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Investigation of Pressure-Induced Changes in the Chromatographic Selectivity of Methyl and Ethyl Orange on Silica Gel

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Summary

With water as the solvent and two azo dyes as the exemplary solutes, the modification of selectivity through pressure-induced changes in column equilibria has been shown to be significant using a chromatograph capable of going to 4000 kg/cm^2. Data from high-pressure conductance, spectroscopy, adsorption chromatography, and steric-exclusion chromatography indicated that the primary contribution to the pressure shifts in selectivity was occurring in the bulk solution. An explanation correlating the various experimental observations is offered.

INTRODUCTION

High-pressure liquid chromatography has been particularly effective in obtaining faster analyses by permitting columns to be packed with very small particles. The smaller particle size results in increased column efficiency due to a reduction of mass transfer for the solute in partitioning between the mobile solvent and the stationary adsorbent. If one wishes to use very small particles, the pressure requirements may sometimes be

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in the range of several thousand kilograms per square centimeter since the pressure required for efficient flow increases with the inverse square of the particle diameter. Theoretical treatments of high-inlet pressures, small particle sizes, and the relation to the van Deemter equation have been offered by Giddings (*1–3*) and Pretorius et al. (*4*).

In our laboratory a column has been successfully packed with 10-μ particles of silica gel and operated under inlet pressures up to 4000 kg/cm^2 to improve the separation of methyl and ethyl orange by adsorption onto silica (*5*). Both the speed of analysis and the resolution were greatly increased over that obtained by conventional approaches. Of further significance was the fact that the selectivity of the column could be changed by pressure. In fact, the distribution ratios for the azo dyes not only went through a minimum at approximately 1500 kg/cm^2 as the inlet pressure was increased but, also, they diverged significantly at higher pressures.

While that report experimentally demonstrated a novel, easy-to-use, and previously unexplored method of controlling selectivity in liquid–solid chromatography, it also raised the question as to the reason(s) for such a large effect of pressure. There is only limited knowledge concerning the effects of high pressure on chemical equilibria in solutions, especially where adsorption is involved. From the thermodynamic standpoint, changes in a column equilibrium, as expressed by the distribution ratio, can be related to changes in the free energy of adsorption. At constant temperature, free-energy changes of the system are governed by the changes in the partial molar volume of the system.

The existence of a reversal in the change of partial molar volume has not often been encountered in high-pressure equilibrium studies (*6*). Such a reversal may indicate that two different pressure-induced phenomena are occurring. As a result, the present investigation was made in order to identify the primary contribution to the pressure-induced equilibrium shifts and to determine whether it was primarily at the interface or in the bulk solution.

The physical properties of two azo dyes, methyl orange and ethyl orange, were examined by spectroscopy in the visible region, conductivity at high pressures, adsorption chromatography in various solvents, at various pressures in aqueous systems, and finally by steric-exclusion chromatography at various temperatures and pressures in different media. As will be seen, the data indicate that the primary contribution to the pressure-induced changes in the column equilibrium was occurring in the bulk solution.

EXPERIMENTAL

Reagents

The azo dyes, methyl orange (MO), ethyl orange (EO), and butyl orange (BO) were purchased from Eastman Organic Chemicals (Rochester, New York) and recrystallized twice from hot distilled water. The purity was checked by two elution experiments, one of which utilized distilled, deionized water as the eluent and the other a 5% (v/v) acetic acid solution. In each case, only one peak eluted from a 1.2-cm i.d. × 25 cm column of Davison Code 950 silica gel (W. R. Grace, Baltimore, Maryland). The unsulfonated analog of methyl orange, *N*,*N*-dimethyl-*p*-phenylazoaniline (DMO) was also purchased from Eastman Organic Chemicals. The dye was recrystallized twice from 95% ethanol. It was purified by elution with toluene from a 2.0-cm i.d. × 20 cm column of Davison Code 950 silica gel followed by a final recrystallization from 95% ethanol [mp 118–119°C; Lit. 117–118°C (*7*)].

For the adsorption chromatography, Davison Code 58 was utilized, the particle sizes being 200–500 μ in the high-pressure experiments and 105–124 μ in the experiments near atmospheric pressure. Controlled porosity glass (Corning Glass Works, Corning, New York) was used for the steric-exclusion chromatography. Various molecular-weight ranges were used; CPG-1250 (40,000–550,000), CPG-700 (15,000–300,000), and CPG-370 (5,000–150,000). All of the glass beads were 125–177 μ in diameter.

For the chromatographic experiments, distilled water was passed through a mixed-bed ion exchanger and then stored in a polyethylene carboy. Water was prepared each week. For conductivity experiments, distilled water was deionized and then doubly distilled from a fused quartz distillation unit immediately before preparation of solutions. Solutions were stored in Pyrex volumetric flasks for a maximum of 3 days of use. Since pH changes can occur with the application of pressure (*8*), buffered solutions were not used.

Dioxane was spectroscopic grade. All other chemicals were reagent grade.

Apparatus

Conventional chromatographic columns were constructed using glass tubing of the same internal diameter as the high-pressure column (0.625 cm). The lengths were 30.5 and 91.5 cm. Teflon fittings (Beckman

Instruments, Fullerton, California) were used as entrance and exit connectors (*9*). A low dead-volume connection between the column and detector was constructed from a No. 501 fitting into which was placed an adaptive tube, 0.508 mm i.d., covered with 10-μ nylon mesh (Glenco Scientific, Houston, Texas) which served as the bed support.

A Milton Roy Minipump (Dayton, Ohio) provided the flow. The detectors for the chromatographic systems were a stabilized Spectronic 20 spectrophotometer (*10*) and R-4 refractometer (Water Associates, Framingham, Massachusetts). The photometer, which had low noise and a drift of less than 0.02% T per hour (at 100.00% T), was suitable for detecting very small concentrations of methyl or ethyl orange. No noise or stability levels were determined for the R-4 refractometer since the performance for trace quantities was much less favorable.

A detailed description of the high-pressure liquid chromatograph has been given elsewhere (*5*). Similarly, the high-pressure conductance equipment has been previously described (*11*).

The resolution of overlapped chromatographic peaks was determined using a DuPont Curve Resolver Model 310 and the deconvolution method described in the DuPont instruction manual.

Recordings of spectra were made using a Cary 14 (Cary Instruments, Monrovia, California) and matched cells.

Procedures and Calculations

All chromatographic columns were packed using vibration while introducing a slurry of the adsorbent which had been previously vacuum-degassed for 1 hr. Elution was carried out using distilled-deionized water that had been thermally degassed, using an IR lamp prior to being pumped into the column.

The general operating procedure for the high-pressure chromatographic instrument has been described (*5*). In the present work, however, the column was operated under a nearly constant pressure drop by means of an exit valve that permitted adjustment of flow rate to a predetermined value regardless of the input pressure. The pressure drop across the column was measured in a separate experiment by placing a "tee" fitting at the exit and attaching a second gauge before the exit valve. The pressure drop was 33 kg/cm^2 or less for all experiments.

The dials of the pressure gauges were marked in units of lb/in.^{2}, but the pressure readings were converted to kg/cm^2 using the equivalency, 1 kg/cm^2 = 14.22 lb/in.^{2}. The resulting values were rounded to the nearest hundred. An uncertainty of 50 kg/cm^2 in the pressure control resulted from refill strokes of the pump, and they lasted less than a half second. In a 15-min run at a flow rate of 0.5 ml/min, there would be approximately 3 strokes.

Because the refill stroke caused slight variations in flow rate, the elution volume was measured by collecting eluent in a buret. The input to the recorder was manually shorted every 0.50 ml to produce an event mark on the chart paper. From the markings, the exact retention volume could be interpolated. This is in contrast to the work near atmospheric pressure where the flow rate was more nearly constant (0.5%).

The chromatographic data obtained near atmospheric pressure were reported in terms of retention volume since the trends were evident without the bias introduced by an estimated calculation of the distribution ratio. However, we wanted to compare the present high-pressure chromatographic behavior with that of the earlier work (*5*), so the distribution ratios were calculated from the chromatogram using

$$V_R = V_i(K + 1) \tag{1}$$

where V_R is the retention volume, V_i is the interstitial volume based on a model of packed spheres, and K is the distribution ratio. The calculation of K also made possible a means of estimating the changes in the partial molar volume as a function of pressure.

The change in partial molar volume with pressure is expressed by the partial differential equation:

$$\frac{\partial(\ln K)}{\partial P} = \frac{-\Delta V}{RT} \tag{2}$$

which is integrated to give

$$\ln \frac{K_2}{K_1} = \frac{-\Delta V(P_2 - P_1)}{RT} \tag{3}$$

In these equations, ΔV is the change in partial molar volume, P is the pressure, R is the gas constant, T is the temperature, and the subscripts refer to the higher and lower pressures. In a strict sense, Eq. (3) is only valid for pressure intervals of about 100 kg/cm^2 (*6*). However, the scatter in the data limits the precision with which one can draw a curve. Therefore, ΔV was not based on the slope of the tangent to the curve but, instead, on the best straight line through the points. Hence, ΔV should be considered as an average value, an approach which has been previously utilized (*6*). Therefore, the term ΔV_{av} will be used to designate the average volume change.

The results of steric-exclusion chromatography are reported in terms of the elution volume, V_e. The elution volume of a species is equal to the sum of the interstitial volume, V_0, and accessible pore volume K_dV_{in}:

$$V_e = V_0 + K_dV_{in} \tag{4}$$

where V_{in} is the internal liquid volume and K_d is the fraction of the pore volume which a molecule encounters ($0 < K_d < 1$). The internal liquid volume, V_{in}, was determined from

$$V_t = V_0 + V_{in} \tag{5}$$

where V_t is the total volume of the mobile phase in the column. The columns operated under atmospheric pressure were calibrated using blue dextran (V_0), sodium chloride (V_t), sucrose (V_t), *p*-nitrophenol (V_t), and the monomer (MW = 66,000) and dimer (MW = 132,000) forms of bovine plasma albumin. When desired, the elution volumes were converted to molecular size in Angstrom units, Å, using Ackers equation (*12*). No attempt was made to calibrate the high-pressure column except to determine V_0 and V_t at 500 kg/cm^2 since the pressure effects on intermediate standards were unknown.

The procedure for using the conductivity instrument has been described elsewhere (*11*). Temperature of the cell was controlled at 25.0 ± 0.1°C. The cell constant of 0.012 cm^{-1} was determined using a 0.01 demal potassium chloride solution. The conductance for water, of the order of 10^{-6} ohms^{-1}, was subtracted from all solute conductances as determined at the various pressures.

Solutions of methyl orange were prepared in conductivity water and stored in Pyrex volumetric flasks for 3 days during the experiments. Solutions were prepared in duplicate, and the average value was taken as the conductance of the solution.

When the conductance data were plotted versus concentration, the break that occurred was considered to indicate a change in an equilibrium process. Based upon similar treatments of Phillips (*13*) and Hamann (*14*) in the investigation of micelle formation, Eq. (6) relates the change in partial molar volume to the concentration at which the break occurred:

$$\frac{\partial \ln(C_{eq})}{\partial P} = \frac{\Delta V}{CRT} \tag{6}$$

where C_{eq} is the conductance at the break and C is a constant, usually in the range of 1.8.

While the use of Eq. (6) and the assumption that $C = 1.8$ was not as firm as one would ordinarily hope, it was useful for making possible the qualitative comparisons between changes in ΔV_{av} obtained in high-pressure chromatography and those obtained in conductance.

In order to determine the amounts of soluble and insoluble silica in the solutions, molybdosilicic acid was formed and determined at 332 nm by the method of DeSesa and Rogers (*15*). Only two standard solutions, 1 and 0.5 ppm of sodium metasilicate ($NaSiO_3 \cdot 9H_2O$), were used.

RESULTS

Effect of Pressure Using Coarse Silica

The prior report (*5*) using 10-μ Syloid as the adsorbent showed that K changed with the inlet pressure(s). However, there was undoubtedly a large pressure drop across the column. In order to insure a low pressure drop, coarse particles (Davison Code 58, 200–500 μ) were packed into the column. Examining the pressure effects on this column had three purposes. First, it would answer the question of the existence of pressure shifts in column equilibria for other preparations of silica. Second, since we had found the thin-layer chromatography separation of MO and EO on larger particles to be incomplete, experimental evidence for the utility of pressure in significantly effecting an improved separation of the dyes should be obtained. Finally, the large particles would allow various flow rates to be attained at one inlet pressure, so that flow rate effects, if any, could be determined with minimal uncertainty due to pressure drop in the column.

Figure 1 shows an elution profile for a mixture of MO and EO at a constant flow rate of 0.6 ml/min. Notice that, as the column pressure was increased, the peak maxima moved apart. The improvement of resolution in going from 520 to 2800 kg/cm^2 was quite dramatic and was clear evidence for pressure-induced changes in the distribution ratios. However, the resolution, even at 2800 kg/cm^2, was not really as good as the earlier results (*5*) due to the present use of the large particles of adsorbent.

A low-pressure-drop column permitted the use of various flow rates even at a low inlet pressure. The data for a given pressure in Fig. 2

were collected by changing the opening of the exit valve between each run. Flow rates ranged from 0.50 to 3.50 ml/min at each pressure. Because the points at each pressure showed no systematic change with flow rate, the spread must represent the normal scatter. Again, the distribution ratios of MO and EO went through a minimum at 1500 kg/cm^2, so the size or type of silica must have had no major effect upon the shape or inflection of the pressure-induced changes in K.

The calculation of the average volume change, ΔV_{av}, for MO over the low-pressure range 530–1500 kg/cm^2 was +5.1 kg/cm^2. After the minimum, the value in the region of 1500–3000 kg/cm^2 was −8.6 cm^3/mole. The respective values for EO were +4.9 and −13.2 cm^3/mole.

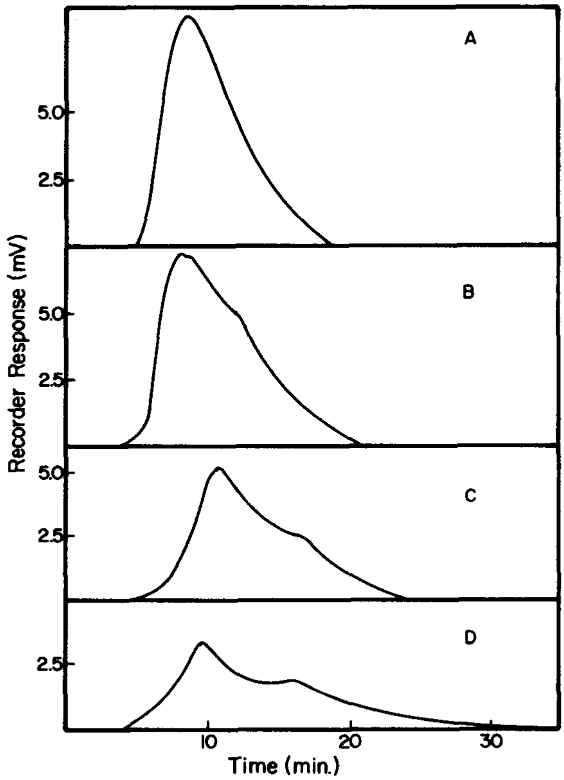

Fig. 1. Mixture of methyl and ethyl orange chromatographed using various pressures at a flow rate of 0.6 ml/min: (A) 560 kg/cm^2, (B) 1400 kg/cm^2, (C) 2100 kg/cm^2, and (D) 2800 kg/cm^2.

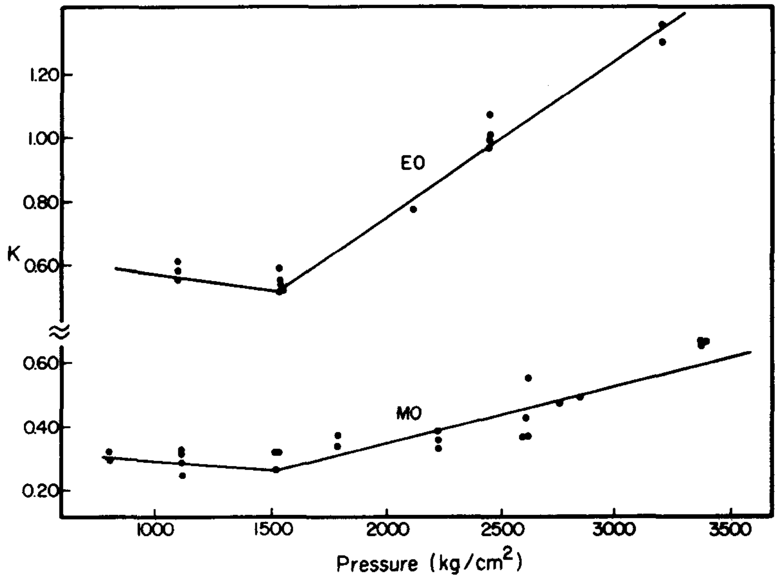

FIG. 2. Effect of pressure upon the distribution ratio of methyl orange (MO) and ethyl orange (EO).

High-Pressure Conductance

On the basis of the chromatography alone, it was not possible to decide whether the changes in the distribution ratios were due primarily to changes in solute–solvent interactions in the bulk liquid or to changes at the interface. To investigate that problem, a high-pressure conductance apparatus was made available by Dr. W. L. Marshall at Oak Ridge National Laboratory.

The study consisted of measuring six duplicate samples of MO solutions in the concentration range of 1.0–10.0 × 10^{-4} M. Each solution was placed in the cell and allowed to equilibrate for 15 min. The pressure was then raised in 500 kg/cm^2 steps. Conductance readings were taken at each pressure after approximately 5 min had elapsed. Each original reading was checked again after 5 min to insure that the value had remained constant. From the individual curves of conductance versus pressure, graphs of concentration versus conductance were made as illustrated in Fig. 3 for 500 kg/cm^2. The solid lines are the least-squared fits to the points. Their intersection is at a point designated as C_{eq}.

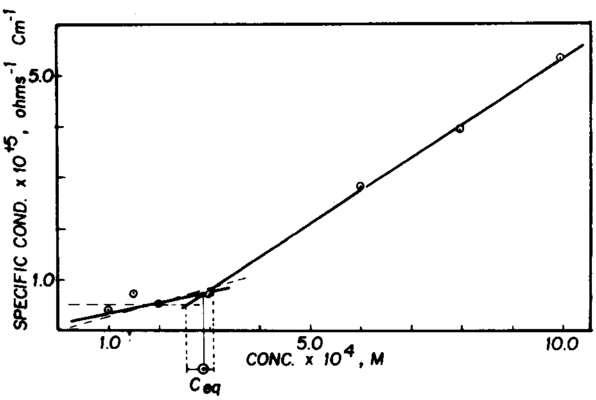

FIG. 3. The specific conductance of methyl orange in aqueous solution at 25°C and 500 kg/cm².

The error limits in C_{eq} were estimated by visually changing the lower line between reasonable limits of uncertainty.

When C_{eq} values at various pressures were plotted in Fig. 4, it became apparent that it, as well as Fig. 2, had an inflection at 1500 kg/cm². Even though those plots broke in opposite directions, the actual changes in the volumes, ΔV_{av}, were the same in sign and approximately the same in size. The values for the conductometric ΔV_{av} were +11.4

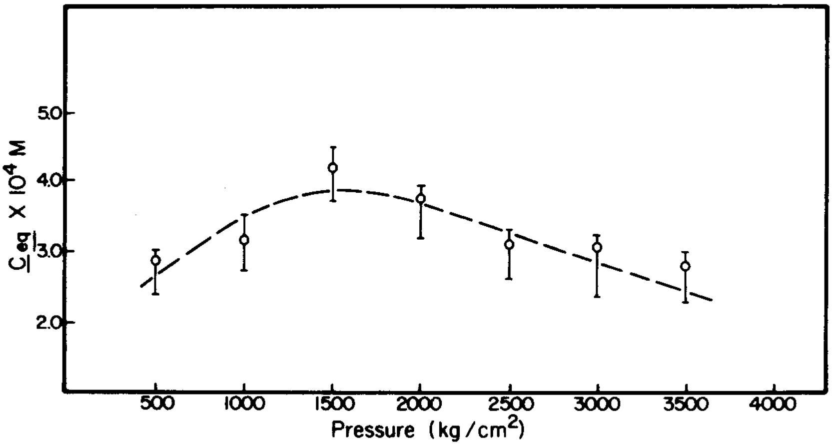

FIG. 4. The variation of C_{eq} with pressure.

cm^3/mole below 1500 kg/cm^2 and −10.6 cm^3/mole above that pressure as compared with +5.1 and −8.6 cm^3/mole from chromatography. Because of these similarities, it appeared that the equilibrium shift, which made the major contribution to pressure-induced chromatographic changes, occurred in the bulk solution.

One of the possible causes of the above changes could be a shift in the ionization of MO and/or the possibility of ion pairing (*16, 17*). In view of the fact that the negative log of the ion product of water changes only from 14.00 (at atmospheric pressure) to 13.35 (at 2000 kg/cm^2) (*18*), it was ruled out as a possible contributor. However, carbon dioxide might lead to greater changes in pH, especially under pressure, though its presence in more than trace amounts was highly unlikely. As a simple, qualitative test for the effect of dissolved carbon dioxide, a Dry-Ice-saturated solution and a solution that had been degassed, both thermally and by nitrogen, were compared as eluents for MO at various pressures. There was no significant difference in the retention volumes on the Davison 58 gel. As a result, carbon dioxide was ruled out as a cause for the large shifts in adsorption with pressure.

Effects of Other Eluents on Adsorption Chromatography

Under atmospheric pressure, MO was not noticeably adsorbed on a 0.625-cm i.d. × 30.5 cm column of Davison Code 58 silica gel, 104–124 μ

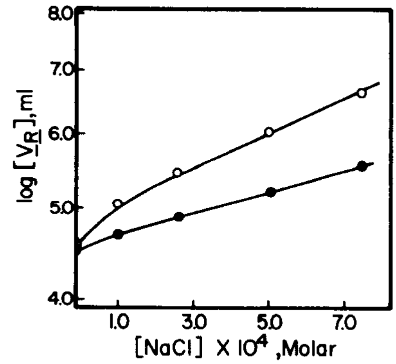

FIG. 5. The effect of sodium chloride concentration in the eluent upon the retention volume, V_R, of methyl and ethyl orange. Column length: 30.5 cm. Column packing: silica gel. Sample size: 5 μl (5.0 × 10^{-5} *M*). Pressure: atmospheric. (●) Methyl orange. (○) Ethyl orange.

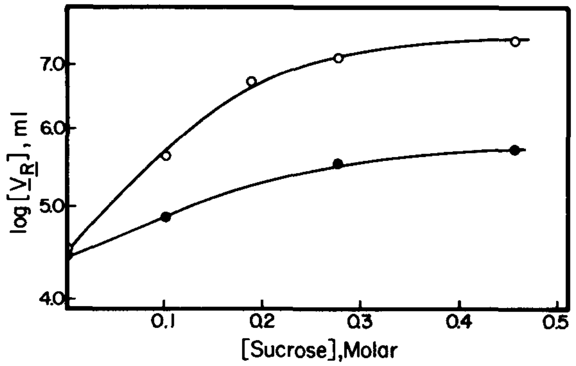

FIG. 6. The effect of sucrose concentration in the eluent upon the retention volume, V_R, of methyl and ethyl orange. Column length: 30.5 cm. Column packing: silica gel. Sample size: 5 μl (5.0×10^{-5} M). Pressure: atmospheric. (●) Methyl orange. (○) Ethyl orange.

in the presence of ethyl alcohol or dioxane at concentrations of 0.01 or 0.1 mole fraction. However, the retention volumes for MO and EO increased as shown in Figs. 5 and 6 when dilute aqueous solutions of sodium chloride or concentrated solutions of sucrose were used as eluents. Each of these two "inert" solutes increased the adsorption. It is especially interesting to note that the presence of a second solute in the aqueous eluent increased the adsorption rather than reducing it as a result of competition for the active sites on the adsorbent.

Steric-Exclusion Chromatography

Highly unusual behavior was observed when solutions of MO were chromatographed near atmospheric pressure using a 0.625-cm i.d. × 30.5 cm column of CPG-370. MO was completely excluded. It was almost totally excluded from the larger pores of a similar column of CPG-700. However, it was only partially excluded using a comparable column of CPG-1250, which had a molecular-weight range of 40,000 to 550,000. Clearly, the MO behaved as though it were a very large species. The calibration of the column is shown in Fig. 7.

The retention volume of MO appeared to change slightly as a function of sample concentration. In order to observe better the concentration dependence, a longer column, 0.625 cm i.d. × 91.5 cm, was packed with CPG-1250. In all cases the peak shapes of MO were Gaussian, and partial penetration of the pores occurred. Although there was no noticeable dependence of retention volume on sample size in the range of 10 to 30 μl, there was a dependence upon sample concentration as shown in Fig. 8. Since the amount of dye injected was of the order of 10^{-9} moles, an overload effect was unlikely. When an attempt was made to check the results using a column of Sephadex, strong adsorption of the dye ruled out any comparison.

Other possible causes for early elution from porous silica could have been charge repulsion, adsorption on colloidal particles of an impurity, or micelle formation. Charge repulsion has been reported to hinder molecular diffusion into pores as indicated by early elution of some ionic phosphates on Sephadex G-25 (*9*). However, that report showed clear evidence of repulsion in the form of peaks that were badly skewed in the forward direction. No such evidence was found in the present

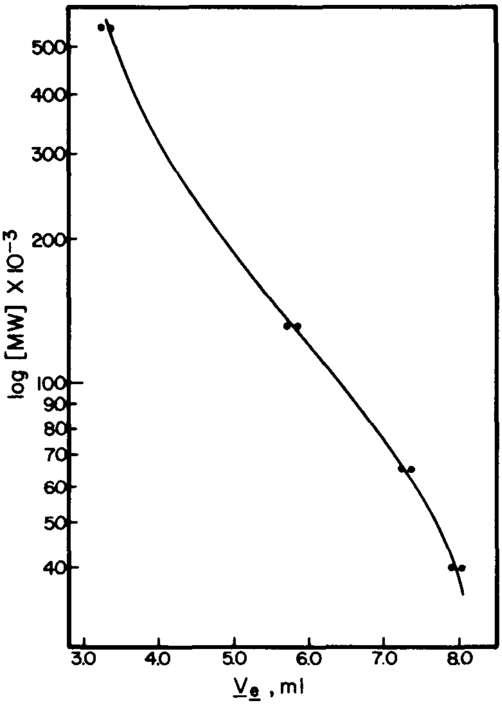

FIG. 7. Calibration curve for the steric-exclusion column. Column length: 30.5 cm. Column packing: CPG-1250.

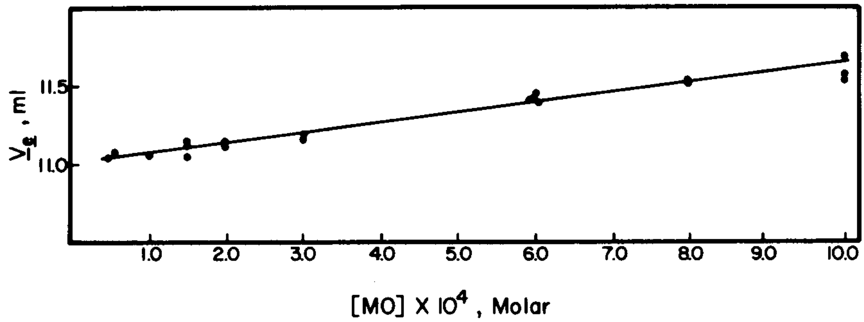

FIG. 8. The effect of methyl orange concentration in the sample upon the elution volume, V_e, of the dye. Column length: 90.5 cm. Column packing: CPG-1250 (V_o = 10.4 ml, V_t = 18.0 ml). Sample size: 30 μl. Pressure: atmospheric.

study. Also, the ratio of the pore diameter of gel to the diameter (molecular weight) of the solute appeared to be too high for a charge effect to be found in the present case.

However, to check that conclusion, other species were eluted. One species, sodium benzenesulfonate, was charged while the other species, DMO, has the same structure as MO but lacks the sulfonate group. The experiments were run on the 30.5-cm column of CPG-1250 (V_0 = 3.50 ± 0.5 ml; V_t = 8.00 ± 0.05 ml) because the solubility of DMO was approximately 10^{-6} M and detectability on the longer column would have been marginal. Even then, in order to insure detection of the DMO peak by the stabilized photometer, a minimum of 100 μl was used on the shorter column. The molecule was almost entirely excluded as indicated by its V_e of 4.0 ml. (V_e for MO was 4.7 ml.) Hence, the charge on MO was not making a significant contribution to its exclusion.

The charge effect was also tested directly using sodium benzenesulfonate. The detectability of sodium benzenesulfonate on the refractometer required a minimum of 5 μl of 1×10^{-3} M. It eluted at the total volume. Therefore, the data indicated that the small elution volume of MO was not the result of a charge–repulsion phenomenon.

Tests for Colloidal Adsorbents

Sources of a colloidal impurity upon which the dye could adsorb were silica from the column or organic polymers from the deionizing bed through which the distilled water had passed. For that reason the deionized-distilled water was redistilled before use as eluent. Again, negative results were obtained and they indicated that organic polymer from the ion exchanger was not a factor.

As a test for silica, the distilled-deionized water, which was used as the eluent, was analyzed both before and after passage through the column of CPG-1250 porous glass beads. This analysis showed no detectable silica (less than approximately 0.05 ppm) in the water before or after passage through the column.

Effects of Solvents in Steric-Exclusion Chromatography

Another check upon the explanation based upon a colloidal silica impurity was made using 0.01 and 0.1 mole fraction ethyl alcohol–water solutions as eluents for exclusion chromatography. As mentioned earlier, the presence of ethyl alcohol eliminated adsorption of MO by a conventional silica-gel column. Hence, if a silica impurity were adsorbing

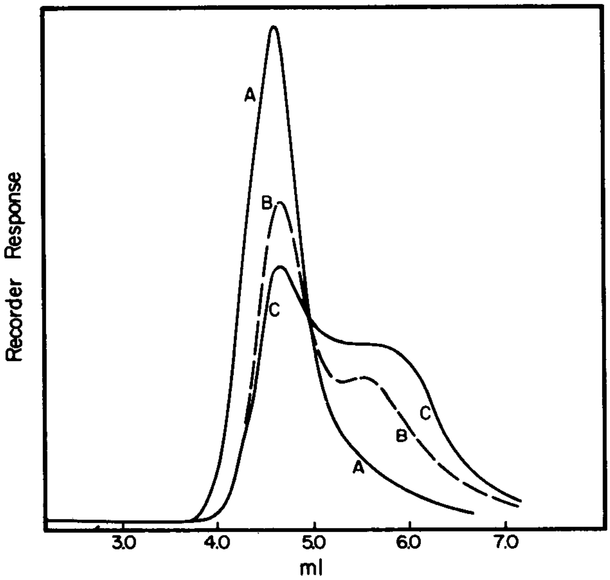

Fig. 9. The effect of dioxane concentrations in the eluent upon the elution profile of methyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 5 μl ($5.0 \times 10^{-5} M$). Pressure: atmospheric. Eluents: (A) 0.01 mole fraction dioxane (identical to water), (B) 0.1 mole fraction dioxane, and (C) 0.8 mole fraction dioxane.

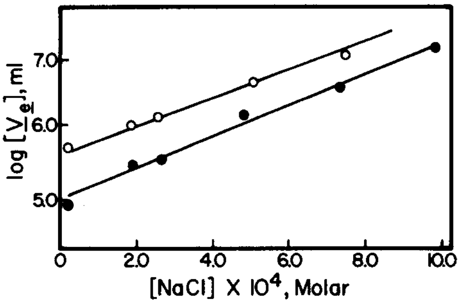

FIG. 10. The effect of sodium chloride in the eluent upon the elution volume, V_e, of methyl and ethyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 5 μl (5.0×10^{-5} M). Pressure: atmospheric. (●) Methyl orange. (○) Ethyl orange.

the dye onto its large aggregates, the presence of alcohol should eliminate adsorption and make the MO behave as a small species. This was not found to be the case. In fact, there was no noticeable effect upon the elution volume of MO or EO in the presence of 0.1 mole fraction alcohol.

Dioxane, on the other hand, behaved differently in steric-exclusion experiments. A 0.01-mole fraction dioxane–water eluent exhibited no effect, but a 0.1-mole fraction dioxane–water solution induced the formation of a second species of MO as implied by the shoulder on the elution curve in Fig. 9. For 0.8 mole fraction dioxane–water eluent, the shoulder increased while the primary peak decreased. The shoulder could not have been due to adsorption because dioxane eliminated chromatographic adsorption, as reported earlier. Nevertheless, the already unusual exclusion behavior of MO was undergoing a further unexpected change.

In an effort to determine whether an impurity or a second form of MO was being separated, the peaks of MO were collected separately, and a visible spectrum of each was taken. For a given concentration of dioxane eluent, each peak and shoulder had the same spectrum. Hence, there might have been a moderately slowly equilibrating mixture which was being partially separated on the column. However, there was a difference between the spectra recorded in 0.1 mole fraction and those in 0.8 mole fraction dioxane–water. Further discussion of the spectroscopic behavior of MO will be postponed until a later section.

Dilute solutions of sodium chloride also had a noticeable effect on the elution volumes of the azo dyes. Unlike the dioxane system, there was only one peak observed. Furthermore, Fig. 10 shows that, as the salt

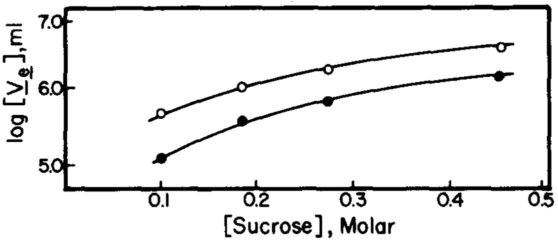

FIG. 11. Effect of sucrose concentration in the eluent upon the elution volume, V_e, of methyl and ethyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 5 μl (5.0 × 10^{-5} M). Pressure: atmospheric. (●) Methyl orange. (○) Ethyl orange.

concentration increased, the elution volume also increased. Sucrose had a similar effect upon the elution volume (Fig. 11), but the concentration of sucrose necessary to obtain an equivalent effect was significantly higher than that for sodium chloride.

Effect of Temperature

As the temperature of the aqueous eluent was increased, the elution volume decreased as shown in Fig. 12. This indicates that the apparent size of MO increased noticeably at the higher temperature.

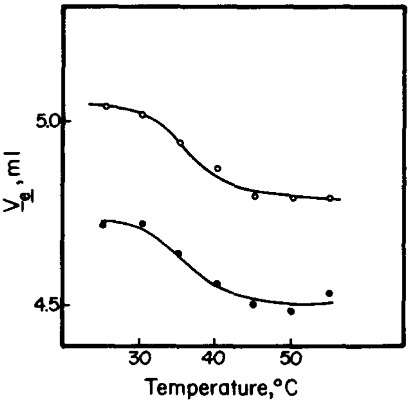

FIG. 12. Effect of temperature upon the elution volume, V_e, of methyl and ethyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 5 μl (5.0 × 10^{-5} M). Pressure: atmospheric. (●) Methyl orange. (○) Ethyl orange.

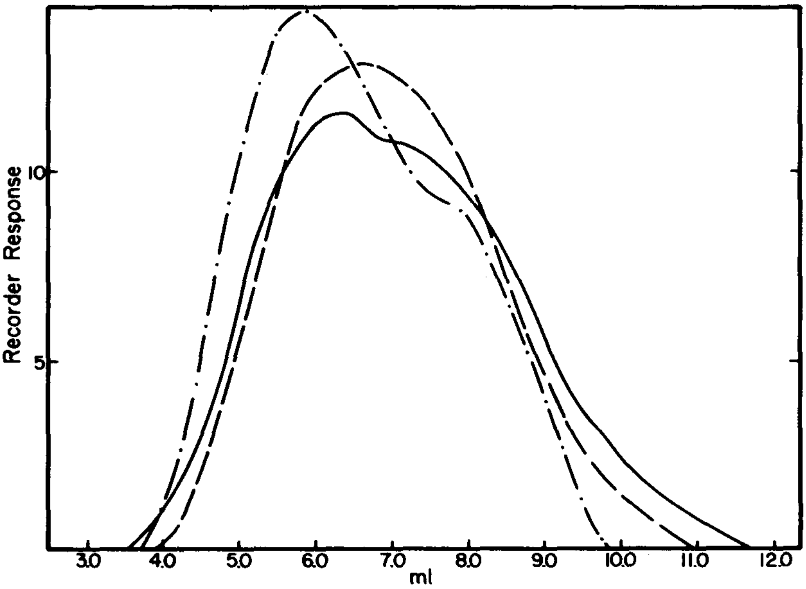

FIG. 13. The effect of pressure upon the peak shape of methyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 30 μl (7.5 × 10^{-4} *M*). Pressure: (- -) 560 kg/cm², (——) 1000 kg/cm², and (–•–) 1400 kg/cm².

High-Pressure Steric-Exclusion Chromatography

MO and EO were eluted from a 0.625-cm i.d. × 30.5 cm column packed with CPG-1250 porous glass beads. The total internal volume of the column was 9.8 ± 0.1 ml and the interparticle volume was 4.0 ± 0.1 ml. The elution profiles for the individual azo dyes are given in Figs. 13–16. Both MO and EO had Gaussian shapes at 500 kg/cm² as they did when eluted near atmospheric pressure, and there was no forward tailing. As the pressure was increased, a shoulder appeared on the peak for each solute and became especially noticeable above the region 1450–1550 kg/cm². Above 1500 kg/cm², the shoulder became more distinct and moved toward smaller molecular weights with increasing pressure. Both the peak and the peak shoulder were collected, and the visible spectrum of each determined. For each dye, the two constituent peaks had the same spectrum which, in turn, was characteristic of that dye.

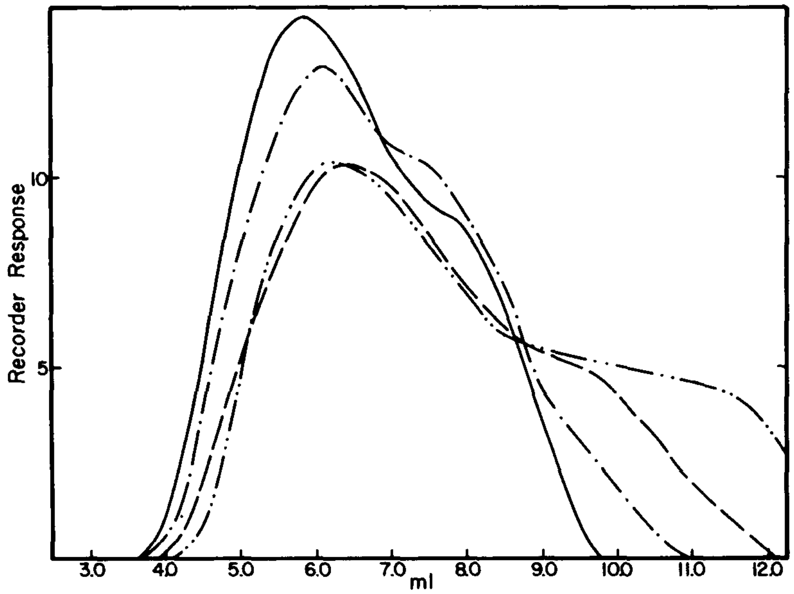

FIG. 14. The effect of pressure upon the peak shape of methyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 30 μl (7.5 × 10^{-4} *M*). Pressure: (–•–) 1400 kg/cm^2, (——) 1800 kg/cm^2, (– –) 2100 kg/cm^2, and (–••–) 2800 kg/cm^2.

At pressures above 560 kg/cm^2 where a shoulder was definitely present, each elution curve could be deconvoluted on the assumption that two Gaussian peaks were present. The peak maxima were reproducible within ±0.33 ml, the average reproducibility being approximately ±0.10 ml. The peak areas were reproducible within ±10 units. The best deconvolution fit came at 1400 kg/cm^2. As shown in Table 1 for MO, the general trend in peak maxima was an increase in V_R with pressure. The second peak was moved the more significantly.

The changes in the peak shapes observed at high pressures were similar to those brought about by dioxane. Clearly, for both MO and EO, physicochemical changes were manifested from perturbations in the bulk solution.

Visible Spectroscopy

If micelles were present in solutions of MO and EO, they might possibly be the reason for the high-pressure chromatographic behavior.

However, MO and EO were found to obey Beer's law from 1.0×10^{-6} to 1.0×10^{-3} *M* at atmospheric pressure, and this confirmed an earlier statement that MO and EO do not form micelles (*19*). On the other hand, butyl orange, which is known to form micelles (*19*), was found to deviate from linearity at 8×10^{-5} *M* with the peak maximum exhibiting a red shift. Hence, the existence of micelles does not appear to be the reason for the unusual behavior of MO.

In dioxane solutions, changes in the spectrum of MO occurred as shown in Fig. 17. The concentrations of MO were chosen to illustrate

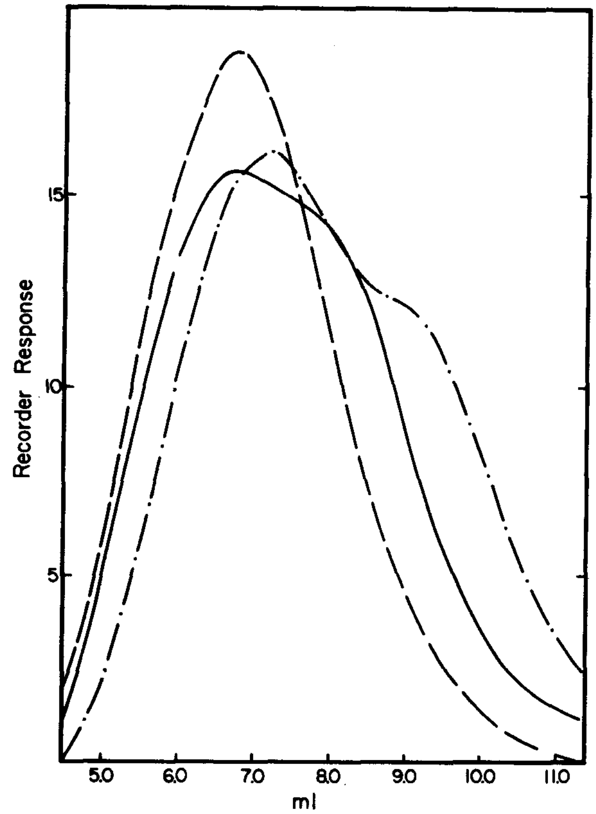

FIG. 15. The effect of pressure upon the peak shape of ethyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 5 μl (7.5×10^{-4} *M*). Pressure: (– –) 560 kg/cm², (——) 1000 kg/cm², and (–•–) 1400 kg/cm².

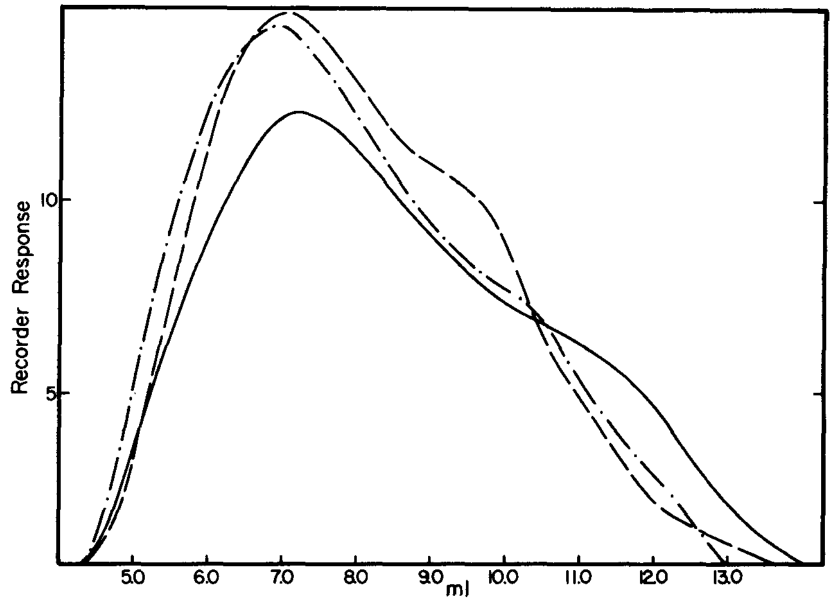

FIG. 16. The effect of pressure upon the peak shape of ethyl orange. Column length: 30.5 cm. Column packing: CPG-1250. Sample size: 5 μl (7.5 × 10^{-4} *M*). Pressure: (- -) 1800 kg/cm², (-•-) 2100 kg/cm², and (——) 2800 kg/cm².

the trends without seriously overlapping. Note that as the dioxane concentration was increased, the wavelength of the maximum shifted to the blue region and the molar absorbance decreased.

DISCUSSION

The improvement of selectivity in chromatography has previously been accomplished by changing the chemical system or the temperature. However, pressures of the magnitude used in the present study provide another major variable. With the use of only pressure, it was possible to obtain faster, improved resolution of MO and EO on both large and very small adsorbent particles. The ease of controlling or changing pressure should be a significant advantage in adsorption chromatography compared to the use of different solvents, a procedure which usually requires lengthy reconditioning between runs.

TABLE 1

Comparison of Elution Volumes for the Deconvoluted Peaks of Methyl Orange at Various Pressures

Pressure (kg/cm^2)	V_{e1} (ml)[a]	% of Total elution curve	V_{e2} (ml)[a]	% of Total elution curve
1000	5.66 ± 0.08 (6)	50 ± 6	7.66 ± 0.12 (6)	50 ± 6
1400	5.76 ± 0.06 (5)	59 ± 5	8.22 ± 0.17 (5)	41 ± 5
2100	6.06 ± 0.10 (4)	69 ± 7	8.63 ± 0.25 (4)	31 ± 7
2800	5.94 ± 0.10 (5)	57 ± 10	8.67 ± 0.33 (5)	43 ± 10
3500	6.05 ± 0.10 (3)	58 ± 10	8.82 ± 0.35 (3)	42 ± 10

[a] Values in parentheses indicate the number of data points used to calculate the standard deviation.

In order to understand better the influence of pressure on the molecular interactions, the azo dye–silica system was studied in detail. The changes in the distribution ratio did not appear to be the result of a change in acidity due to the presence of trace carbon dioxide or to the slight increase in the ionization of water with pressure. A major change in pH would have been required to alter the extent of ionization of MO ($pK = 3.2$) or EO ($pK = 3.3$) and thereby change their adsorption equilibria. However, the unusual data from the steric-exclusion experiments plus data from experiments in which eluents other than water were used have suggested other possibilities.

Recall that at atmospheric pressure the apparent sizes of MO and EO were a thousand times larger than one ion of dye. As the concentration of the dye increased, the apparent size decreased. However, since the spectral absorbances of MO and EO were linear with concentration upon to 10^{-3} M, the presence of an agglomerate or micelle is not a likely cause of the two anomalies.

One obvious explanation could be based upon the presence of colloidal impurities upon which the dyes could be adsorbed. If that were the case, as the concentration of dye increased the relative amount of adsorbed dye would decrease, and the apparent size would also decrease. The presence of salt or sucrose would be expected to displace the adsorbed dye and lead to changes in the retention volume of the dye. That line of reasoning has several shortcomings. First, the use of distilled-deionized-redistilled water should eliminate any impurity from the ion exchanger as well as any silica that was present before redistillation, but it did not. Second, the presence of silica was found to be insignificant in the aqueous eluent (distilled-deionized water) before and after elution through the exclusion column. Also, it seems unlikely that MO would adsorb onto trace silica in the eluent and not onto the silica packing in the column, the most likely source of silica. Third, ethyl alcohol solutions, which desorbed MO from silica in an adsorption column, had no effect upon the elution volume of the dye in exclusion chromatography. However, those eluents which increased the adsorption of dye onto a silica column (sodium chloride and sucrose), also decreased the apparent size of the dye. Therefore, adsorption of MO and EO by trace colloidal impurities does not satisfactorily account for the size anomaly.

An explanation based upon hindered diffusion into the pores of the column due to charge repulsion was also examined and found not to apply: the absence of peak-skew of the front edge of the MO or EO peaks; the fact that the pore size was several orders of magnitude larger than the ion; and finally, DMO which is an uncharged MO (without the sulfonate group) behaved like MO, whereas benzenesulfonate ion entered the pores, as evidenced by its large retention volume.

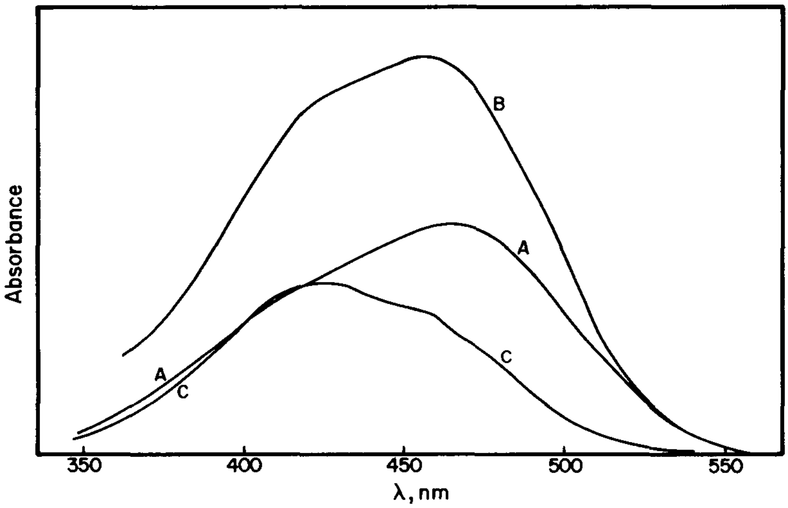

FIG. 17. The effect of solvent upon the visible spectrum of methyl orange. (A) Methyl orange (4×10^{-5} *M*) in water. (B) Methyl orange (8×10^{-5} *M*) in 0.1 mole fraction dioxane. (C) Methyl orange (4×10^{-5} *M*) in 0.8 mole fraction dioxane.

Another laboratory has reported evidence that also recently points to to a size anomaly. Using a solution containing a high concentration of electrolyte that would minimize charge repulsion by Sephadex, Ueno et al. (*20*) have observed changes in the elution volume of several oxo acids of phosphorus. They suggested that the elution change was due to a variation in the hydration of the ion. An extension of this concept may be applied to the present experimental data.

Before discussing a possible explanation of the data, it will be helpful to review the behavior of MO under pressure. In both chromatographic and conductometric experiments, ΔV_{av} was found first to be positive and then to reverse its sign at approximately 1500 kg/cm^2. This indicates that the lower-pressure form of MO had a smaller compressibility than the higher-pressure form. This type of behavior has also been found for the variation of critical micelle concentrations for a number of long-chain aliphatics (*14*, *21*) and other micelle-forming species (*22*, *23*). To account for the change in sign of the partial molar volume, explanations have been offered that the positive ΔV_{av} results from the loss of bound water in the hydration sphere of the ion (*14*, *23*) and the negative value results from internal freezing of the micelle at high pressures (*14*). A reversal in the sign of ΔV_{av} has also been found in the phase diagrams of hydrophobic solutes, such as nicotine and 4-methylpiperidine, in dilute aqueous solutions (*24*). All of these reversals in sign have occurred in the pressure region of 1500–2500 kg/cm^2. In brief, these systems have involved a variety of organic solutes in water. While similar changes may indeed be taking place in all of the solutes, it seems more reasonable to suspect the water.

For the present discussion, each azo dye is considered to be a highly hydrated monomer. In order to explain the steric data, the "hydration shell" would have to be relatively stable as well as very large. While such a model may not be appealing at first, there is evidence in the literature which supports it (*25–32*). First, when a hydrophobic molecule such as a hydrocarbon is dissolved in water, the limit of solubility is very low; there is a high positive free energy that results from a large decrease in the entropy and an enthalpy change which is zero or slightly positive (at room temperature). The large negative entropy of solution implies that a lattice-ordering of water occurs around the solute (*25*). The resulting structural entities are envisioned not as stable, frozen chunks, but are referred to as flickering clusters (*26*). The flickering cluster is a dynamic situation with the vicinal water structure exhibiting various changes. This structure was first believed to be similar to the tetrahedral arrangement of ice and was referred to as the "iceberg" surrounding a hydrocarbon. Recently, however, the structure has been shown to be different from ice (*32*). Terms such as "hydration of the second kind" (*33*) and "β form" (*34*) have been used to refer to and distinguish it from ice structure. The β-form is stable with respect to temperature and pressure (*34*). The size of the β-form cluster is not known, but it is believed to extend many layers into the bulk water. In fact, the structure of water at many aqueous interfaces, which is believed similar to the β-form, has been reported to range from 50 Å on clay surfaces up to 0.1 μ on mica (*35*).

MO and EO are primarily large hydrophobic species so the existence of hydration is very likely. If the dye served as a stabilizing nucleus for a structural form of water, it would explain the large molecular size in the steric-exclusion chromatography. The size of the hydrated monomer of MO was calculated utilizing Ackers equation (*12*) and the specific pore size of CPG-1250 equal to 1310 Å ± 10% as reported by the manufacturer. Assuming that one layer of water is 2.76 Å thick (*41*), the calculated size indicates that approximately 85 layers of water surrounded one molecule of dye. This figure indirectly reflects the fact that the probability for entrance into a pore falls very rapidly once the diameter of the diffusing species exceeds 0.1–0.2 of the pore diameter (*44*). The concept of hydration spheres has been extended by Diamond (*36*) who applied it to long-range cluster-ordering by ions. He stated that if an ion ties up an increasing number of water molecules in its coordination shell as the concentration of the ion increases, there will be less and less "free" water. Hence, it will become increasingly difficult to complete the coordination shell for all of the ions. This concept has also been used to explain changes in activity coefficients for solutes (*37*, *38*).

The decrease in apparent size of MO with an increase in its concentration and the effects of sodium chloride and sucrose are consistent with this viewpoint in that competition for water would be involved. The ionic salt distorts the water structure more readily due to electrostriction and, hence, is effective at lower concentrations. Because higher concentrations of these solutes favored greater adsorption of MO and EO, it appears that adsorption was inversely related to the hydrated size. On the other hand, the effect of dioxane, which is a water-structure breaker, is believed to favor the formation of ion pairs as the dielectric constant drops (*39*, *40*).

Increased temperature caused the apparent size of the dyes to increase. This effect is difficult to accept since it is known that the structure of bulk water breaks down at higher temperatures. However, the observation that the size increased somewhat with temperature does agree with the statement that the β-form is stable with respect to temperature (*34*). Even more interesting is the striking similarity between the decreasing curves for temperature versus elution volume of the dye systems and that for temperature versus surface tension of the *n*-hexane–water interface as reported by Franks and Ives (*30*). If a similarity exists between the water structured in the β-form and that in the interface, the two phenomena may imply that the hydration sphere of MO expands as the temperature increases but not enough to break the hydration cluster.

The concept of the hydrated monomer can also be applied to the high-pressure behavior of the MO (and EO) where it appears that more than one form of each dye existed. In high-pressure experiments that involved chromatography and conductance, an equilibrium mixture appeared to be present. Because the changes in the average molar volume, ΔV_{av}, with pressure were roughly the same for both sets of experiments, we have attributed the major contribution to pressure-induced changes in an equilibrium in the bulk solution.

The question that remains is the nature of the change. The peak shapes for MO under high-pressure were very similar to those obtained at one atmosphere for dioxane–water mixtures. Hence, if dioxane leads to formation of an ion pair, the high pressures may be doing the same (*16*, *36*). The difference between the high pressure-induced and dioxane-induced ion-pairs would then be only the source of the driving force. As has been pointed out, the dioxane-induced form is now commonly attributed to electrostatic attraction (*36*), while a structure-enforced ion-pair is aided by the water itself in trying to minimize the disturbance to its structure (*16*, *36*). High pressures force large cations or anions to ion-pair (structure-enforced type) and the ion-association results in a volume decrease (*16*, *36*). The idea of a structure-enforced ion-pair parallels one by Wen and Sato (*42*) who proposed "structural salting-in" of large tetraalkylammonium ions in certain cases.

Another phenomenon, which may also be occurring under pressure, is destruction of the bulk-water structure. Above 1500 kg/cm^2, the bulk structure appears to collapse, as evidenced by plots of viscosity versus pressure (*43*). Since ΔV is made up of contributions by both the solute and solvent, both destruction of the bulk water and structure-enforced ion-pairing could be important. A pressure increase could force the cation and anion together into a single cavity (ion-association) in order to decrease the disturbance to the bulk water. This association should release a considerable amount of bound solvent into the bulk solution, so that the transition to the ion-paired state would increase the volume of the solution. At pressures of 1500 kg/cm^2 and above, where it has been suggested that the bulk water is destroyed, ion-association is highly favored but can only be accomplished by rearrangement of the tightly packed water and a resultant decrease of the average volume. This explanation is also consistent with the concept of a stable hydration sphere and its behavior with respect to pressure.

In conclusion, the present experimental framework offers substantial evidence which corroborates the role of water as an active participant in high-pressure chromatography. Although the characteristics of the MO– and EO–silica systems appear, for the most part, to be consistent with such a picture, work must be done on other systems to determine the generality of the behavior. However, even in the present primitive state of knowledge, the practical use of pressure-induced changes in adsorption chromatography appears to be promising.

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We wish to thank W. L. Marshall and L. B. Yeatts of Oak Ridge National Laboratory who made available their equipment for high-pressure conductance measurements. We are indebted to L. A. Hanlein of Corning Glass Works for supplying the porous glass beads. Financial support from the U.S. Atomic Energy Commission under Contract AT(11-1)-1222 is gratefully acknowledged.

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