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# POLARITY OF SOME CHROMATOGRAPHIC MATERIALS INTENDED FOR THE SEPARATION OF BIOPOLYMERS: SPECTROSCOPIC LABEL TECHNIQUE

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## SUMMARY

By using a bound spectral polarity-detecting label, we investigated the solvation power of the solvent near the polymeric matrix of some polar chromatographic media intended for the separation of biopolymers in aqueous solutions. It was found that with highly polar materials such as cellulose and cross-linked polydextran gels (Sepharose, Sephadex) the solvation power of water molecules is reduced and that the polarity detected in the vicinity of the sorbent is much lower. The chromatographic materials investigated (Sephadex derivatives for gel and ion-exchange chromatography, Sepharose derivatives for gel and hydrophobic chromatography, cellulose, Spheron, Dowex 1-X2 and ion-exchange materials based on cellulose, starch, Spheron and Glycophase-coated porous glass) were compared with respect to the magnitude of the difference between the polarity of the microenvironment of sorbents and the polarity of water. Effects that influence this difference are discussed.

## INTRODUCTION

The development of biochemistry in last two decades has been considerably accelerated by the introduction of chromatographic materials and carrier media based on cellulose, agarose, cross-linked polydextrans and starch. These materials are widely used in many separation techniques, including affinity and ion-exchange chromatography, and are also utilized in the immobilization of enzymes.

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The bioaffinity interaction not only depends on the character of interacting materials, but also is affected to a certain extent by the properties of the support, that is, by the interacting compounds bound on the support or coming into close contact with it. If we neglect diffusion effects in the support, two main influences remain, namely the electrostatic effect, if the matrix carries a charge, and the effect due to the polarity of the support. The former effect, that of long-range electrostatic interactions on the function (*i.e.*, mainly on the pH activity profile) has been examined in detail[1]. The latter effect is caused by the differences between the polarity of the polymer chain of the chromatographic material and the polarity of the surrounding solvation medium (water in most instances, or aqueous salt solutions with a lower ionic strength); for other types of chromatographic materials, such as glycophase-coated porous glass, the effect is due to the difference between the polarity of surface groups, which come into contact with the solvent, and the polarity of bulk solution. The larger, "more hydrophobic" molecules, such as polymer chains, affect the structure of the solvent in their vicinity and sterically hinder the solvation of compounds or groups situated in their neighbourhood. This makes the polarity in this region appear to be lower than that of the bulk solution. As a result, various processes that depend on the polarity of the surrounding medium, *e.g.*, some chemical reactions and binding and ion-exchange equilibria, are also influenced by this phenomenon.

The decrease in the polarity of the microenvironment of synthetic polymers in solution[2,3] and the effect exerted by this factor on some reactions, such as ion equilibria, solvolytic splitting of esters, isomerization of stilbene and ion activities, have been discussed in earlier papers[4,5]. It may be expected that the effect observed with soluble polymers will be even more pronounced for densely cross-linked polymeric chromatographic materials, or even for porous coated inorganic materials.

In a previous study the polarity of the microenvironment of soluble polymers was measured by using a spectroscopic label bound at low concentration on polymer chains[2,3]. The spectroscopic label used in both this and the previous study has a solvatochromic charge-transfer (CT) band in its visible spectrum; the position of the band characterizes the polarity or solvation power of the surroundings of the label (reporter). This principle, *i.e.*, observation of the shift (energy) of the CT band in some molecules having a high dipole moment in various solvents, has been employed in the characterization of the polarity of solvents and in the formulation of semi-empirical scales of the polarity of solvents[6]. We have now used the same method for insoluble chromatographic materials. We recorded the absorption (for highly transparent chromatographic materials) or reflection spectra of chromatographic materials modified to a very low degree by molecules of the spectroscopic label. Using the position of the CT band, it was possible to characterize the polarity of the medium near the sorbent. By comparison with the calibration graph obtained for an unbound label in aqueous solutions, it is possible to estimate an approximate value of the local polarity for a certain sorbent (on a semi-empirical scale), or the value of the local dielectric constant useful for, *e.g.*, calculations of ion equilibria and activities. The method may also be employed for comparison of materials intended for the separation of biopolymers on the basis of non-polar interactions.

## EXPERIMENTAL

### *Materials*

Sephadex G-10, G-25 and G-50, SP-Sephadex C-25, CM-Sephadex C-25, DEAE-Sephadex A-25, QAE-Sephadex A-25, Sepharose 4B, CL 4B and CL 6B and octyl-Sepharose CL 4B were products of Pharmacia (Uppsala, Sweden). Octyl-Sepharose CL 4B II (with a higher degree of substitution) was prepared from Sepharose CL 4B according to ref. 7. Cellulose (Genuine Standard) and DE-32 Microgranular Cellulose were manufactured by Whatman (Balston, Maidstone, Great Britain). Spheron P-300 (20–40 $\mu$m) was obtained from Lachema (Brno, Czechoslovakia). DEAE-Spheron (capacity 2.0 mequiv./g) was prepared from Spheron P-300 in our laboratory[8]. Glucose-coated Spheron P-300 was obtained by courtesy of Dr. Čoupek. DEAE-starch was kindly provided by Dr. Bayer. DEAE-Glycophase-coated porous glass and CM-Glycophase-coated porous glass (74–125 $\mu$m) were obtained from Pierce (Rockford, IL, U.S.A.) and lysozyme (salt-free) from Worthington (Freehold, NJ, U.S.A.).

### *Methods*

All chromatographic materials were modified by reaction with 1-(4-bromobutyl)-4-(3-ethoxy-4-hydroxystyryl)pyridinium bromide (BPB). The BPB molecule bound on a chromatographic support (BPB I) can be seen in Fig. 1, which also shows the other related compounds (chromophores) used in this study as polarity reporters. BPB was prepared by a reaction between 4-(3-ethoxy-4-hydroxystyryl)-pyridine and 1,4-dibromobutane in absolute methanol at 60°C for 60 min[9] and

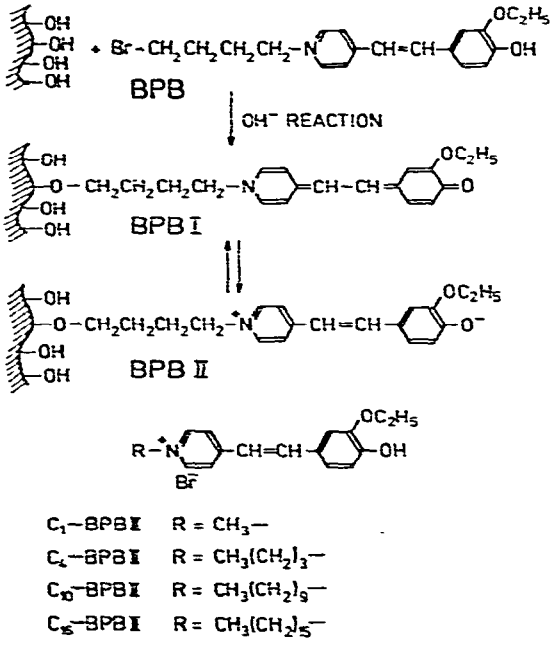


Fig. 1. Bond formation between 1-(4-bromobutyl)-4-(3-ethoxy-4-hydroxystyryl)pyridinium bromide (BPB) and carrier, and structures of the other related chromophores used.

recrystallized three times from methanol. The BPB was bound on the chromatographic materials by treating them with an aqueous alkaline solution or dioxan–water (1:1) solution of BPB at a concentration between 0.1 and 2 mg per 10 ml at 40–60°C for 60–240 min. The chromatographic material was then washed with water, 0.5 *M* hydrochloric acid, 0.5 *M* sodium hydroxide solution, water, methanol, ethanol, acetone and water, until all excess of BPB was removed. After washing with 0.5 *M* hydrochloric acid and water, the products were frozen and stored in the dark. The high molar extinction coefficient of the label[2] allowed us to carry out the reaction so as to attain only a very low degree of substitution and to preserve the character of the material. $C_1$-BPB II, $C_4$-BPB II, $C_{10}$-BPB II and $C_{16}$-BPB II were prepared in a similar manner to BPB using methyl, *n*-butyl, *n*-decyl and cetyl bromide, respectively.

At alkaline pH, the BPB I label is converted into BPB II (the p*K* of this transition in water is 8), the absorption band of which is more sensitive to the polarity of the solvent (Fig. 1). At pH 9.15, at which the measurements were performed, the largest part of the label present in the aqueous solution is in the form II. At this pH, the label bound on various matrices exhibits a different ratio between the concentrations of forms I and II (Fig. 2). The calibration graph was obtained by

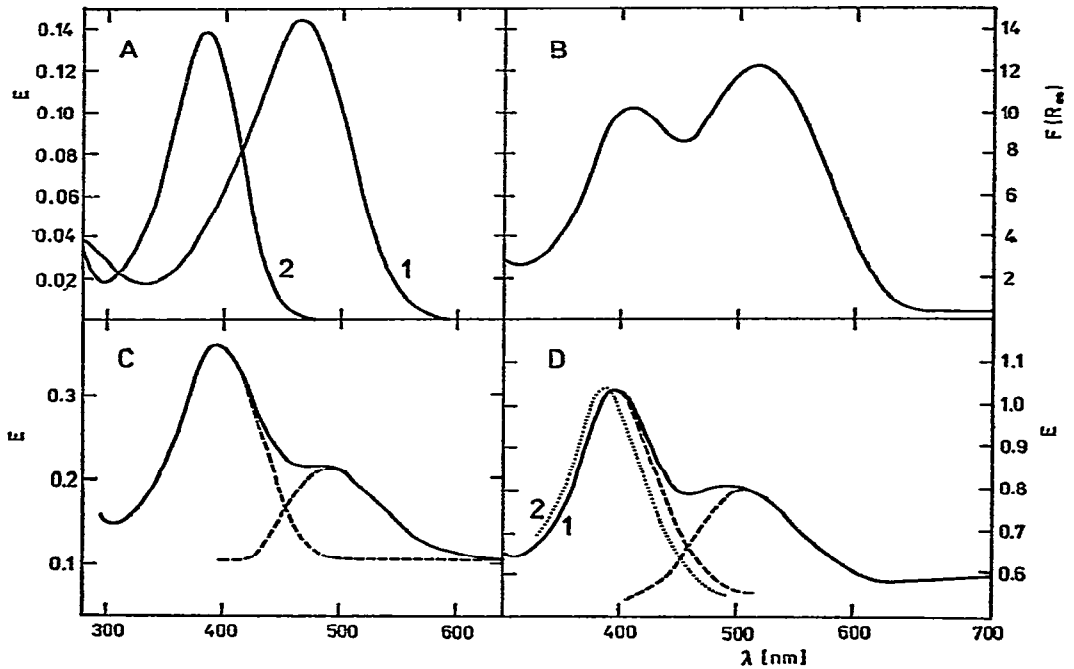


Fig. 2. (A) Absorption spectrum of the $C_1$-BPB label in solution: (1) in 0.1 *M* borax buffer (pH 9.15) (form II); (2) in distilled water (form I). (B) Diffusion reflection spectrum of $C_1$-BPB bound on Spheron P-300, 0.1 *M* borax buffer (pH 9.15). [$F(R_\infty) = 2R_\infty/1 - R_\infty$; $R_\infty$ = diffusion reflectance]. (C) Absorption spectrum of $C_1$-BPB bound on Sephadex C 25, 0.1 *M* borax buffer (pH 9.15) (the broken lines are used to indicate separated bands of forms I and II; band of form I coincides with the spectrum recorded in water). (D) Absorption spectrum of $C_1$-BPB bound on Sepharose 4B; (1) in 0.1 *M* borax buffer (pH 9.15); (2) in water (the broken lines are used to indicate separated bands of forms II and I).

measuring BPB II and other derivatives, in which the nitrogen atom in the pyridine ring is substituted with methyl ($C_1$-BPB II), *n*-butyl ($C_4$-BPB II), *n*-decyl ($C_{10}$-BPB II) and *β*-methacryloyloxyethyl groups[2,3,7,9], in water and in water–organic solvent (methanol, ethanol, 1-propanol, 2-propanol, *tert.*-butanol, acetone and dioxan) mixtures. No differences in the position of the maximum of the solvatochromic band could be observed for variously N-substituted derivatives, even with a bulky hydrophobic substituent ($C_{10}$).

The optical spectra were recorded with a Hitachi Perkin-Elmer 340 spectrophotometer with a reflecting attachment with one integrating sphere. The samples, swollen in water, were rendered alkaline prior to the measurement by washing with 0.1 *M* borax buffer (pH 9.15) until the stationary state was reached, in which the ratio of the intensities of the absorption bands of forms I and II did not vary. The stationary state characterized by the same spectrum was also reached after preliminary treatment with 0.1 *N* sodium hydroxide solution followed by washing with borax buffer (pH 9.15). The samples were then sedimented into cells filled with a 0.1 *M* borax buffer (pH 9.15). From the optical viewpoint, in all instances the samples were heterogeneous and multiple-light scattering. The intensity of the light scattering of the individual carriers differed considerably, obviously with respect to the magnitude and distribution of the domains of the polymer gel and free solution, and depending on the difference between the refractive indices of these domains. Strongly diffusively reflecting samples (Spheron and cellulose types) were placed in the integrating sphere and the spectra of diffusion reflectance, $R$, were measured relative to magnesium oxide as a standard (the samples, 1 and 2 mm thick, do not transmit any light and their reflectance is considered as the reflectance of an infinitely thick layer, $R_\infty$). Relatively transparent samples were placed closely before the integrating sphere, and the absorption spectra with respect to a reference buffer solution were measured. As is illustrated by several examples in Fig. 2, the absorption bands of forms I and II are not sufficiently separated in the spectra (the half-width of the absorption CT bands in the spectrum of the label was found to increase after binding on the matrix, the more so the larger was the difference between the polarities of the polymer and the solvation medium[3]). The absorption bands of form II were separated so that the recorded spectra were modelled on a curve analyser from two absorption bands, the model for the band of form I being the spectra of form I obtained by an independent measurement of non-alkali-treated samples in water. With this procedure, the accuracy with which the position of the maximum is determined is affected by the following factors.

(a) Depending on the carrier, the absorption band of form I is more or less shifted towards longer wavelengths with increasing pH. For this reason, in the separation of bands on the curve analyser in some instances the band of form I obtained by measurement in water had to be shifted towards longer wavelengths (by 7 nm at most) so as to make its position coincide with that of the band of form I in complex spectra recorded at pH 9.15.

(b) With transparent samples, absorption spectra were used in the separation of the band of form II. For multiple-light scattering samples, however, absorbance is not an additive quantity as it is if the Lambert–Beer law is valid. This error did not apply to samples investigated by the diffusion reflection method, where the separation was carried out with spectra re-calculated using the Kubelka-Munk[10] function, $F(R_\infty)$:

$$F(R_\infty) = \frac{(1 - R_\infty)}{2R_\infty} = \frac{K}{S} \quad (1)$$

where $R_\infty$ is the diffusion reflectance of an infinitely thick sample layer, $K$ is absorbance and $S$ is the scattering factor. The spectrum recorded at pH 9.15 is in this instance a real arithmetic sum of the spectra of forms I and II:

$$F(R_\infty) = F(R_\infty^{I}) + F(R_\infty^{II}) = \frac{K^{I} + K^{II}}{S} \quad (2)$$

where $K^{I}$ and $K^{II}$ are the absorbances of forms I and II, respectively, and $S$ is the scattering factor given by the carrier.

(c) The position of the maximum in the spectra is affected by the dependence of the light scattering on the wavelength.

The effect of factors (b) and (c) given by light scattering was tested by measuring samples of various thickness (0.1, 0.2, 0.5 and 1 cm) and by diluting samples with carriers without any bound chromophore. The accuracy of the determination of the position of the maximum of the absorption band of form II, given by the magnitude of the correction for factors (a), (b) and (c) was estimated in our experiments to be $\pm$ 3 nm.

## RESULTS AND DISCUSSION

Various chromatographic materials employed in the separation and immobilization of biopolymers were compared, such as loosely cross-linked gels based on natural polysaccharide polymers (Sephadex, Sepharose, etc.), medium cross-linked polystyrene ion exchanger (Dowex 1-X2), highly cross-linked hydrophilic macroporous heterogeneous gel (each of the 3–4 monomeric units is cross-linked), Spheron P-300 (ref. 11) and porous glass coated with a hydrophilic monolayer[12].

The local polarities near these chromatographic materials and a comparison of the hydrophilicities of these materials are obtained by comparing wavelengths or the corresponding energies of the solvatochromic CT band of the BPB II spectroscopic label, which is bound on these materials through an oxytetramethylene spacer (Fig. 1). The wavelengths of the maxima of solvatochromic CT bands after binding on the sorbents are given in Table I. As the polarities of solvents on semiempirical scales are mostly given as the transition energies of sensitive spectral bands, the respective energies, $E_T$, of these transitions calculated from the equation[6]

$$E_T\,(\text{kJ/mole}) = N_A h\nu = \frac{1.197}{\lambda_{max}} \cdot 10^4 \quad (3)$$

where $N_A$ is Avogadro's number, $h$ is Planck's constant, $\nu$ is frequency and $\lambda_{max}$ (nm) is the wavelength of the maximum of the solvatochromic band, are also given.

Table I also presents values measured with an unbound label in an aqueous solution containing only buffer. These values ($E_T$ 255.2 and 256.3 kJ/mole) characterize the polarity of the pure solvent. If the maxima of the solvatochromic band of modified BPB II bound on chromatographic materials suspended in this solvent are shifted towards longer wavelengths (lower energies), they indicate a limited solvation of the chromophore near the chromatographic material and a lower local polarity.

TABLE I

WAVELENGTHS OF THE MAXIMA FOR THE SOLVATOCHROMIC CT BAND ($\lambda_{max}$ nm) AND THE RESPECTIVE TRANSITION ENERGIES ($E_T$ kJ/mole) FOR THE BPB II LABEL BOUND ON VARIOUS CHROMATOGRAPHIC MATERIALS, FOR A FREE LABEL WITH THE SUBSTITUENTS $C_1$, $C_4$ AND $C_{10}$ AND FOR A LABEL WITH THE SUBSTITUENTS $C_4$, $C_{10}$ AND $C_{16}$ SORBED ON SPHERON P-300

All measurements were performed in 0.1 *M* borate buffer (pH 9.15) by recording transmission or reflection spectra.

| *Material* | $\lambda_{max}$ *(nm)* | $E_T$ *(kJ/mole)* |
|---|---|---|
| Sephadex G-10 | 510 | 234.7 |
| Sephadex G-25 | 502 | 238.4 |
| Sephadex G-50 | 500 | 239.4 |
| Sepharose 4B | 499 | 239.9 |
| Sepharose CL 4B | 505 | 237.0 |
| Sepharose CL 6B | 509 | 235.2 |
| Octyl-Sepharose CL 4B | 506 | 236.6 |
| Octyl-Sepharose CL 4B II | 516 | 232.0 |
| Cellulose | 493 | 242.8 |
| Spheron P-300 | 520 | 230.2 |
| Glucose-coated Spheron P-300 | 533 | 224.6 |
| SP-Sephadex C-25 | 486 | 246.3 |
| CM-Sephadex C-25 | 490 | 244.3 |
| QAE-Sephadex A-25 | 496 | 241.3 |
| DEAE-Sephadex A-25 | 502 | 238.4 |
| DEAE-cellulose | 498 | 240.4 |
| DEAE-starch | 494 | 242.3 |
| DEAE-Spheron P-300 | 528 | 226.7 |
| DEAE-Glycophase-coated glass | 502 | 238.4 |
| CM-Glycophase-coated glass | 500 | 239.4 |
| Dowex 1-X2 | 515 | 232.4 |
| $C_1$-BPB II in buffer | 467 | 256.3 |
| $C_4$-BPB II in buffer | 469 | 255.2 |
| $C_{10}$-BPB II in buffer | 467 | 256.3 |
| $C_4$-BPB II after sorption on Spheron P-300 | 524 | 228.4 |
| $C_{10}$-BPB II after sorption on Spheron P-300 | 534 | 224.2 |
| $C_{16}$-BPB II after sorption on Spheron P-300 | 534 | 224.2 |

In pure buffered aqueous solutions without sorbent, three compounds were measured, containing a methyl ($C_1$-BPB II), an *n*-butyl ($C_4$-BPB II) and an *n*-decyl ($C_{10}$-BPB II) substituent on the nitrogen atom of the chromophore. All of these compounds exhibited approximately the same maxima for the solvatochromic CT band. Hence it can be concluded that the mere size of the substituent, even non-polar aliphatic substituents (up to the size of *n*-decyl), does not perceptibly affect the solvation of the dipole of the chromophore. As it has been proved that chemical modifications to one of the more remote carbon atoms of the substituent are also not reflected in the spectrum[2], it is obvious that the shift of the solvatochromic band after binding on chromatographic materials is due only to the different polarities of the medium near the chromatographic material.

It can be seen from Table I that in all materials under investigation the solvation of the bound label is hindered and affected to some extent; hence, the polarity detected in a region near the chromatographic material is lower than the

polarity of bulk solution. None of the chromatographic materials investigated in this work, even with a matrix consisting of relatively very polar polysaccharide chains which, being uncross-linked, are very readily soluble in water (*e.g.*, Sephadex), is polar enough to restrict the solvation of bound molecules of the reporter. Not only the lower polarity of the polymer backbone of the chromatographic material but, compared with water molecules, also the limited mobility of the matrix participate in this effect. A similar effect has been observed with some soluble synthetic polymers[2,3] and has been examined in a similar way for natural macromolecules having a rigid conformation[13,14].

The lower local polarity of the microenvironment of the chromatographic material compared with the surrounding bulk medium may be reflected in the retention of less polar compounds, and also in the influence on some chemical equilibria and reactions, through local effective activities which depend on the local dielectric constant.

The most hydrophilic material revealed by a comparison between the chromatographic materials under study (Table I) is Sepharose 4B ($E_T$ 243.3 kJ/mole), with cellulose ($E_T$ 242.8 kJ/mole) next to it with respect to polarity. However, both materials exhibited a difference in polarity compared with the buffer in which they were swollen and measured. If Sepharose 4B or cellulose is swollen in water, the polarity near the bound BPB II reporter corresponds approximately to that of water–ethanol containing 25% (v/v) of ethanol (Fig. 3).

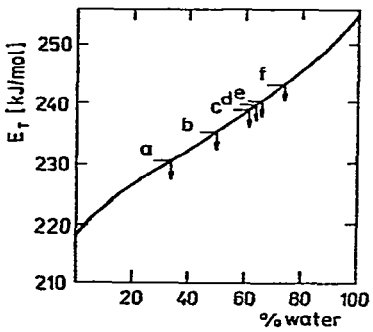


Fig. 3. Comparison of the polarity of the microenvironment of chromatographic supports expressed by the CT band energy ($E_T$, kJ/mole) of BPB II bound on (a) Spheron P-300, (b) Sephadex G-10, (c) Sephadex G-25, (d) Sephadex G-50, (e) Sepharose 4B and (f) cellulose with the polarity of water–ethanol mixtures[3]. For conditions of measurement, see Experimental.

An even lower polarity was measured for all types of Sephadex (G-10, G-25 and G-50), which differ in their exclusion limits. Certain differences, although almost at the limit of the accuracy of optical measurements, were observed depending on the degree of cross-linking. The polarity measured for the most highly cross-linked Sephadex G-10 was the lowest ($E_T$ 234.7 kJ/mole), which (Fig. 3) corresponds approximately to that of water–ethanol containing 50% of ethanol. The local polarity near the cross-linked polysaccharide matrix Sephadex G-10 may also be roughly characterized by the dielectric constant of this mixture. For Sephadex G-25, with a lower degree of cross-linking, the polarity was higher ($E_T$ 238.4 kJ/mole),

and may be compared with that of water–ethanol containing slightly less than 40% of ethanol. Sephadex G-50, with a higher exclusion limit and a lower degree of cross-linking, was even more polar. The differences revealed by the measurements are not large but are easy to understand. The lowest polarity was measured for Sephadex G-10, with the highest degree of cross-linking, which contains the largest amount of short cross-linking non-polar alkyl groups and for which the retention of less polar groups has often been recorded (*e.g.*, ref. 15).

Roughly the same polarities as for Sephadex were recorded for cross-linked Sepharoses ($E_T$ 237 kJ/mole for Sepharose CL 4B and 235.2 kJ/mole for more cross-linked Sepharose CL 6B).

Spheron P-300 is the least polar material studied. It is a macroporous rigid copolymer[11] of 2-hydroxyethyl methacrylate and ethylene dimethacrylate. Owing to its hydroxylic group, the basic monomer confers upon the polymer a certain hydrophilicity; the material is therefore wetted with water and swells in it. The high degree of cross-linking (20–30%), the limited mobility of the polymeric backbone and the aliphatic character of the basic polymeric backbone are the causes of the lowest measured $E_T$ (226.7 kJ/mole). The polarity detected in the vicinity of Spheron is the same as in water–alcohol mixtures containing 65% of ethanol (Fig. 2) or 88–89% of methanol. Such a polarity is relatively low, and it is not surprising, therefore, that even if Spheron is wetted with water and swells in it, it interacts in aqueous solutions and in solutions of salts with non-polar groups on the protein surface, causing their retention[16]. The material has been suggested for some biochemical applications[17] and for the hydrophobic chromatography of proteins[16]. The decrease in polarity in the vicinity of Spheron P-300 compared with water is larger (20.9 kJ/mole) than that observed with linear poly-(2-hydroxyethyl methacrylate)[2,3], where the difference measured against the bulk solution in most solvents was only 4–8 kJ/mole. Hydrophobic interactions of proteins with the surface of Spheron P-300 can be reduced by modifying the sorbent surface with sugar units (glucose). As has been demonstrated by chromatographic experiments, and as also follows from the very low $E_T$ for glucose-coated Spheron P-300, such a modification does not cause any essential changes in the hydrophobic binding sites for small molecules.

Another two materials prepared by introducing non-polar octyl moieties into the polar Sepharose gel and intended for the hydrophobic chromatography of proteins were also compared with Spheron P-300. The increased hydrophobicity of the chromatographic material is more pronounced with octyl-Sepharose CL 4B II, *i.e.*, only at a higher degree of substitution, which means a higher density of octyl groups in the gel. With octyl-Sepharose CL 4B, which has a very low density of the polarity reporter groups and a relatively low density of octyl groups, the relatively low fraction of octyl groups is so close to the reporter groups that it is able to affect their solvation. The effect would certainly be more pronounced if the reporter molecules were localized only in the vicinity of hydrophobic octyl substituents. More probably, however, they are distributed randomly throughout the gel structure. For this reason, polarity detected with octyl-Sepharose CL 4B is very close to the value observed with the originally unsubstituted polymer (Fig. 4). For octyl-Sepharose CL 4B II, where a higher degree of substitution was attained (the retention of proteins on this material is approximately three times greater than on commercial octyl-Sepharose CL 4B), a distinct decrease in polarity compared with unsubstituted Sepharose CL

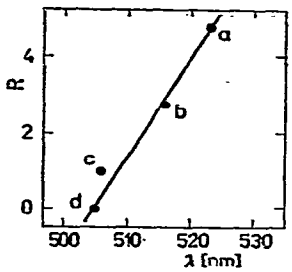


Fig. 4. Dependence of retention, $R$ (defined as $R = (V_{el} - V_0)/V_0$, where $V_0$ is the gel permeation elution volume of lysozyme when retained by hydrophobic interactions and $V_{el}$ the elution volume of lysozyme in 0.5 $M$ NaCl) on the polarity of the chromatographic material). (a) Spheron P-300; (b) octyl-Sepharose CL 4B II; (c) octyl-Sepharose CL 4B; (d) Sepharose CL 4B for hydrophobic chromatography of lysozyme. The polarity is expressed through the wavelength of the solvatochromic band of the label bound on these materials. Chromatographic analysis was performed in a 0.5 $M$ NaCl–0.05 $M$ phosphate buffer (pH 5.5) at 20°C.

4B was been observed. The polarity thus measured ($E_T$ 232 kJ/mole) corresponds to that of water–ethanol containing 60% of ethanol.

Table I also presents measured local polarities for ion-exchange derivatives of Sephadex, cellulose, Spheron, starch, Glycophase-coated porous glass and Dowex 1-X2. The polarity measured for all ion-exchange derivatives of Sephadex, with the exception of DEAE-Sephadex A-25, was higher than that of a comparable unsubstituted material without a major number of charged groups, such as Sephadex G-25. The introduction of ionized polar groups, *viz.*, sulphopropyl (SP), carboxymethyl (CM) and quaternary triethylammoniumethyl (QAE), into the original matrix leads to a higher net local concentration of charge and counter ions, reflected in a rise in the local polarity near gel chains. Only the polarity of weakly basic diethylaminoethyl (DEAE) derivatives of Sephadex, Spheron and cellulose, ionized to a small degree at pH 9.15, is close to the values for the original unsubstituted materials.

The polarity of DEAE-starch is higher than that of DEAE-Sephadex A-25; the local polarities measured for ion exchangers prepared on the basis of Glycophase-coated porous glass (DEAE and CM derivatives) are roughly the same. As the last two materials possess a very low total capacity, and hence also a lower local charge concentration, it can be assumed that in these instances the local polarity is not affected to any great extent by ion groups.

The polarity determined for Dowex 1-X2 is rather low ($E_T$ 232.4 kJ/mole) and corresponds to that of water–ethanol containing slightly less than 60% of ethanol. Dowex 1-X2 is an anion exchanger, the matrix of which consists of very hydrophobic styrene and divinylbenzene units. The local polarity measured near the gel structure of the ion exchanger in an aqueous suspension is much lower than the polarity of the surrounding medium, *i.e.*, water. In this instance the difference between the polarity of water, in which the ion exchanger is swollen, and that of the microenvironment of the ion exchanger is reduced by the presence of a large number of polar and charged groups (capacity 4.3 mequiv./g, *i.e.*, approximately one group per two nuclei).

The method of comparison of the polarity of chromatographic materials described above differs from methods described earlier (*e.g.*, ref. 18). The polarity of

the microenvironment of some synthetic polymers has been measured in the same way; the local microenvironment on the surface of globular proteins has been measured by means of spectral labels (reporters) for many years[13,14]. The measurement of the local polarity on the surface of some of the chromatographic materials described here is better suited for more polar materials having preferably a microhomogeneous structure in an aqueous medium and in polar solvents and mixtures. The sorbent must possess reactive groups, such as hydroxyl, amine and thio groups. This measurement gives direct information about how the difference between the polarities of the sorbent and the solvent affects the solvation power of the solvent near the sorbent, and also provides a means for the numerical expression of this effect in the form of the local dielectric constant, which, together with a simple comparison of the polarities of chromatographic materials, may also be employed in calculations of the local activities, ionic equilibria and other charge interactions near the sorbent. Of all these applications, the possibility of calculating the effective pH value is the most important.

One should bear in mind that generally the local polarity calculated from the spectra of the label need not be identical with that operative in reactions of other molecules on the chromatographic support. The supports are inhomogeneous, and the measured CT band is a superposition of the absorption bands of the label bound in various regions. The determined maximum of the absorption band and the local polarity calculated therefrom are a mean values characterizing the distribution of the label in the support. This distribution is given by the conditions of the binding reaction, *i.e.*, by the magnitude of physical interactions of the label with the particular regions and with the reaction solvent, by the rate of diffusion to reactive groups and by the kinetics of the binding reaction. An example can be seen in the low $E_T$ values (Table I) calculated from the spectra of the $C_{10}$-BPB II and $C_{16}$-BPB II labels adsorbed on Spheron (Spheron was placed in a solution of the label in methanol, then thoroughly washed with water and measured in the usual way in borax buffer, pH 0.15). The label, which in this instance is bound on the matrix by hydrophobic forces through *n*-decyl and cetyl hydrocarbon chains, is obviously preferentially localized in less polar domains of the heterogeneous carrier. In contrast, a label without the hydrophobic chain ($C_1$-BPB II) did not exhibit any tendency to be sorbed preferentially in non-polar regions, and it could be readily washed out from the supports with water. Fig. 4 shows a correlation of the local polarity calculated from the spectra of the bound label with the retention, $R$, for the hydrophobic chromatography of lysozyme on Spheron and octyl-Sepharose with various degrees of substitution. The relatively good agreement obtained on two different types of materials, *viz.*, aerogel and xerogel, shows that the spectra of the bound label predominantly reflect the polarity in the surface regions of the support, which is important for an interaction between the latter and macromolecules.

Together with the accuracy of the determination of the maximum of the solvatochromic band, the reliability of measured data depends mainly on the condition that specific interactions between chromophore and matrix, or solvent, or the so-called sorting effects caused in mixed solvents by the molecule of the reporter, should be lowest for the chosen reporter. Semi-empirical scales of the polarity of solvents based on the energy of solvatochromic bands, chromophores with a high dipole moment and with a structure similar to BPB II range among the most

universal scales of polarity[6,7]. Good correlation over a wide range of solvents has been found not only with the dielectric constant, but also with various solvation-sensitive processes[6,19]. This suggests that specific interactions with solvent and solvent-sorting effects are not important, and that the same may be assumed for specific interactions with various organic sorbents. A simple interpretation is the advantage given by this type of label compared with, for example, fluorescence measurements, which offer similar possibilities. As may be expected and as has also been demonstrated for soluble polymers[9], the difference between polarities detected in the vicinity of the polymer in bulk solution decreases with the distance of the chromophore from the binding site.

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