chloride molecule. It is pertinent in this regard that the benzolysis of trityl chloride in a non-polar solvent is more sensitive in rate to the capacity of the medium to provide for electrophilic than for nucleophilic solvation.[11,12] It has been demonstrated in this and the previous[2] study of chloroacetolysis that when there is free acid in the medium (when $[HA]_t/[Am]_t > 2$), a term appears in the rate law which involves the product of the concentrations of the 2:1 salt and the acid. Presumably $Am(HA)_2$ and the free acid may function, respectively, as nucleophile and electrophile either in a concerted or a stepwise (eq. 11) attack on trityl chloride.

(11) M. F. Hawthorne and D. J. Cram, *J. Am. Chem. Soc.*, **76**, 3451 (1954).

(12) See C. E. Boozer, J. D. Robinson, A. Soldatos, J. C. Trisler and C. Wiley, *ibid.*, **78**, 3428 (1956), for a discussion of the relative importance of these two types of solvation in solvolytic reactions of halides.

$$(C_6H_5)_3CCl + HOOCR \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (C_6H_5)_3C^+Cl^-\cdots HOOCR$$

$$\xrightarrow[k_2\ (\text{slow})]{R_1R_2\overset{+}{N}H_2\ \overset{-}{O}COR.RCOOH} (C_6H_5)_3COCOR + R_1R_2NH_2^+Cl^- + RCOOH \quad (11)$$

The reaction which occurs when $[HA]_t/[Am]_t = 2$ is most interesting. Under these conditions a single 2:1 salt molecule must function both as the nucleophile and the electrophile which are required to accomplish displacement readily. The detailed course of the reaction which occurs under these circumstances cannot be formulated at this time.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Chromatography on Columns Packed with a Non-polar Material[1]

BY IZCHAK Z. STEINBERG[2] AND HAROLD A. SCHERAGA

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A method is described for the preparation of polystyrene particles of high adsorptive surface area. The chromatography of several organic acids (in aqueous solution) on the polystyrene thus prepared illustrates the dependence of the strength of hydrophobic bonds on the nature and size of the interacting non-polar groups. The separation of mixtures of some organic acids could thus be accomplished by chromatography on the large-surface polystyrene. Urea seems to decrease the strength of the hydrophobic bonding to the non-polar surface. The general effect of urea on the adsorption process is shown, however, to be somewhat complicated.

Introduction

The thermodynamic parameters for the interactions of non-polar groups of proteins in aqueous solution (hydrophobic bonds) have recently been calculated by a statistical mechanical treatment,[3] based on earlier considerations of the properties of water and of aqueous solutions of hydrocarbons.[4-7] It is known from experiment[4-7] that the transfer of a non-polar substance from a non-polar phase into water is accompanied by a decrease in both enthalpy and entropy (near room temperature); the predominance of the entropy effect over that of the enthalpy makes the process endergonic, that is, unfavorable. Presumably, the non-polar group has an ordering effect on the water molecules near it,[4] thereby increasing the degree of hydrogen bonding among the water molecules. Non-polar groups in an aqueous medium tend to associate; this association minimizes their contact with water and is accompanied by a decrease in the degree of hydrogen bonding and an increase in the freedom of the water molecules which are thus liberated from the neighborhood of the non-polar groups. Near room temperature, the formation of a hydrophobic bond between a pair of side-chain groups in a protein is accompanied by a decrease in free energy and an increase in both enthalpy and entropy; the predominance of the entropy effect over that of the enthalpy makes the process exergonic. The actual numerical values[3] depend on the nature of the side-chain groups and on their extent of overlap.

Adsorption experiments on non-polar surfaces may provide useful information about the properties of hydrophobic bonds in aqueous solution which were predicted in the previous calculations.[3] Such experiments can be carried out by chromatographic methods. Rieman[8] has recently reviewed his work and that of his collaborators showing that mixtures of some organic substances can be separated on ion exchange resins; these experiments utilized the varying degree of binding of non-polar substances to the non-polar parts of the resins. However, it was reported[8,9] that no adsorption or separation could be effected on columns of polystyrene free of ionic groups because the surface area of the polystyrene particles was too small. For the purposes of the present

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(2) On leave from the Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel, 1961–1962.

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paper, it was advantageous for the packing material of the column to be entirely non-polar and of large surface area. Such material will be of importance if columns are to be utilized in research on hydrophobic bonding, where it is desired to eliminate electrostatic interactions; it may also offer possibilities of analytical applications.

In the present paper we describe a method for the treatment of crosslinked polystyrene particles to increase their surface area considerably and illustrate their use in the separation of some model compounds on columns. The strength of hydrophobic bonding of these compounds to the column packing is found to be sensitive to the size of their non-polar portion. Urea is shown to affect the strength of hydrophobic bonding to these columns.

Experimental

Materials.—In the preliminary experiments polystyrene (Dow Chemical Co., PS9) was used. For the quantitative experiments, 2% crosslinked polystyrene particles (a copolymer of styrene and divinylbenzene in the ratio of 98:2) of 200–400 mesh was obtained through the kindness of Dr. E. T. Dumitru of the Dow Chemical Co.

The following substances were used in the experiments: Baker reagent grade phenol and acetic acid, Matheson, Coleman and Bell, *p*-dioxane (m.p. 10–11°) and *n*-caproic acid (m.p. −3° to −5°), Mallinckrodt USP benzoic acid, Eastman butyric and isobutyric acid (reagent grade), Mallinckrodt analytical reagent methanol, ammonium sulfate, KOH and urea. Urea solutions were neutralized to pH 7 with dilute HCl.

We are grateful to Dr. J. A. Rupley of this department for the preparation of ribonuclease A and oxidized ribonuclease A and to Dr. J. Kurtz of the Weizmann Institute for the gift of poly-L-proline of mol. wt. 15,000.

Preliminary Experiments.—The packings used in the preliminary experiments consisted of a fine powder of non-crosslinked polystyrene. The powder was prepared by slowly adding a mixture of water–methanol (85:15, v./v.) to a 3% solution of polystyrene in phenol with vigorous shaking. The polymer was washed with distilled water by decantation. This material (when packed in a column) was capable of adsorbing organic acids. For example, benzoic acid had a distribution coefficient[10] C of about 1.2; however, there was considerable spreading of the peak. Other difficulties included: long time required to pack the column; slow rate of flow of liquid; formation of bubbles in the column when gas pressure was used in the packing or running of the columns. This material was, therefore, abandoned in favor of swollen crosslinked polystyrene.

Swelling of Crosslinked Polystyrene.—In order to increase the surface area of the crosslinked polystyrene, 10.5 g. of the material were swollen in 75 cc. of dioxane for several hours at room temperature; dioxane is a good solvent for polystyrene but only swells the crosslinked particles. The suspension of swollen particles in dioxane was frozen rapidly by pouring it slowly into a flask whose outside surface was cooled in a mixture of Dry Ice and ethoxyethanol. The solid dioxane was leached out with methanol which had been precooled in the same refrigerant mixture. The methanol solution was then decanted, and the particles were washed by decantation with distilled water. The particles were not permitted to dry, since it was difficult to re-wet them if they had been dried.

Presumably the particles prepared in this manner retained their swollen configuration, at least partially. This material had considerable adsorptive capacity, probably because of its high surface area. For example, an aqueous solution of saturated methyl red was rendered practically colorless when 2 cc. were shaken with about 20 mg. of the material.

Columns were packed with this material for chromatographic experiments.

Analysis of Effluents.—The substances run on the columns packed with the porous polystyrene particles were, for the most part, organic acids, since their detection and analysis could be carried out easily with a pH-stat. The effluent liquid from the column was run into a vessel of about 15 cc. volume, which was hermetically sealed with a rubber stopper and contained a stirring bar which was operated magnetically. A combined calomel–glass electrode (Radiometer type GK2021B) and three narrow polyethylene tubes passed through four separate holes in the rubber stopper. The three polyethylene tubes were used to: (1) carry the effluent from the column into the vessel, (2) carry off the liquid from the vessel when it passed a height covering the tip of the electrode, and (3) carry a standard solution of KOH from an Agla micrometer syringe driven by a Radiometer type TTTla pH-stat. The volume of base necessary to titrate the acid in the effluent liquid was recorded on the Radiometer recorder, type SBR2a, attached to the pH-stat. In this way, automatically recorded chromatograms were obtained, the ordinate being the total amount of acid eluted from the column and the abscissa the time (*i.e.*, volume) of flow. The volume of liquid flowing through the column was checked from time to time by observing the volume in a graduated cylinder which was the outflow container from the titration vessel. The water used for eluting the sample off the column was passed through a mixed ion-exchange bed just before entering the top of the column. The pH-stat was set to titrate the acids coming off the column at approximately pH 7.

From the pK's of the acids used as samples, it may be calculated that, at the concentrations used, the acids are only partially ionized. The state of ionization of the adsorbed molecules is not known.

All experiments were conducted at room temperature (about 22°). Rates of flow were about 15 cc./cm.^{2}/hr.

Other methods of analysis were used for the macromolecules which were investigated. Ribonuclease A and oxidized ribonuclease A were detected by measuring the light absorption of the effluent liquid at 278 mμ and poly-L-proline by measuring the optical rotation of the effluent.

Results and Discussion

Columns of various sizes were tried; however, it was found that very small columns were sufficient for a separation of butyric acid from caproic acid. Figure 1 illustrates such a separation on a column 5 cm. long and 0.5 cm. inner diameter. It may be added that benzoic acid (not shown in Fig. 1) is eluted from the column between butyric and caproic acid, closer to the latter and partly overlapping it. The concentrations usually used were 0.001 M; however, butyric acid was run also at concentrations of 0.01 M, giving a sharp peak with the same C-values as at lower concentrations, showing that the surface is not saturated even at the higher concentration. The acids run on the columns were not always recovered quantitatively; the per cent recovery ranged between 80 and 100.

The interstitial volume of the column used to obtain the data of Fig. 1 was 0.9 cc. From this, the following C-values were calculated: acetic acid, 0; butyric acid, 1.1; isobutyric acid, 1.3; benzoic acid, 6.1; caproic acid, 10. The magnitudes

(10) The distribution coefficient C is defined as the ratio of the quantity of sample constituent adsorbed on the solid at any plate of the column to the quantity of the same constituent in the interstitial solution of the same plate at equilibrium.[8,11] It is calculated from the formula

$$C = U^*/V - 1$$

where U^* is the volume of eluate collected from the addition of the sample to the peak of the elution pattern, and V is the interstitial volume of the column packing. The value of V was estimated by running a solution of HCl through the column. This acid presumably runs with the front of the flowing solution. In the evaluation of U^* and V a correction is made for the dead volume between the base of the column and the titration vessel (see below).

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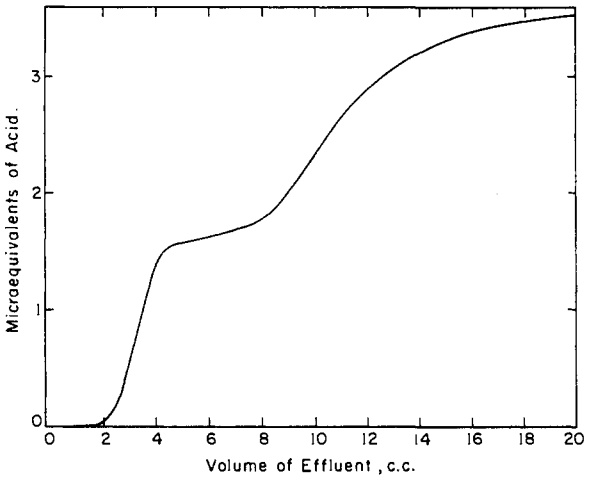

Fig. 1.—Separation of a mixture of butyric and caproic acids on crosslinked polystyrene. The column (5 cm. long, 0.5 cm. inner diameter) was charged with 2 cc. of solution (0.0010 *M* in butyric acid and 0.0011 *M* in caproic acid). Rate of flow was 3 cc./hr.; temp. 22°. The caproic acid comes off after the butyric acid, as determined in separate experiments in which single components (in water) were added to the column. Also, the *C*-values are unaffected by the presence of a second acid component.

of *C* for these substances follow the same order as the standard free energy of formation of hydrophobic bonds between non-polar groups similar to those contained in these substances, as calculated previously.[3] Similar *C*-values were obtained on several different columns containing the same packing.

The effect of urea on the *C*-value was determined by dissolving the solute in urea and eluting with the same solvent. In 8 *M* urea the *C*-value of caproic acid is 4.0, compared to the value 10 in water. This result is in accord with the current hypothesis[6,12] that urea weakens hydrophobic bonds. On the other hand, 5 and 8 *M* urea had little effect on the adsorption of butyric acid on the columns, the *C*-values being several per cent. *higher* in the urea solutions. It is conceivable that, in pure water, the carboxyl group interferes somewhat with the adsorption process and that urea removes this interference. This effect of urea on the carboxyl group (in the case of butyric acid) seems to be nearly balanced by the weakening effect of urea on the hydrophobic bond; in the case of caproic acid, however, the hydrophobic bond is stronger and overshadows the effect of the carboxyl group in the adsorption process. Evidently, urea may produce a variety of effects, and care must be exercised in interpreting its effect on such complicated systems as proteins.

It was also of interest to see if the non-polar groups of native and denatured proteins and polyamino acids would lead to an adsorption of such macromolecules on the polystyrene particles. For this purpose, ribonuclease A, oxidized ribonuclease A (both isoionic in ammonium sulfate at concentrations ranging from 0 to 1 *M*) and poly-L-proline II in pure water were run through a column 12 cm. long and 0.3 cm.^{2} cross section; the column was charged with 1 cc. of solution containing about 5 mg. of sample. Unfortunately, these substances eluted with the front. Presumably, the pores of the polystyrene particles are not accessible to the macromolecules. However, it is hoped that some variations in the column packing (*e.g.*, decreasing the degree of crosslinking in the polystyrene) may increase the area of non-polar surface accessible to macromolecules. This may provide a tool for exploring the availability of accessible non-polar groups in native and denatured proteins. The information thus obtained may be of considerable interest, since the non-polar groups in proteins presumably contribute to the stabilization of certain conformations of proteins and lead to some association reactions involving proteins.[6,13–16]

It is worth pointing out that this technique should be applicable to further studies of the properties of hydrophobic bonds. For example, the effect of temperature and salt on the *C*-values can provide quantitative data on the influence of these variables on the strengths of hydrophobic bonds between various non-polar groups.

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