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Utilization of a Model Copolymer to Evaluate the Contribution of Hydrophobic Bonding in Drug Binding

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Abstract □ A copolymer, having an overall composition of 1 unit of vinylpyridine to 26 units of vinylpyrrolidone, was synthesized. Several alkyl copolymers were made by quaternizing the nitrogen of vinylpyridine units of the copolymer with normal alkyl bromides, ethyl through hexyl. Using an equilibrium dialysis procedure, the interaction of *p*-toluene sulfonic acid sodium with each alkyl copolymer was studied over a temperature range of 15–45°, and the binding constants and thermodynamic parameters were determined. The quaternized vinylpyridine units of the alkyl copolymers constituted the binding sites for the *p*-toluene sulfonate ions. The stability of the complex formed in the reaction increased with an increase in temperature from 15–37° but decreased at 45°. The binding process was found endothermic and associated with positive entropy effects, indicating that the polymeric model system described here demonstrated properties expected for hydrophobic bonding. The effect of urea on the binding of sulfonate ion by one of the alkyl copolymers was studied and found to be in keeping with the claim that urea breaks hydrophobic bond.

Keyphrases □ Vinylpyridine–vinylpyrrolidone copolymers—synthesis □ Equilibrium dialysis—copolymer–*p*-toluene sulfonic acid sodium interaction □ Binding sites—copolymer–*p*-toluene sulfonate ions □ Urea effect—alkyl copolymer binding sulfonate ions □ UV spectrophotometry—identity

During the past 3 decades, several interactions have been reported which involved binding of drugs by plasma proteins; notable examples are penicillins (1), sulfonamides (2–5), methyl orange (6–7), and short- and long-chain fatty acids (8–14). In connection with these binding studies, it has been reported that the interactions primarily take place through ionic forces, but a further contribution to the stability of protein–drug complex is made by the hydrophobic part of a drug molecule. It has also been noticed that the larger the hydrophobic group of a drug molecule, the more stable is the complex. The contributions of hydrophobic groups is attributed to van der Waals forces. However, a close examination of the thermodynamic data has revealed that van der Waals interactions alone cannot account for the stabilizing effect of hydrophobic groups. It is felt that hydrophobic bonding probably plays an important role in drug–protein complexing.

Hydrophobic bonding is a concept introduced by Kauzmann (15), who postulated its thermodynamic properties by extrapolating the behavior of small-size hydrocarbons in an aqueous medium. A hydrophobic bond is defined as the tendency of hydrophobic groups, mainly the hydrocarbons, to adhere to one another in an aqueous solution. The adherence of hydrophobic groups in an aqueous medium is not thought to be merely a manifestation of van der Waals forces; but the structure of water in close proximity to hydrophobic groups is believed to play a significant role, since such adherence processes are accompanied by entropy effects. The concept of hydrophobic bonding was originated to indicate its contribution in stabilizing the folded configuration of globular proteins. On the basis of a physical model, Nemethy and Scheraga (16) have shown that a hydrophobic bond can be formed between two isolated side chains attached to a rigid peptide backbone of protein. Attempts have been made to estimate the thermodynamic contribution of hydrophobic groups to form a drug–protein complex, but the simultaneous contribution of protein molecules due to their "configurational adaptability" obscured such evaluation (17, 18).

It is known that in the event of drug–protein interaction, the thermodynamic activity of a drug in the body is reduced, the biological action of a drug is influenced, and even the metabolism and excretion are hindered. The extent of participation of hydrophobic bonding in exerting such effects is not completely understood. However, one may expect that the concept of hydrophobic bonding can be applied to advan-

tage in drug formulation for purposes of: (*a*) enhancing the stability of certain drugs by complexation with biologically suitable agents capable of forming hydrophobic bonds with drug molecules, and (*b*) manipulating the release rate of drugs incorporated in a sustained-release dosage form. This necessitates extensive understanding of the properties of hydrophobic bonding. Recently, certain model systems have been studied to determine the thermodynamic parameters of hydrophobic bond formation. These model systems consisted of the adsorption of short-chain fatty acids by polystyrene resin at 4° (19), dimerization of small-chain fatty acids at 25° (20), and the interaction of phenol with short-chain fatty acid anions at 25° (21, 22). None of these model systems includes a water-soluble polymeric system containing definite sites for hydrophobic bond formation with suitable small molecules. It was the purpose of this study to devise such a model polymeric system that would make possible the evaluation of the contribution of hydrophobic bonding.

MODEL SYSTEM

The proposed model system consists of *p*-toluene sulfonic acid sodium (PTSAS) salt and a series of relatively simple, water-soluble copolymers containing definite sites for hydrophobic bonding. The interactions of this system are intended to be studied by equilibrium dialysis. Since the model copolymers considered here are not available commercially, they were synthesized in the laboratory. This research project therefore constitutes two distinct phases: (*a*) synthesis and characterization of a model copolymer and its derivatives, and (*b*) binding studies to determine thermodynamic functions of hydrophobic bonding for the model system.

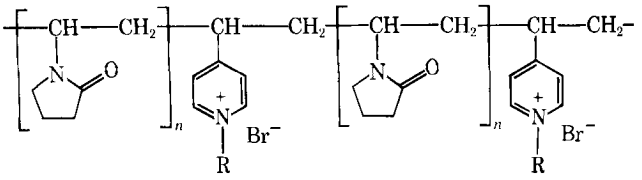
The copolymer is made from two types of monomers, 4-vinylpyridine (hereafter called vinylpyridine) and *N*-vinyl-2-pyrrolidone (hereafter called vinylpyrrolidone); its overall composition is designed to contain about 30 units of vinylpyrrolidone to 1 unit of vinylpyridine. Several alkyl copolymers are prepared by quaternizing the nitrogen of vinylpyridine units of the copolymer with an appropriate *n*-alkyl bromide of a homologous series. The alkyl bromides used are bromoethane through bromohexane. The quaternized units of the copolymer would be expected to constitute the binding sites.

The interactions of PTSAS with each alkyl copolymer were studied at different temperatures using equilibrium dialysis, and the thermodynamic constants were determined. From such data the energy contribution of the methylene groups of the alkyl chain is estimated.

The choice of the model system used was made on the basis of the following considerations. PTSAS is a salt of a strong acid and strong base and will remain completely ionized in aqueous solution. Being an aromatic compound, the complicating feature of micelle formation which occurs with long-chain, aliphatic, water-soluble species (23) can be avoided. Also, PTSAS is amenable to spectrophotometric assay.

Since the phenomenon of hydrophobic bonding has to be studied in aqueous solution, the copolymer employed for this purpose must be water soluble. A copolymer composed mainly of polyvinylpyrrolidone might be expected to be water soluble even when a large fraction of binding sites is occupied. Since both monomers, vinylpyridine and vinylpyrrolidone, are equally reactive, the composition of a copolymer formed should approximate the composition of the feed of reaction (24), which in this case is 1 part of vinylpyridine to 30 parts of vinylpyrrolidone. It may then be reasonably expected that, in this copolymer, any 2 vinylpyridine units would be separated by about 25–35 units of vinylpyrrolidone (Structure I).

It is reasoned that the interaction of negatively charged *p*-toluene sulfonate ions takes place only with positively charged quaternized vinylpyridine units of the copolymer. Thus, the quaternized units represent the binding sites. Electrostatic binding is designed to bring the sulfonate ions sufficiently close to the quaternized units to facilitate hydrophobic bonding taking place between the hydrophobic



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portion of the sulfonate ion and the alkyl group of a quaternized vinylpyridine unit. [See Structure I for a schematic representation of the structure of a copolymer molecule showing two vinylpyridine units (quaternized with R = ethyl through hexyl bromide) separated by about 26 units (*n*) of vinylpyrrolidone.] The binding energy involved in such interactions is due to two factors, electrostatic binding and hydrophobic bonding. Since all alkyl copolymers are prepared from the same parent copolymer, the charge density of each alkyl copolymer is probably not significantly different, and the energy contribution of electrostatic binding in all cases should remain the same. Therefore, the difference in the binding energy that may be shown in the binding of *p*-toluene sulfonate ion to various alkyl copolymers can be attributed to the difference in the hydrophobicity of alkyl chains attached to the quaternary nitrogen of vinylpyridine units.

The distance between adjacent binding sites on the polymer is such that the binding of a sulfonate ion to one site does not significantly influence the binding of another ion to the next site.

A potential complicating factor in the interpretation of energetics of binding is a possible secondary interaction of the sulfonate ion with the backbone of a copolymer which is primarily composed of polyvinylpyrrolidone. However, the difference in affinity of the anions for the quaternized site and for the copolymer backbone probably would be sufficient to permit isolation of the respective interactions. It is further believed that this tendency of secondary binding will be considerably reduced if the sulfonate ion, like other anions of similar molecular size (23), requires participation of as many as 10 units of vinylpyrrolidone for its binding.

The purpose of studying the system in 5 *M* urea solution was to provide additional means to test the validity of the present model system, since urea is known to weaken hydrophobic bonding (15, 25, 26).

SYNTHESIS AND CHARACTERIZATION OF A MODEL COPOLYMER AND ITS DERIVATIVES

The main characteristics desired of the model copolymer are: (*a*) it should be water soluble; (*b*) its molecular size should be large enough to render it nondiffusible to the cellophane membrane employed during the binding studies; and (*c*) its molecular composition is such as to contain about 30 units of vinylpyrrolidone to 1 unit of vinylpyridine.

Through a series of systematic experiments, the conditions necessary to yield the copolymer of desired properties were determined. Accordingly, 2.7 moles of vinylpyrrolidone and 0.095 mole of vinylpyridine (distilled at 45° under reduced pressure) were dissolved in about 260 g. of alcohol. To this solution was added 7.4 g. of α,α-azobis-isobutyronitrile previously dissolved in 40 g. of acetone. The reaction mixture was contained in a 2.5-l. capacity bottle with a bakelite stopper. Nitrogen gas was bubbled into the reaction mixture for 15 min. and the bottle was stoppered. The copolymerization was carried out with constant stirring at 60° using a heating plate equipped with a magnetic stirrer. When the reaction mixture became quite viscous in about 4 hr., the stirring was discontinued, but the heating was continued at 50° for another 36 hr. The reaction mixture was diluted with about 1600 ml. of acetone and fractionated by adding ether. Three fractions of the copolymer were obtained subsequent to the addition of about 850-, 750-, and 500-ml. quantities of ether, in that order. The second fraction was collected, dissolved in 2.5 l. of distilled water, filled into several cellophane tubes, and placed in a tank containing 8 gal. of distilled water for dialysis. The water in the bath was being constantly stirred and was replaced with freshly distilled water every 24 hr. Dialysis was continued for 4 days until no copolymer was detected in the water outside the tubes. The tubes were removed from the bath, and the contents were concentrated by blowing warm air over the bags. The concentrated copolymer was dissolved in a

mixed solvent containing 1230 ml. of acetone and 150 ml. of ethanol. The copolymer was precipitated with 1500 ml. of ether, which was added to the copolymer solution in 100-ml. portions with constant stirring. The viscous mass thus obtained was separated by decantation of ether. The polymer phase was further precipitated by adding to it 500 ml. of ether with constant stirring. The ether layer was decanted. The copolymer precipitate was then washed twice with ether, using 500 ml. of ether each time. The copolymer was dried at 60° under reduced pressure.

Analysis of Copolymer—Polyvinylpyrrolidone, when present in an acidic medium, does not absorb in the UV region. However, the copolymer shows a characteristic absorption maximum at 254 mμ. Therefore, it was necessary to synthesize polyvinylpyridine, which would serve as a reference compound for the quantitative determination of the vinylpyridine content in a known amount of copolymer by a spectrophotometric method. Polyvinylpyridine was synthesized by polymerizing vinylpyridine (freshly distilled at 45° under reduced pressure) using the conditions similar to those used for the synthesis of the copolymer. A linear relationship observed between the concentration of polyvinylpyridine and absorbance is shown in Fig. 1*a*. This offered an effective analytical tool to quantitate the amount of vinylpyridine present in a given quantity of copolymer. The Beer plot obtained for the copolymer is shown in Fig. 1*b*. With reference to the standard plot (Fig. 1*a*), it was calculated that the copolymer contained 26 units of vinylpyrrolidone to 1 unit of vinylpyridine. For the sake of convenience, the composition of the copolymer may be expressed as 1:26.

Alkyl Copolymers—Twenty grams of copolymer (1:26) was dissolved in 120 g. of nitromethane, and an appropriate quantity of alkyl bromide was added. The quantity of each alkyl bromide, from 1-bromoethane through 1-bromohexane, used was 16, 18, 30, 34, and 40 g., respectively. The reaction was carried out at 70° with constant stirring for 4 days in a closed glass container. The reaction mixture was brought to room temperature and poured in a thin stream within 15 min. into 1 l. of ether to precipitate the alkyl copolymer. The mixture was constantly stirred during the precipitation. The ether was decanted and the precipitate was dissolved in a mixture of 200 ml. of acetone and 20 ml. of ethyl alcohol. The solution was added to 500 ml. of ether to precipitate the alkyl copolymer. The ether was decanted, and the alkyl copolymer thus obtained was washed three times with 200-ml. portions of ether and dried at 50° under reduced pressure.

Assay of Alkyl Copolymer—The bromide content of the quaternized copolymer was quantitatively determined by a potentiometric titration method using 0.01 *N* silver nitrate solution as a titrant (27). From knowledge of the bromide content in a given quantity of alkyl copolymer, the proportion of quaternized units of vinylpyridine was calculated.

The composition of each alkyl copolymer is expressed in terms of units of vinylpyrrolidone present to 1 quaternized unit of vinylpyridine. The composition of various alkyl copolymers is recorded in Table I. In those cases where the alkyl copolymers are shown to contain more than 26 units of vinylpyrrolidone to 1 unit of vinylpyridine, all the vinylpyridine units were not quaternized by the respective alkyl bromide. However, it was experimentally demonstrated that the binding sites are represented solely by the quaternized units of vinylpyridine. Since the difference between the molecular weight of vinylpyridine (mol. wt. 105) and that of vinylpyrrolidone (mol. wt. 111) is small, and since the unquaternized vinylpyridine unit does not constitute a binding site for the sulfonate ion, it is not unreasonable to equate an unquaternized vinylpyridine unit to a vinylpyrrolidone unit while determining the composition of the alkyl copolymers in terms of units of vinylpyrrolidone present to 1 quaternized unit of vinylpyridine, as shown in Table I.

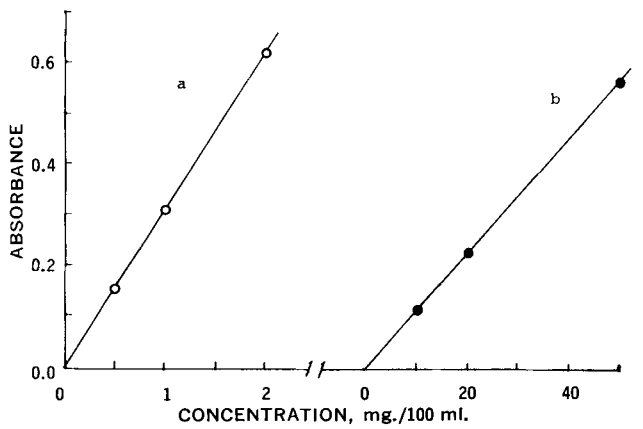

Figure 1—*The Beer plots of absorbance against concentration of synthesized polyvinylpyridine (a) and 1:26 copolymer (b) in diluted hydrochloric acid at 254 mμ.*

Table I—Composition of the Alkyl Copolymers Expressed in Terms of Number of Vinylpyrrolidone Units to One Quaternized Vinylpyridine Unit

Copolymer	Composition
Ethyl	1:26
Propyl	1:29
Butyl	1:29
Pentyl	1:30
Hexyl	1:27

Copolymers of composition 1:20 and 1:34 were also synthesized, and only the hexyl derivatives were prepared.

EXPERIMENTAL

Materials—Ethyl copolymer (1:26), propyl copolymer (1:29), butyl copolymer (1:29), pentyl copolymer (1:30), and hexyl copolymer (1:20, 1:27, and 1:34) were synthesized in the laboratory. *p*-Toluene sulfonic acid sodium salt[1] was recrystallized from acetone–water solution. Sodium chloride (reagent grade); urea USP; and cellophane membrane[2] were used.

Solution of Alkyl Copolymer—A 2% (w/v) solution of each alkyl copolymer was prepared in 0.1 *M* sodium chloride solution (distilled water). A 2% (w/v) solution of pentyl copolymer was also prepared in 5 *M* urea.

Solution of *p*-Toluene Sulfonic Acid Sodium (PTSAS) Salt—The 0.01, 0.015, 0.02, 0.025, and 0.03 *M* solutions of PTSAS were prepared in 0.1 *M* sodium chloride solution. The ionic strength of each solution was adjusted to 0.13 with NaCl. Similar solutions were also prepared with 5 *M* urea.

Dialysis Procedure—Each dialysis cell consisted of two Plexiglas blocks, 6.3 × 6.3 × 2.6 cm., each half with a cavity having a capacity of 20-ml. Threaded Plexiglas plugs provided access to the cell cavities. To assemble the cells, a cellophane membrane previously freed of water-soluble material was clamped between the two symmetrical halves. Solutions were pipeted into each cavity as required; the stoppers, fitted with polyvinyl chloride washers, were screwed in tightly. The cells were then rotated at 9 r.p.m. in a water bath maintained at 15, 22, 30, 37, or 45 ± 0.2°. The system attained equilibrium within 44 hr. The cells were then removed from the bath, and the contents from the nonpolymer side of each cell were immediately transferred to a 20-ml. container provided with a cap. After the solution attained room temperature, each solution was analyzed for PTSAS by a spectrophotometric method.

Assay Method—PTSAS was assayed with a Beckman DU spectrophotometer at 261 mμ. Two milliliters of the sample solution was diluted to 50 ml. with distilled water and its absorbance noted using water as a blank. To correct for any spectral contribution of trace quantities of diffused macromolecule, dialysis cells were set up containing the solution of an appropriate alkyl copolymer on one side of the membrane and 0.13 *M* sodium chloride solution on the other side.

RESULTS

To ascertain that only the quaternized vinylpyridine units of an alkyl copolymer represented the binding sites, the interaction of 0.02 *M* PTSAS with each of three hexyl copolymers of composition 1:20, 1:27, and 1:34 was studied. The concentration of each hexyl copolymer was expressed in terms of its hexyl-4-vinylpyridinium

[1] Eastman Organic Chemicals, Rochester, N. Y.
[2] Visking Cellulose Casing, Viscing Corp., Chicago, Ill.

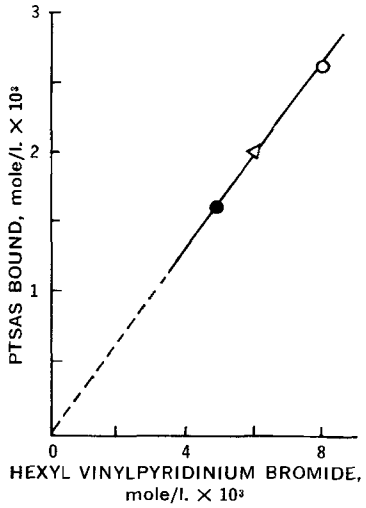

Figure 2—*Binding of 0.02 M PTSAS by hexyl copolymers of three different compositions, 1:34, ●; 1:27, △; and 1:20, ○; at room temperature.*

bromide content. (This practice of expressing concentration was also adopted with other alkyl copolymers.) A plot of concentration of hexyl copolymer *versus* moles of bound PTSAS was made as shown in Fig. 2. The linear plot obtained clearly indicates that the binding of sulfonate ions by the alkyl copolymer is a function of the concentration of quaternized vinylpyridine units of the latter.

The binding data obtained at various temperatures for each alkyl copolymer derivative were treated according to Eq. 1 (6):

$$1/r = 1/nKa + 1/n \qquad \text{(Eq. 1)}$$

where r is the number of moles of PTSAS bound per mole of an alkyl copolymer, n is the number of binding sites per mole of the alkyl copolymer, K is the binding constant (liter/mole), and a is the molar concentration of free PTSAS at equilibrium. From the slope ($1/nK$) and intercept ($1/n$), the binding constant, K, was evaluated. The straight lines of the plots were obtained by the method of least squares. Typical Langmuir plots obtained in the studies are shown in Fig. 3. The binding constants are listed in Table II.

The resulting straight lines indicate that each sulfonate anion binds to an identical group present in the alkyl copolymer and that the electrostatic repulsion between sulfonate ion already bound to one site and that bound to the next binding site is negligible.

It was noticed that in none of the cases was the intercept of Fig. 3 equal to unity, indicating that all the binding sites of an alkyl copolymer are not available for interaction with the sulfonate ions. It is likely that a few binding sites remain buried within the macromolecule, because the positively charged binding sites are so widely spaced that absolute rigidity of the macromolecule cannot be attained to prevent it from coiling completely. It also appears that the extent of availability of the binding sites partly depends on temperature, since out of every 10 binding sites, 7 to 8 sites at 15° and 22°, 8 to 9 binding sites at 30° and 45°, and 6 to 7 binding sites at 37° are available for the interaction. Although the number of available binding sites was not the same at all the temperatures

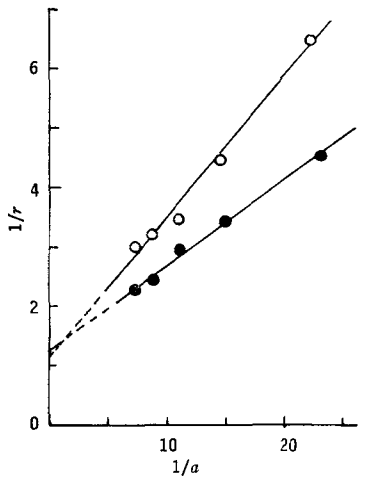

Figure 3 — *Typical Langmuir plots obtained for binding of PTSAS by ethyl copolymer, ○; and hexyl copolymer, ●; at 30°.*

Table II—Binding Constants for the Interaction of PTSAS with Various Alkyl Copolymers over the Temperature Range of 15–45°

Copolymer	Binding Constant, K, l./mole				
	15°	22°	30°	37°	45°
Ethyl	46.00	—	46.66	50.00	32.70
Propyl	61.00	—	85.70	102.14	61.30
Butyl	52.84	54.56	55.00	63.28	42.56
Pentyl	62.80	—	111.00	127.20	58.00
Hexyl	63.36	85.84	94.80	108.08	68.60

studied, the number of binding sites available at any one particular temperature was essentially the same for all the alkyl copolymers.

Thermodynamic Functions—From the data in Table II (excluding the data obtained at 45°) a plot of log K *versus* $1/T$ was made (Fig. 4). The linear curves (least squares) obtained indicated that over the range 15–37° the enthalpy of binding is constant. The data obtained at 45° are discussed later. From the slope ($-\Delta H/2.303$) of the linear curve, the enthalpy of binding, ΔH, was calculated. The free energy of binding, ΔF, was calculated from the following relationship:

$$\Delta F = -RT \ln K \qquad \text{(Eq. 2)}$$

Finally, the entropy of binding, ΔS, was calculated from the following expression:

$$\Delta S = \frac{\Delta H - \Delta F}{T} \qquad \text{(Eq. 3)}$$

The thermodynamic constants obtained for the alkyl copolymers are listed in Table III.

When the corresponding binding constant, K, is plotted against the alkyl copolymer of the ascending homologous series, an ascending zigzag curve is obtained (Fig. 5). The binding constants of the alkyl copolymers with an odd number of carbon atoms in the alkyl chain are higher than those of the alkyl copolymers with the next

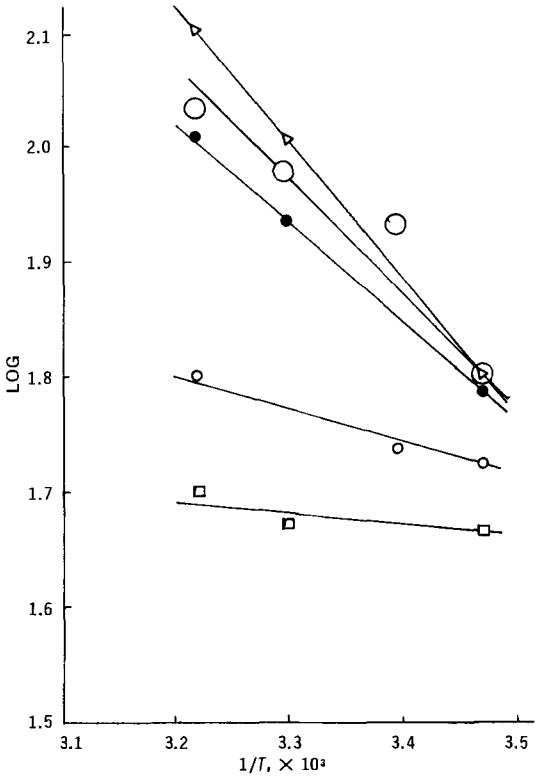

Figure 4—*Plots of logarithm of binding constant* vs. *reciprocal of absolute temperature for the interaction of PTSAS with ethyl copolymer, □; propyl copolymer, ○; butyl copolymer, ●; pentyl copolymer, △; and hexyl copolymer, ○; over the temperature range of 15–37°.*

Table III—Thermodynamic Parameters of Binding for the Interaction of PTSAS with Various Alkyl Copolymers

Copolymer	ΔF, cal./mole 15°	22°	30°	37°	45°	ΔH, cal./mole Temp. Range 15–37°	ΔS, e.u. Temp. Range 15–37°
Ethyl	2193	—	2316	2414	2205	458	9.21
Propyl	2354	—	2683	2853	2602	4068	22.32
Butyl	2272	2345	2430	2558	2372	1373	12.64
Pentyl	2372	—	2837	2987	2567	5611	27.77
Hexyl	2377	2613	2743	2888	2674	4728	24.41

Table IV—Binding Constants and Thermodynamic Parameters of Binding for the Interaction between PTSAS and Pentyl Copolymer in the Absence and in the Presence of 5 *M* Urea over the Temperature Range of 15–37°

5 *M* Urea	K, l./mole 15°	30°	37°	ΔF, cal./mole 15°	30°	37°	ΔH, cal./mole	ΔS, e.u.
Absent	62.8	111.0	127.2	2372	2837	2987	5611	27.77
Present	29.2	40.0	44.0	1932	2221	2331	3433	18.62

even number of carbon atoms. This zigzag pattern is seen to exist at all the temperatures studied. Consequently, the values of thermodynamic functions determined for the series of alkyl copolymers also reflect the same pattern. However, the values of the binding constants, and for that matter those of the thermodynamic functions, increase with increasing alkyl chain length when a series of alkyl copolymers, containing an odd or even number of carbon atoms in the alkyl chain, is considered.

From the comparative data presented in Table IV and Fig. 6, it is clearly seen that the binding of the sulfonate ion by the pentyl copolymer has been influenced very significantly in the presence of 5 *M* urea. It is also noticed that the binding of the sulfonate ion by the pentyl copolymer increased with increase in temperature from 15–37°. The plot of log K against $1/T$ (Fig. 7) gives a straight line (least squares), which again suggests that the enthalpy of binding for the system is constant over this temperature range. In the presence of 5 *M* urea, the free energy of binding for this system became less negative by 400 cal./mole at 15°, 616 cal./mole at 30°, and 556 cal./mole at 37°. Similarly, the enthalpy of binding became less positive by 2200 cal./mole and the positive entropy of binding reduced by about 9 entropy units.

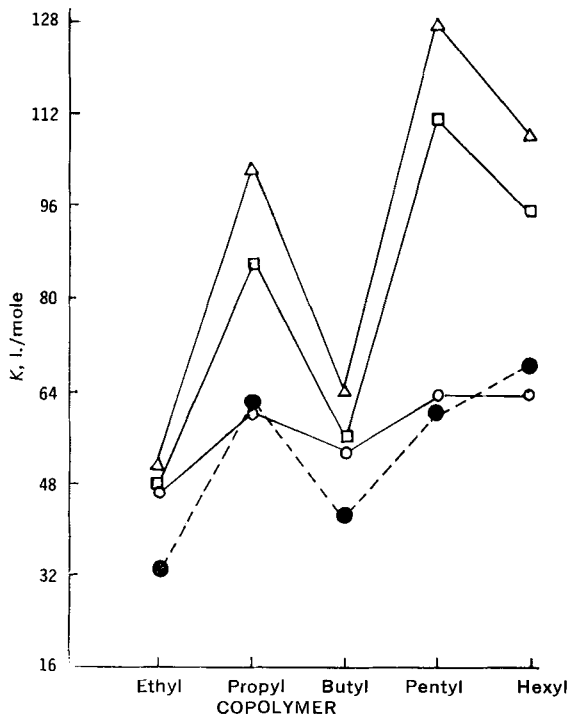

Figure 5—*Plots of binding constant* vs. *alkyl copolymer at 15°, ○; 30°, □; 37°, △; and 45°, ●.*

Another feature that becomes discernible is that the number of binding sites, which remain available on the pentyl copolymer molecule for binding of the sulfonate ions, is practically the same whether or not urea is present in the system. This may be noted by visual inspection of the intercepts of the plots in Fig. 6. This feature may serve to indicate that the intrinsic nature and behavior of the alkyl copolymers are not significantly modified in the presence of 5 *M* urea.

DISCUSSION

An examination of the data shows that the stability of the complex formed between an alkyl copolymer and the sulfonate ion increased with increasing temperature over the range 15–37° and that the binding process was endothermic. More importantly, the binding was associated with an increase in entropy, indicating that the model system demonstrates thermodynamic behavior in common with that described for hydrophobic bonding.

From the data of the composition of alkyl copolymers, it can be seen that the charge density of each alkyl copolymer is not significantly different. Therefore, the energy contribution of electrostatic binding of each alkyl copolymer is considered the same. The difference shown by these alkyl copolymers in the values of their respective thermodynamic constants can be attributed to the contribution of the methylene, $=CH_2$, groups in the alkyl chains.

Despite the fact that the enthalpy of binding, which may be called enthalpy of hydrophobic bonding, was positive and therefore unfavorable, the bond formation was strengthened at relatively higher temperature. The free energy required to strengthen hydrophobic bond was derived from the substantial gain in entropy during the binding process. In aqueous solution, according to the concept of iceberg formation (28), the hydrocarbon groups of alkyl copolymers and *p*-toluene sulfonate ion are considered to be surrounded by one or more layers of water molecules, which are highly ordered with better hydrogen bonding than the molecules in ordinary liquid water. This brings about a lowering of configura-

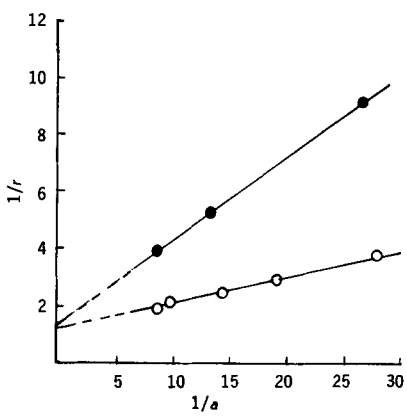

Figure 6—*Comparison of Langmuir plots for binding of PTSAS by pentyl copolymer in the absence of urea, ○, and in the presence of 5 M urea, ●, at 30°.*

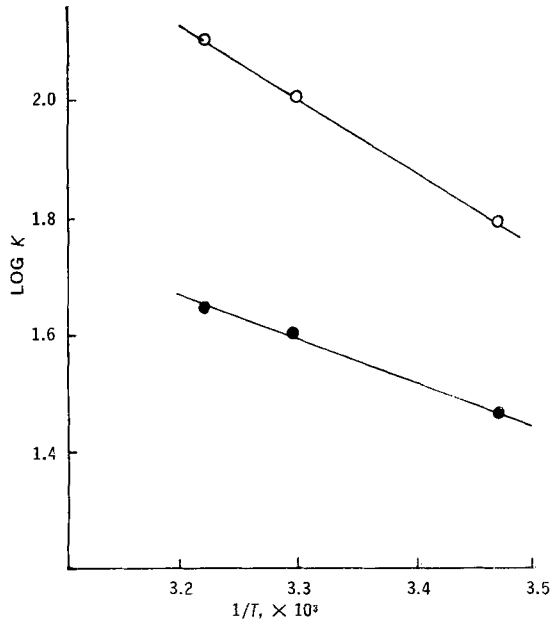

Figure 7—*Comparison of plots of logarithm of binding constant vs. reciprocal of absolute temperature for the binding of PTSAS by pentyl copolymer in the absence of urea, ○, and in the presence of 5 M urea, ●.*

tional entropy of the hydrocarbon groups. There is also a true hydration about the sulfonate group of the anion and the quaternary nitrogen of the vinylpyridinium groups of the alkyl copolymer. When binding occurs between the reactants concerned, the icebergs become either less ordered or contain fewer water molecules. As a result, the water molecules are released from the ordered structure, producing proportional increase in entropy due to the release of the configurational entropy of the hydrocarbon groups of the reactants.

Although the hydrophobic bonding might be expected to become continually stronger with a rise in temperature, there is a limit of maximum temperature for iceberg structure (16). The limit of maximum temperature depends upon the aliphatic or aromatic nature of the hydrophobic group. Since the π-electron orbitals of aromatic hydrocarbon react with the highly hydrogen-bonded water species, the extent of partial "icecage" formed around the aromatic hydrocarbon is smaller than that formed around the aliphatic hydrocarbon (29). Consequently, the lowering of configurational entropy of the aromatic hydrocarbon group is less than that of the aliphatic hydrocarbon groups. Nemethy and Scheraga (16) have theorized that, when the interaction takes place between the aliphatic side chains, the hydrophobic bond becomes stronger up to 58°; but when the interaction takes place between aromatic hydrocarbons, the hydrophobic bond becomes stronger up to 42°. According to this theory, the lowering of configurational entropy of the phenyl group would be negligible at 45°. Therefore, in the present system, when binding takes place at 45°, the contribution to the total entropy gain by the phenyl group of the sulfonate ion due to the release of its configurational entropy would be negligible. In view of this theory, it is conceivable that the decreased stability of the hydrophobic bond in the model system at 45° is due to the possible breakdown of the partial ice cage around the phenyl group of the sulfonate ion (Table III).

As shown in Fig. 5, the zigzag pattern exhibited in the values of the binding constants is intriguing. This pattern points to the possible role the number of contacts or closeness (established between the interacting hydrophobic groups within the Van der Waals radii) plays to determine the strength of hydrophobic bond (29). If the concept that the carbon atoms of the alkyl chain assume an extended zigzag arrangement in the liquid state (30) is extended to explain the zigzag pattern observed in Fig. 5, it seems probable that the terminal methyl group of the propyl and pentyl chain (containing odd numbers of carbon atoms) orient much closer to the phenyl group of the target sulfonate ion than the terminal methyl group of the butyl and hexyl chain (containing even numbers of carbon atoms).

Also, in view of the fact that in a normal hydrocarbon the angle formed by the two consecutive C—C bonds is approximately 110° and the C—C bond length is 1.54 Å (31), it can be estimated that the terminal methyl group of odd carbon alkyl chain, as compared to that of the even carbon alkyl chain, would be closer to the aromatic ring of the sulfonate ion by a geometric distance of about 0.88 Å. It is, therefore, conceivable that more water molecules are released by complexing the propyl group with the sulfonate ion than by complexing the butyl group, thereby resulting in substantial gain in entropy. Similarly, the pentyl group forms a more stable complex with the sulfonate ion than does the hexyl group.

It is seen that the increment in the binding energy with the increase of two methylene groups in an alkyl chain is not uniform and depends on the temperature of the interaction. The increment of the binding energy varied from −18 to −105 cal. at 15°, −97 to −329 cal. at 30°, and −134 to −330 cal. at 37°, depending on what two alkyl chains were considered. Similar nonuniformity in the increment of free energy has been reported by Nemethy and Scheraga (16) even on the basis of a physical model. Such nonuniformity may be attributed to the fact that the stability of hydrophobic bond is governed by several factors, such as the number of contacts and the closeness of contact between the interacting hydrophobic groups, the structure of iceberg, and the extent of partial ice cage around the hydrocarbons involved. A significant variance in the iceberg structure around the alkyl groups of various alkyl copolymers can be expected, since a variance in the iceberg structure around the lower hydrocarbons, methane, ethane, propane, and butane, has been reported by Clausen and Polgase (32) from solubility studies in water. They also observed a nonuniformity in the decrease of entropy values for the solubility of these hydrocarbons of a homologous series. The decrease in entropy with the additional $-CH_2-$ group from methane to butane was 1.4, 6.4, and 1.0 e.u., respectively.

The effect of urea on the binding of sulfonate ion by the pentyl copolymer is in keeping with the claim that urea breaks hydrophobic bond. From Fig. 7 it is noted, however, that the thermodynamic behavior of the present model system in the presence of 5 *M* urea remained qualitatively the same. This suggests that the iceberg structure in the immediate vicinity of the alkyl chains of the alkyl copolymers and phenyl group of the sulfonate ion is not completely disrupted but has been significantly modified by the urea molecules, which are known to undergo hydrogen bonding with water.

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Determination of Phenoxymethyl Penicilloic Acid and Phenoxyethyl Penicilloic Acid in Urine in the Presence of the Parent Penicillins

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Abstract □ Penicillin and the corresponding penicilloic acid are extracted by chloroform from an acidified sample of urine which has been partly saturated by ammonium sulfate. A portion of the extract is dried and redissolved in a small volume of acetone. A measured aliquot is chromatographed on a silica gel thin-layer plate by acetone–acetic acid (19:1). The separated penicilloic acid is detected by starch–iodine spraying. The zones are transferred into glass tubes, nitrated, and neutralized by ammonia. The absorbance of the yellow supernatant, which is proportional to the concentration of penicilloic acid, is measured spectrophotometrically.

Keyphrases □ Penicillin, penicilloic acid in urine—penicilloic acid determination □ Phenoxymethyl penicilloic acid—determination in urine □ Phenoxyethyl penicilloic acid—determination in urine □ TLC—analysis □ Starch–iodine spray—TLC spot identification □ Colorimetric analysis—spectrophotometer

Due to their instability in solution, penicillins taken orally or administered parenterally undergo chemical changes, and the breakdown products are excreted from the body in the urine.

Walkenstein *et al.* (1) found that between 30 and 60% penicillin G in urine remains biologically active, the major degradation product being penicilloic acid. The data were obtained by comparison of radioassays and bioassays of urine. Penicillins can be estimated directly by several methods such as iodometric, hydroxylamine, or biological, but penicilloic acid in the presence of penicillin is usually found by difference. Pan (2) gives a method for the determination of penicilloic acid in penicillin G fermentation broth, which is based on extraction at different pH values and subsequent colorimetric determination. Phenoxymethyl penicillin (penicillin V) and phenoxymethyl penicilloic acid can be separated by paper chromatography; Roehr (3) gives R_f values in four different systems.

McGilveray and Strickland (4) use TLC for identification of about 10 penicillins in four systems.

It was observed that using silica gel G and acetone–acetic acid (19:1) in TLC penicillin V and phenoxyethyl penicillin (phenethicillin) can be separated from the corresponding penicilloic acids. The separated penicilloic acids, containing a phenolic group in the side chain, may be determined subsequently by a colorimetric method (5) by nitration, neutralization by ammonia, and spectrophotometric measurement. Based on this observation, a method has been developed that permits direct determination of penicilloic acid, in the presence of penicillin, in urine.

The sample, partially saturated by ammonium sulfate, is extracted by chloroform at pH 2, using a 1:5 ratio of water to chloroform. The chloroform extract is dried and redissolved in acetone, and an aliquot is taken for chromatography on silica gel using acetone–acetic acid (19:1). The zones of separated penicilloic acid, located by starch–iodine spraying, are transferred into glass tubes and nitrated in the presence of the silica to form quinoid radicals, which are yellow in color in the presence of ammonia. The silica, which does not interfere in this process, is separated by centrifugation, and the supernatant is used for spectrophotometric measurements.