	None	0.9984	0.0357 ± 0.0057	236.2 ± 2.56	295.1
	Propylene glycol (10:1)	0.9969	0.0367 ± 0.0081	236.2 ± 3.54	
	Glycerol (5:1)	0.9993	0.0006 ± 0.0037	277.4 ± 1.84	
Propylparaben	None	0.9979	0.0093 ± 0.0040	746.2 ± 8.56	1096.5
	Propylene glycol (10:1)	0.9975	0.0267 ± 0.0061	730.9 ± 10.66	
	Glycerol (5:1)	0.9933	0.0067 ± 0.0075	747.1 ± 15.33	
Butylparaben	None	0.9989	0.0106 ± 0.0048	2048.9 ± 17.13	3715.4
	Propylene glycol (10:1)	0.9940	0.0034 ± 0.0112	2221.1 ± 43.13	
	Glycerol (5:1)	0.9998	0.0033 ± 0.0020	2345.5 ± 8.60	

[a] Molar ratio of competitor to preservative is shown in parentheses (r^2, n_1, and C are derived from r *versus* D_f plots). [b] Octanol–water partition coefficient data from Ref. 27. [c] All parameter uncertainties are expressed as standard errors.

represent a constant value of $r/[D_f]$ (*i.e.*, a horizontal asymptote) at large r values. The partitioning phenomenon may be envisioned as a process whereby preservative molecules, in an attempt to lower their free energy, migrate from a thermodynamically less stable aqueous environment in the bulk phase to a more stable (less polar) micellar phase, with the degree of penetration and affinity being proportional to their lipophilicity.

The data also indicate that the values of C observed for the parabens are in excellent agreement with their reported (27) octanol–water partition coefficients (Fig. 5), which further substantiates that the binding process described by the term C is a type of partitioning phenomenon. The coefficient of determination for the regression of the calculated values of C on the partition coefficients was 0.9968. This excellent agreement between C and the lipophilicity of the preservative molecules also supports the notion that the binding sites are less polar than their surrounding aqueous environment.

The values of C obtained for each of the four parabens are also illustrated in Fig. 6 since the values of C plotted as the ordinate in Fig. 5 are equal to the slopes of the respective lines plotted in Fig. 6. This plot illustrates that the relative binding affinities of the four parabens for polysorbate 80 are in the order butyl > propyl > ethyl > methyl. This relative binding order was previously noted by other investigators (28–30).

In addition to the simple partitioning mechanism described, Higuchi and Lach (31) proposed that phenolic compounds that possess an acidic hydrogen could associate with electrophilic atoms such as oxygen, found in the polyether ethylene oxide chains of nonionic surfactants. They suggested that this type of interaction could be enhanced by the "squeezing together" of the hydrophobic portions of the interacting molecules by water molecules. This rationale provides a possible explanation for the progressive increase in binding to polysorbate 80 observed as the nonpolar character of the paraben was increased from the methyl to the butyl esters. The relative binding affinities based on the data of Fig. 6 (*i.e.*, the slopes of each plot) are in the order 1:2.5:7.8:21.5, from the methyl to the butyl esters, respectively.

Barr and Tice (4) and deNavarre (32) postulated that the inactivation of phenolics by nonionic surfactants was due to this type of complex formation. Other investigators, however, postulated that these interactions may be explained adequately in terms of solubilization within surfactant micelles (33). Some investigators (34, 35) suggested that, because inactivation occurs between preservatives and surfactants of extremely diverse chemical structure, solubilization is a more probable explanation than specific complex formation.

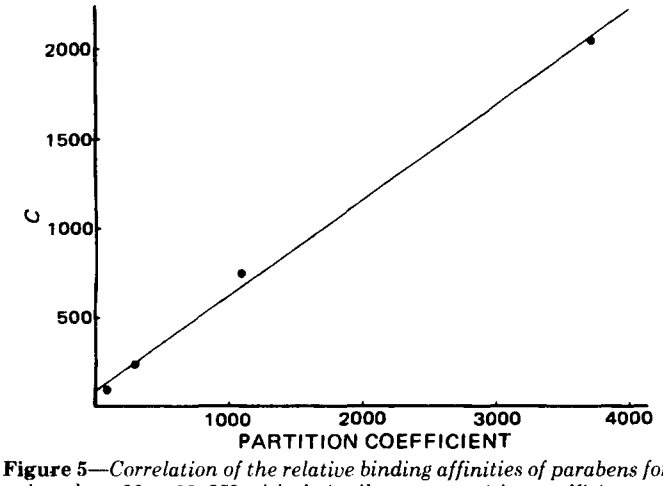

Figure 5—*Correlation of the relative binding affinities of parabens for polysorbate 80 at 23–25° with their oil–water partition coefficients.*

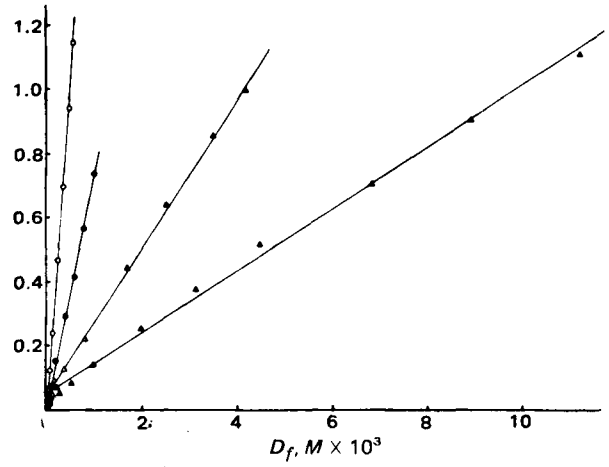

Figure 6—*Relative binding affinities of parabens to polysorbate 80 at 23–25°. Key: ▲, methylparaben; △, ethylparaben; ●, propylparaben; and ○, butylparaben. All data points represent the average of duplicate determinations.*

The literature indicates that a significant preservative–surfactant interaction requires micelles regardless of which mechanism is operative. In fact, micelles of nonionic surfactants probably provide an environment highly suited for both hydrogen bonding and micellar solubilization to operate simultaneously.

The r *versus* $[D_f]$ plots (Figs. 1–4) that illustrate the binding of the various paraben derivatives to polysorbate 80 also indicate that neither propylene glycol or glycerol displaced significant amounts of bound preservative from the polysorbate micelle. This result is shown by the virtual superimposability of these plots and the close agreement of the binding parameters (Table I) in the presence and absence of either polyol. These polyols probably are too polar to partition deeply enough into the polysorbate 80 micelle to displace the bound preservative. The previously reported synergistic antimicrobial effects of these polyols with the parabens apparently occur *via* a mechanism other than the simple displacement of the preservative molecules from their micellar binding sites. The antimicrobial activity enhancement associated with these agents possibly may be due to an osmotic effect or to their ability to hydrogen bond preferentially with available water, thereby preventing microorganism metabolism (5, 36–38) even though the polyol concentrations used (<6% w/v for propylene glycol and <5% w/v for glycerol) were considerably less than the levels (20–50%) described by Barr and Tice (37). An alternative possibility is that the polyol concentrations were too low to produce a measurable displacement of the bound paraben species.

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trans-Cinnamic Acid–α-Cyclodextrin System as Studied by Solubility, Spectral, and Potentiometric Techniques

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Abstract □ Complex formation in aqueous solutions of *trans*-cinnamic acid or *trans*-cinnamate ion (the substrate, S) and α-cyclodextrin (the ligand, L) can be described quantitatively as the 1:1 and 1:2 complexes, SL and SL_2. The solubility, spectral, and potentiometric data over a wide range of ligand concentrations yielded consistent estimates of the complex association constants. For cinnamic acid at 25°, $K_{11} = 2260\ M^{-1}$, $\Delta H^\circ_{11} = -9.3$ kcal/mole, and $\Delta S^\circ_{11} = -8$ e.u.; and $K_{12} = 60\ M^{-1}$, $\Delta H^\circ_{12} = -12$ kcal/mole, and $\Delta S^\circ_{12} = -26$ e.u. For cinnamate ion at 25°, $K_{11} = 110\ M^{-1}$, $\Delta H^\circ_{11} = -1.9$ kcal/mole, and $\Delta S^\circ_{11} = +11$ e.u.; and $K_{12} = 15\ M^{-1}$, $\Delta H^\circ_{12} = -9$ kcal/mole, and $\Delta S^\circ_{12} = -15$ e.u. (all entropy changes are unitary quantities). Thermodynamic cycles for the complexes, using solubility data, reveal that complex formation in the solid phase is thermodynamically spontaneous but that complex stability is greater in aqueous solution than in the solid phase.

Keyphrases □ Complexation—*trans*-cinnamic acid with α-cyclodextrin, thermodynamic analysis, stoichiometry, stability equilibria, determined by spectral, solubility, and pH measurements □ Thermodynamics—complexation analysis, determined by spectral, solubility, and pH measurements, *trans*-cinnamic acid–α-cyclodextrin complex □ *trans*-Cinnamic acid—analysis of complexation with α-cyclodextrin, thermodynamics □ α-Cyclodextrin—analysis of complexation with *trans*-cinnamic acid, thermodynamics □ Solubility measurements—complexation of *trans*-cinnamic acid with α-cyclodextrin □ UV spectrometry—complexation of *trans*-cinnamic acid–α-cyclodextrin □ pH measurements—complexation of *trans*-cinnamic acid–α-cyclodextrin

The chemical and physical properties of *trans*-cinnamic acid and its derivatives make these compounds highly useful substrates[1] in studies of molecular complex formation (1–7). Preliminary studies in this laboratory on the *trans*-cinnamic acid–α-cyclodextrin (cyclohexaamylose) system in aqueous solution showed that simple 1:1 stoichiometry in the complex does not adequately describe the equilibrium system. This observation seemed to be worth pursuing because cinnamic acid reportedly forms a 1:1 complex with α-cyclodextrin (8) and because this substrate does not seem to be an atypical one in such studies; hence,

[1] The substrate is the compound whose measured properties constitute the dependent variable; the ligand is the substance (α-cyclodextrin in this case) whose concentration is the independent variable.

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