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Sorption Effects in Gel Filtration: I. A Survey of Amino Acid Behavior on Sephadex G-10

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Summary

1. Sephadex G-10, as supplied by the manufacturer, behaves as a weak cation exchanger. The cation-exchange capacity can be nearly eliminated by washing the gel with 1 *M* aqueous pyridine.

2. The behavior of amino acids and other small solutes on pyridine-washed columns under defined conditions is very reproducible.

3. The behavior of a solute on Sephadex G-10 fundamentally depends on its effective molecular size. Charged solutes tend to be excluded in media of low ionic strength, but the effective size and hence the degree of exclusion of such molecules can be decreased substantially by including high concentrations of small electrolytes in the medium.

4. Salt can increase the adsorption of potentially adsorbable charged molecules in a passive way by increasing the amount of gel available to them. Salt might actively promote the adsorption of hydrophobic molecules by causing them to seek out regions within the gel where there is no salt, e.g., in the immediate vicinity of the gel matrix.

5. Any pair of small water-soluble molecules that differ with respect to size, charge, content of delocalized π electrons, or hydrophobic character (e.g., the length of a linear hydrocarbon chain) should be separable on Sephadex G-10. The gel should therefore be very useful for purifying small water-soluble synthetic products.

The molecular-sieving properties of Sephadex and several other types of hydrophilic xerogels, both natural and synthetic (*1*), have been exploited with enormous success by the use of the simple gel filtration technique in the few years that have elapsed since Porath and Flodin (*2*) first reported that separations based on differences in molecular size could be achieved by elution chromatography on

columns packed with swollen grains of cross-linked dextran gels.

The gel filtration behavior of many globular proteins and various oligo- and polysaccharides on Sephadex gels of different water regain are accurately accommodated by one or more of four derived mathematical expressions relating the elution volume of a molecule, expressed in some reduced form, to its effective molecular radius, i.e., usually the diffusional Stoke's radius or, in the case of flexible homopolymers, the calculated radius of gyration. The simple relationships of Squire (*3*), Porath (*4*), and Laurent and Killander (*5*) all involve the assumption that the partition of the solute between the interstitial liquid of the column and the imbibed solvent present in the contiguous gel grains is determined entirely by steric hindrance to diffusion involving perfectly elastic collisions between the solute and the hydrated gel matrix. The different forms of the equations reflect the use of different assumptions regarding the nature and geometry of the spaces or elements responsible for the differential exclusion effect. Ackers (*6*) has proposed that a somewhat more complicated expression based on a model in which frictional drag as well as steric hindrance to diffusion is considered must be used to account accurately for the behavior of large solutes on very loose gels such as Sephadex G-200.

Siegel and Monty (*7*) have recently reported that some elution data obtained on Sephadex G-200 for a number of proteins with molecular radii ranging from about 20 to more than 100 Å fit all the above-mentioned expressions, except that of Squire, equally and remarkably well. Apparently the molecular radii of proteins that do not adsorb to the gel can be estimated very accurately from gel filtration data obtained on Sephadex G-200 by the use of any one of the three applicable expressions.

Although the agreement between observed elution behavior and the "ideal" behavior defined by one of the above-mentioned sterical models is best in the case of gels of high water regain, the expressions of Porath (*4*) and Laurent and Killander (*5*) also seem to work well for some solutes on such tightly cross-linked gels as Sephadex G-25. In fact, the former expression was derived specifically to account for the elution behavior of cellodextrans (*8*) on G-25. Smooth, inverse relationships between elution volume and molecular size have been observed for such simple neutral homologs as lower oligomers of ethylene glycol* and lower polyols, even

* Descriptive pamphlet on Sephadex G-10 and G-15, Pharmacia.

on the very dense Sephadex G-15 and G-10 gels and the similar DVS-9 dextran gel studied by Marsden (*9*). Apparently, all the Sephadex gels, from G-200 to G-10, can, under suitable conditions, function as passive molecular sieves for certain types of molecules. The fact that the Laurent-Killander model also works for polyacrylamide gels (*10*), which are totally different chemically from the dextran gels, indicates that the sieving properties of hydrophilic xerogels in general can be explained in terms of physically meaningful steric parameters of the matrix without reference to its particular chemical nature.

Although none of the simple sterical models upon which the theoretical expressions discussed above are based can possibly be completely accurate with regard to the precise microscopic mechanism of the sieving process, the wide applicability of the expressions themselves seems to provide ample theoretical justification for the use of suitably calibrated gel filtration columns to estimate the sizes and/or molecular weights (*11*) of molecules, provided, of course, that nonsterical solute-gel interactions are absent. The reliability of such estimates are limited in practice by the fact that nonsterical interactions often do occur, and the dilemma is that the departures from "ideal" behavior that result from such interactions are sometimes difficult to recognize unless the size and shape of the molecule are already known.

Departures from ideal behavior can be either positive or negative, since nonsterical exclusion and adsorption processes can cause a molecule to elute either earlier or later, respectively, than it would in their absence. One fairly simple and sometimes effective means of testing the involvement of nonsterical effects is afforded by the fact that the elution volume of a molecule whose elution behavior is governed by steric factors alone should be insensitive to changes in conditions that do not change the shape or state of aggregation of the molecule or alter the water regain of the gel. Certain types of nonsterical effects can thus be detected by manipulating the ionic composition or pH of the medium. If the molecule has a very rigid structure or if the preservation of native structure is not essential, more drastic tests can be applied. Synge and coworkers (*12,13*) have shown that the elution behavior of peptides and proteins that are prone to adsorb to Sephadex can be "normalized" by performing the gel filtration in a solvent composed of equal parts of phenol, acetic acid, and water. Apparently, this medium abolishes almost all solute-solute and solute-gel interac-

tions other than those involved in the sieving process. Since this solvent alters the sieving range of the gel, the column must be calibrated in the same medium if size estimates are to be made. Carnegie (*14*) has reported that the molecular dimensions of many peptides with molecular weights in the range 400–2000 can be determined accurately by gel filtration on Sephadex G-25 in the above medium. Presumably, strong urea solutions could be used for the same purpose (*15*).

Although it thus seems possible to conduct gel filtration experiments under conditions where adsorption almost certainly does not occur, such conditions are likely to be far too drastic for many applications. Unless some completely reliable means of detecting adsorption can be developed, size estimates derived from gel filtration data obtained in simple aqueous media for substances containing structures favorable for adsorption (e.g., polypeptides and proteins containing aromatic amino acids) must always be confirmed by other methods.

Ion exclusion and adsorption effects often cause solutes to behave in a way that cannot conceivably be explained on the basis of steric factors alone, and in such cases their involvement is obvious. For example, any substance whose gel filtration behavior is solely determined by sterical selection processes must show an elution volume less than the total volume of solvent present in the column. A K_d greater than 1 is thus unequivocal evidence for the involvement of adsorption. In fact, since some of the water imbibed by a hydrophilic xerogel is undoubtedly bound firmly to the gel matrix as a hydration layer and is therefore not available as solvent to other molecules, the limiting K_d value for a molecule that does not itself "solvate" or adsorb to the gel must always be somewhat less than 1. Lathe and Ruthven (*16*) reported that wet starch contains 0.25 g of hydration water per gram, and in view of the obvious chemical similarity between the two substances it seems reasonable to assume that Sephadex is hydrated to the same extent. Accordingly, the "limiting" K_d values for Sephadex G-10, G-15, G-25, and G-50 would be 0.75, 0.83, 0.90, and 0.95, respectively.

The occurrence of nonsterical interactions between certain types of solutes and the gel matrix was unambiguously demonstrated in two early exploratory investigations of the sorption properties of Sephadex (*17,18*). Gelotte (*18*) divided the nonsterical effects into two categories on the basis of different underlying mechanisms.

In the first category are the direct electrostatic exclusion and adsorption effects that occur with anionic and cationic substances, respectively, and which are caused by the small number (10–30 μeq/g) of fixed carboxyl groups that are present in the gel matrix. In distilled water or buffers of very low ionic strength, the repulsive and attractive electrostatic forces operate over long distances and can therefore influence or dominate the behavior of a charged molecule of any size. Very small anions can thus be completely excluded, while cationic solutes of sizes well above the sterical exclusion limits of the gel can be bound and retained. Since all the Sephadex gels seem to have about the same carboxyl content, the charge density in the swollen gels is roughly inversely proportional to the water regain. The effects are therefore most pronounced with the tightest gels. An ionic strength of 0.05 or less is adequate to eliminate electrostatic effects with small monovalent ions on G-25, but higher salt concentrations might be required to prevent electrostatic binding of very basic polypeptides and proteins (*19*). These electrostatic effects can also be eliminated by working at a pH where the carboxyl groups involved are not significantly ionized, as was done by Cruft (*20*), who used 0.02 *N* HCl as eluant for the fractionation of very basic histones on Sephadex G-75.

A distinctly different type of adsorption occurs with aromatic substances, conjugated polyenes, urea, and other solutes containing extended coplanar systems of π electrons. This type of adsorption has not so far been identified with any particular "sites" in the gel structure, and possibly involves some type of generalized molecular orbital interactions. This short-range "aromatic" adsorption requires intimate contact between the solute and the gel and is therefore usually observed only with penetrant species.

Another type of adsorption has been proposed recently by Marsden (*9*) to account for the fact that the K_d values obtained for series of normal aliphatic alcohols and alkane diols on a very tightly cross-linked dextran gel increased with increasing length of the unbroken series of methylene groups in the hydrocarbon chain. This aliphatic or "hydrophobic" adsorption occurs with a positive enthalpy change, indicating that such substances, unlike aromatic ones where the enthalpy change is negative, have no real affinity for the gel matrix. Apparently the water in the immediate vicinity of the dextran chains differs in structure from the water elsewhere

in the gel and the hydrocarbon molecules are more readily accommodated by the former.

The interplay of nonsterical interaction and fundamental sieving effects in gel filtration is not confined to the cross-linked dextrans. All the hydrophilic xerogels that have been tested extensively so far in this application seem to be at least qualitatively similar to Sephadex in their adsorption properties, particularly with regard to the tendency to adsorb aromatic substances; and, like Sephadex, all seem to contain small amounts of charged or ionizable groups and therefore display some small capacity for ion exchange and electrostatic ion exclusion.

From the standpoint of separation work, nonsterical interactions are undesirable only when they interfere with separations that could be accomplished in their absence. Very often adsorption effects allow separations that could not be achieved on the basis of steric factors alone. The aromatic effects, and even the electrostatic effects discussed above, have already been exploited deliberately to solve particular separation problems.

The cation-exchange properties of Sephadex are of very limited value in separation work because the very low capacity generally does not provide the linear isotherms required for useful elution chromatography. However, Miranda et al. (*19*) were able to isolate some basic toxins from scorpion venom by adsorbing them to Sephadex. After eluting the nonbasic, nontoxic constituents with distilled water, the toxins were displaced with a salt gradient.

The aromatic adsorption property is considerably more useful because the isotherms are linear over a wide range of concentrations and symmetrical peaks are consequently obtainable by elution development. Perhaps the most elegant example of the use of relatively mild aromatic adsorption properties of Sephadex to solve an otherwise difficult separation problem is the work of Ruttenberg et al. (*21*), wherein the cyclic decapeptide antibiotics, tyrocidines A, B, and C, were separated on the basis of differences in tryptophan content by chromatography on Sephadex G-25 in 10% acetic acid.

Obviously, the value of hydrophilic xerogels in all applications would be increased enormously if the nonsterical effects could be manipulated or eliminated at will, according to the demands of the particular problem at hand. The common amino acids together

represent all the structural features (e.g., charged groups, aromatic rings, and aliphatic chains) that are known to be susceptible to some type of nonsterical interaction with the matrix material of Sephadex gels, and for these small molecules all such interactions should be particularly pronounced on a very dense gel. Therefore, we decided to investigate the influence of solvent environment on the behavior of various amino acids on Sephadex G-10. A great practical advantage of this model system is that very accurate elution profiles can be obtained by the use of an amino acid analyzer. Some results obtained in a general, preliminary survey are presented below.

MATERIALS AND METHODS

Preparation of Column

A 2-kg batch of Sephadex G-10 ($W_r = 0.96$) was fractionated in the dry state by air elutriation. The finest fraction obtained (75 g), which consisted of particles smaller than 40 μ in diameter, was used for the experiments described below.

The gel was suspended in 400 ml of 50% (v/v) acetic acid in a 500-ml suction flask and the mixture was degassed thoroughly with a water pump. The gel was allowed to settle and the "fines" were decanted. The procedure was repeated three times and the gel was washed once with deionized water and finally equilibrated with 0.2 *M* acetic acid containing 1% NaCl. After degassing, the gel was allowed to stand overnight to ensure complete swelling.

The gel slurry was then packed into a 1 × 150-cm Perspex chromatographic tube equipped with adjustable plungers at both ends. The column design and the packing procedure used are those described by Porath and Bennich (22). The packing medium was then displaced by passing 200 ml of degassed 0.2 *M* acetic acid through the packed gel bed, the top plunger was inserted, and the latter solvent was pumped through the column at 10 ml/hr. When the gel bed ceased to decrease significantly in height (about 2 days), the top plunger was adjusted to touch the gel surface and the chromatography experiments were begun. The packed column contained about 44 g of Sephadex.

Preparation and Application of Samples

Stock solutions of the various solutes studied were prepared in deionized water. Chromatographic samples were made up by pipetting aliquots of the appropriate stock solutions corresponding to 0.5, 1.0, or 2.0 μmoles of the desired solutes into a small conical test tube. Larger amounts (4–8 μmoles) of urea were used because of its low ninhydrin color. If ribonuclease was to be included in the run, 0.5–1.0 mg of the dry substance was added directly to the sample solution. For reasons which will become clear later, it was particularly important to apply the samples to the column in a medium as close as possible in composition to that with which the column was equilibrated. The use of dialysis for this purpose was precluded by the small size of the solutes, but satisfactory solvent matching could be accomplished in the manner illustrated by the following examples. When the solvent composition desired was 0.2 *M* acetic acid–0.5 *M* NaCl, 250 μl of 0.8 *M* acetic acid–2.0 *M* NaCl was added to the sample mixture and the volume was adjusted to 1.0 ml with deionized water. When the sample solution was required to be 2.0 *M* in NaCl (solvent medium C), 200 μl of 1 *M* acetic acid and 117 mg of solid NaCl were added and the volume was adjusted as before.

A sample volume of 1.0 ml was used in all the experiments described herein. Sometimes the sample was applied to the column directly through the roller-type peristaltic pump used for elution. Apart from the 20-cm length of elastic Tygon tubing (i.d., 1.65 mm; o.d., 3.2 mm) used in the pump, all the lines leading to and from the pump and column were polyethylene capillary tubing (i.d., 0.85 mm; o.d., 2 mm). Connections made simply by inserting the latter tubing into the former to a depth of about 5 mm withstood easily the backpressure created by the column and analytical system used when the flow rate was 10 ml/hr. Whenever samples were applied through the pump, the sample segment was bracketed between two small air bubbles (ca. 10 μl each) to ensure clean flow through the pump. Bubbles of this size always became trapped in the tiny conical space above the membrane in the plunger at the upper end of the column and did not interfere with the formation of sharp starting zones at the top of the gel bed. Since the polyethylene tubing and the Perspex plunger were transparent, the moment at which the sample zone reached the membrane could be accurately determined simply by observing the progress of the

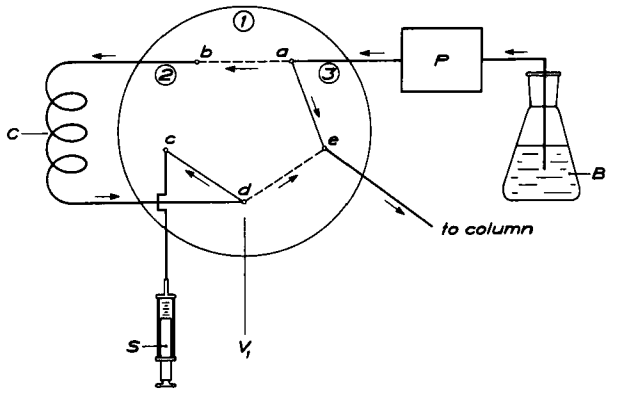

FIG. 1. Schematic diagram illustrating the use of a recycling valve [see Fig. 3 of (22)] as a sample injector. P denotes the peristaltic pump. C is the sample coil of capillary tubing. a–e designate the five valve access cannulas. The dashed and solid straight lines drawn between adjacent cannulas indicate alternative pairs of paths inside the valve.

bubbles. The trapped bubbles always dissolved rather quickly in the deaerated buffers used for elution and did not accumulate from one run to the next.

Although good starting zones were always obtained when samples were applied by the latter procedure, it was often more convenient to use a semiautomatic sample injector which had been adapted, as illustrated in Fig. 1, from a selector valve of the type devised by Porath and Bennich (22) for use in recycling chromatography. The sample coil, C, consisting of 2 m of polyethylene capillary tubing (see above) with a total capacity of 1.14 ml, was connected between the valve access cannulas b and d. The dashed and solid lines between adjacent cannulas indicate the paths that are open within the valve when the selector handle is in positions 1 and 3, respectively. In the former case the sample coil is included in the flow path between the pump and the column. The arrows indicate the normal direction of flow.

The sample coil could be charged with fresh sample at any time when the selector handle was in position 3, even during a run. The following procedure was used to charge the coil. With the selector handle in position 3, the coil was disconnected from cannula b. A 2-ml syringe was filled with the desired solvent, and after expelling any bubbles, the filled syringe was connected to cannula c and its contents were flushed slowly through the coil. With the syringe still

in place, the free end of the coil was immersed in the sample solution and exactly 1 ml was drawn slowly into the coil by pulling back the plunger of the syringe. The coil was then reconnected to cannula b. Meanwhile, buffer could be pumped to the column through the bypass until steady-state flow conditions were established in the system. Then the sample was introduced into the column simply by turning the valve selector handle to position 1. At a flow rate of 10 ml/hr, less than 10 min was required for the sample to clear the coil. At any convenient time after this, the valve could be returned to the coil bypass position if desired.

Some general principles and advantages of automatic or semiautomatic sample injection have been discussed by Crestfield (23).

Operation and Analysis

The column was eluted with downward flow at a linear rate of 12.7 cm/hr (10.0 ml/hr). The flow rate was maintained constant with a peristaltic pump fitted with Tygon tubing. Experiments were performed at 25 ± 1° unless otherwise indicated. Since all but two of the solutes studied are ninhydrin-positive, the column effluent was analyzed directly with a Spinco Model 120 amino acid analyzer and the ninhydrin profile was automatically recorded. With the ninhydrin pump operating at 10 ml/hr, the total flow rate in the reaction coil was 20 ml/hr. When the standard reaction coil (about 30 m long) was used, this flow rate provided a reaction time of 36 min. Good strip-chart records with stable base lines were obtained under these conditions with all the eluting media used. Base-line compensation was difficult only with the tris buffer, which gives a strong background color even with the normal 15-min reaction time.

However, continued use of the standard coil with this excessive reaction time led to the formation of a hard white crust on its inner surface. After about 20 runs, the deposit began to flake off and obstruct the coil. Possibly the deposit would not have formed if the buffers used in the G-10 experiments had contained the BRIJ detergent, which is always present in the standard amino acid buffers, but the use of such a detergent would have introduced an unwanted parameter in the present studies. Therefore, the deposition problem was eliminated by using reaction coil only 12.5 m long, which provided a reaction time of 15 min at a total flow rate of 20 ml/hr. Since the shorter reaction coil permitted operation of the ninhydrin pump

at the normal rate of 15 ml/hr without excessive signal attenuation, the analyzer could be used alternately for both amino acid analysis and the Sephadex experiments without readjusting the ninhydrin pump. At the resulting combined flow rate of 25 ml/hr, the reaction time in the short coil was 12 min.

The auxilliary reaction coil was made by wrapping Teflon capillary tubing (AGW22 TW Natural, Pennsylvania Fluorocarbon Co., Inc., Philadelphia, Pa.) around a 6 × 16-cm cylinder of stainless-steel screen. This assembly was placed in the reaction bath inside the regular reaction coil, and the ends were led to the outside through a tubing outlet (Beckman part No. 313241) placed in an unused opening in the glass reaction-bath lid. The ends of both coils were fitted with the standard Beckman swivel fittings so that either one, or both, could be incorporated into the circuit as desired. The connections were made with small Teflon cylinders which had been drilled through longitudinally. The cylinders were recessed and threaded at both ends to accommodate the swivel fittings in a leakproof seal. The ends of the unused coil were connected together in the same way to keep it from drying out. A pair of slide valves would simplify the task of switching from one coil to the other, but only 1 min is required to change the connections manually.

Glucose and acetic acid are the only ninhydrin-negative substances whose elution positions were determined in this study. When glucose was run, some ninhydrin-positive substances (lysine, ammonia, and usually glucosamine) whose effluent positions has been determined previously with the recorder were included in the sample. The effluent from 35–80 ml was collected in fractions of 1 ml, and separate aliquots from each fraction were analyzed manually with the orcinol and ninhydrin procedures. The use of the internal standards increased the significance of the small changes observed in the elution position of glucose and provided a valuable check on the accuracy of the volume scale in the recorder analyses.

The elution position of acetic acid was determined by running 1 ml of 1 *M* acetic acid through a column equilibrated with 0.2 *M* acetic acid. The acetic acid peak was detected by passing the effluent through the analyzer of a Beckman Spectrochrome programmed to record transmission at 230 mμ.

Experiments were performed in the different media used in the

TABLE 1

K_d Values of Amino Acids and Other Substances on Sephadex G-10[a]

Substance	Solvent medium[b]									
	A	A′	B	C	D	E	F	G	H	I
Ribonuclease	0	0	0	0	0	0	0	0	0	0
Bacitracin	0	0	0.26	—	0	—	—	—	—	—
Tetraglycine	0.33	0.10	0.38	0.42	—	0.18	—	—	—	—
Leu-Gly-Gly	0.38	—	—	—	—	—	—	—	—	—
Gly-Asp	—	0.27	—	—	—	—	—	—	—	—
Val-Try-Arg	—	0.42	—	—	—	—	—	—	—	—
Lysine	0.20	0.06	0.29	0.35	0.17	0.17	0.16	0.19	0.14	0.24
Histidine	0.31	0.07	0.34	0.39	—	—	0.32	—	—	—
Ornithine	—	0.07	—	0.37	—	—	—	—	—	—
Arginine	—	0.08	0.48	0.60	0.27	—	—	—	—	—
Threonine	—	0.28	—	0.51	—	—	—	—	—	—
Valine	0.38	0.28	0.50	0.69	—	—	—	—	—	—
Proline	—	0.29	0.38	0.47	—	—	—	—	—	—
Glycine	0.44	0.29	0.48	0.54	0.34	0.36	0.35	—	0.35	0.32
Serine	—	0.30	—	—	—	—	—	—	—	—
Isoleucine	—	0.30	0.57	0.86	—	—	—	—	—	—
Leucine	0.40	0.32	0.58	—	—	0.39	—	—	—	—
Norleucine	—	0.32	0.59	0.90	—	—	—	—	—	—
Methionine	0.40	—	—	0.58	—	—	—	—	—	—
Glutamic Acid	0.44	0.41	0.49	0.61	—	—	0.29	0.14	—	0.13
Aspartic acid	—	0.53	—	0.54	—	—	—	—	—	0.15
Phenylalanine	0.86	0.64	1.11	1.83	0.71	0.81	0.65	0.56	0.74	0.61
Tyrosine	1.28	0.93	1.73	2.85	0.96	1.16	0.88	0.61	1.11	0.38
Tryptophan	—	2.74	—	—	—	—	—	—	—	2.88
Ammonia	0.56	0.13	0.68	0.79	0.46	0.39	0.43	0.67	0.38	0.75
Urea	1.09	1.06	1.09	1.17	1.03	1.07	0.95	0.94	1.02	1.06
Glucosamine	—	0.07	0.49	0.51	—	—	—	—	—	—
Glucose	—	0.40	0.49	0.51	—	—	—	—	—	—
Acetic acid	—	1.0	—	—	—	—	—	—	—	—

[a] Column parameters: 1 × 142-cm column, bead size < 40 μ; bed volume (V_b) 110 ml; void volume (V_0) 41 ml; gel volume (V_g) 69 ml; inner volume (V_i) 43 ml.

[b] Solvent media: A, 0.2 *M* acetic acid, pH 2.7 (freshly poured column); A′, 0.2 *M* acetic acid, pH 2.7 (after exposure of gel to all other media); B, 0.2 *M* acetic acid with 0.5 *M* NaCl; C, 0.2 *M* acetic acid with 2.0 *M* NaCl; D, 0.1 or 0.3 *M* pyridine–acetic acid (equimolar), pH 5; E, 0.35 *M* sodium citrate, pH 5.28 (short column buffer for amino acid analyzer); F, 1.0 *M* pyridine with 0.03 *M* acetic acid, pH 6.7; G, 1.0 *M* pyridine with 0.5 *M* NaCl, pH 8.5; H, 0.05 *M* tris-H_2SO_4 buffer, pH 8.07; I, 0.01 *M* NaOH.

order indicated in Table 1, with the exception that the values listed under A′ were obtained upon returning to 0.2 *M* acetic acid as eluent after exposure of the column to all the other media. Upon changing from one medium to another, at least two full column volumes of the new medium was always pumped through the column before applying a sample.

Calculation of K_d Values

K_d values were calculated from the relationship

$$K_d = (V_e - V_0)/V_i$$

where V_e is the elution volume corresponding to the maximum of an effluent peak and V_0 the interstitial volume or void volume of the column. V_0 was determined with Blue Dextran 2000 (Pharmacia). $V_i = gW_r$, where g is the dry weight of the gel present in the column and W_r its water regain in grams per gram. For the gel used in these experiments, $W_r = 0.96$. Since the column used for most of the experiments described below was not prepared from a weighed amount of gel, g was calculated from the relationship

$$g = V_g/v_g$$

where $V_g = V_t - V_0$ and corresponds to the volume in the column occupied by the swollen gel particles. V_t, the total bed volume, was determined by filling the chromatographic tube to the appropriate level with water. v_g is the volume (not including interstitial liquid) occupied by 1 g of gel after swelling, and was determined as $(V_t - V_0)/g$ with two columns prepared from exactly 33.0 and 25.0 g of Sephadex G-10 (30–60 μ fraction) from the batch used for the experimental column. The average value of v_g was 1.55 ± 0.05, which would indicate a wet density, in water, of 1.26 g/ml.

RESULTS

All the patterns shown in Figs. 2–5 and all the K_D values recorded in Table 1 were obtained with the same 1 × 142-cm column of Sephadex G-10. Figure 6 is a pattern obtained on a G-25 column of the same dimensions and is shown for comparison.

Before we attempt to offer a possible physical explanation for the selectivity observed, it seems appropriate to discuss some technical aspects of the separations. Since the chromatograms shown

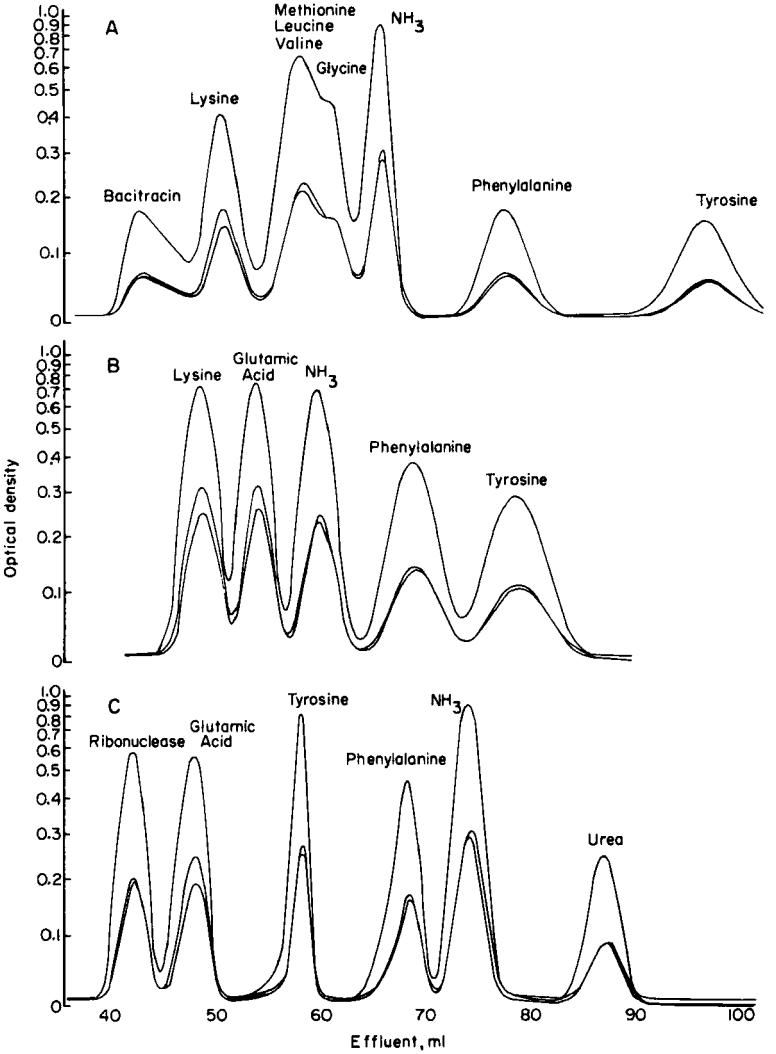

FIG. 2. Patterns obtained with amino acids and other ninhydrin-positive substances on a 1 × 142-cm column of Sephadex G-10 in three different eluants. In pattern A the eluant is 0.2 *M* acetic acid, pH 2.7; in B the eluant is 1 *M* pyridine–0.03 *M* acetic acid, pH 6.7; and in C it is 0.01 *M* NaOH, pH ca. 12. The ordinate is ninhydrin color as recorded continuously with a standard Spinco Model 120 amino acid analyzer. The flow rate through the column was 10.0 ml/hr in all cases.

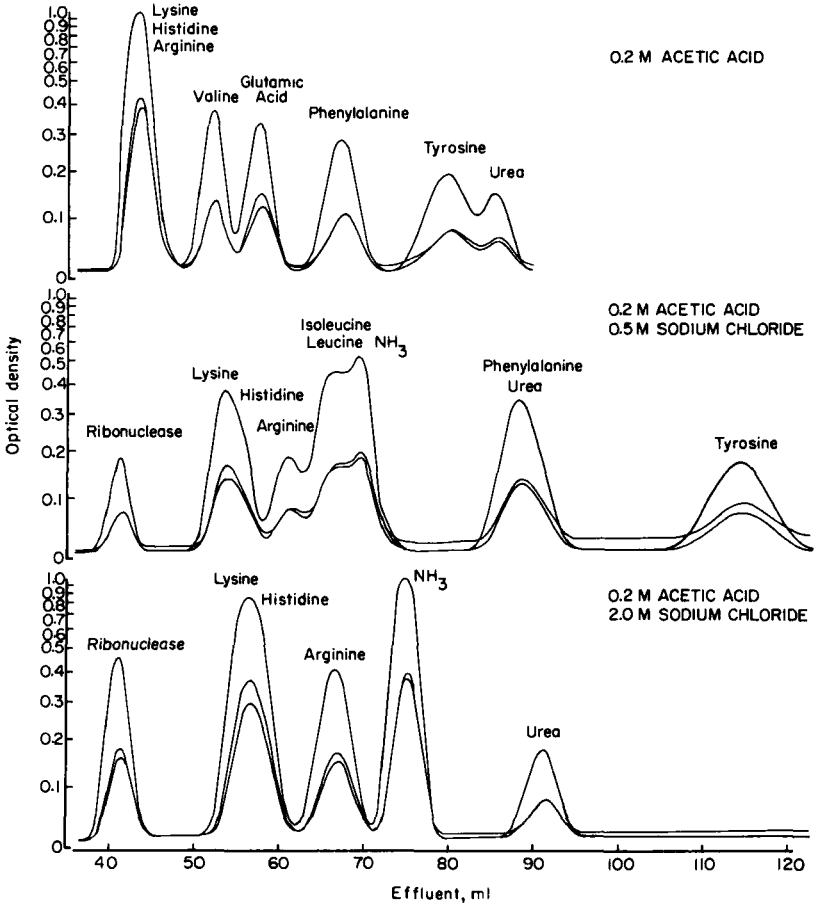

FIG. 3. Patterns showing the effect of salt on the elution behavior of amino acids and other ninhydrin-positive substances on a 1 × 142-cm column of Sephadex G-10. The chromatogram at the top was obtained in 0.2 *M* acetic acid in the absence of salt. The middle and bottom patterns were obtained in 0.2 *M* acetic acid containing 0.5 *M* and 2.0 *M* NaCl, respectively. Flow rate: 10.0 ml/hr. Ordinate: ninhydrin color.

suggest that the use of slender columns of Sephadex G-10 in conjunction with the amino acid analyzer might be of considerable value in the quantitative analysis of mixtures of small ninhydrin-positive substances on a micro scale, we should also like to mention a few important points of technique and to evaluate in meaningful terms the performance of the column.

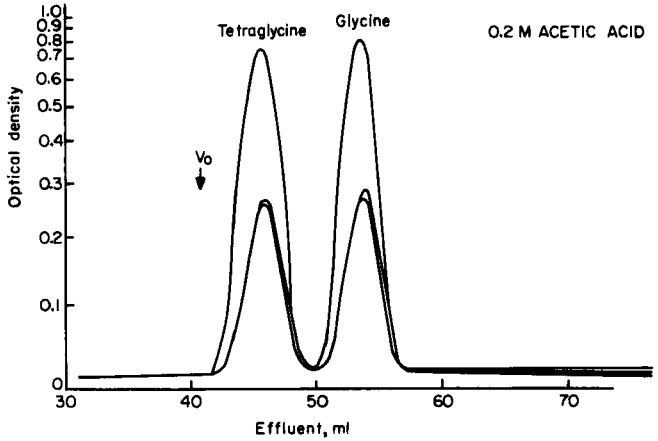

FIG. 4. Separation of tetraglycine (2 μmoles) and glycine (1 μmole) on a 1 × 142-cm column of Sephadex G-10 in 0.2 *M* acetic acid. Flow rate: 10.0 ml/hr. Ordinate: ninhydrin color.

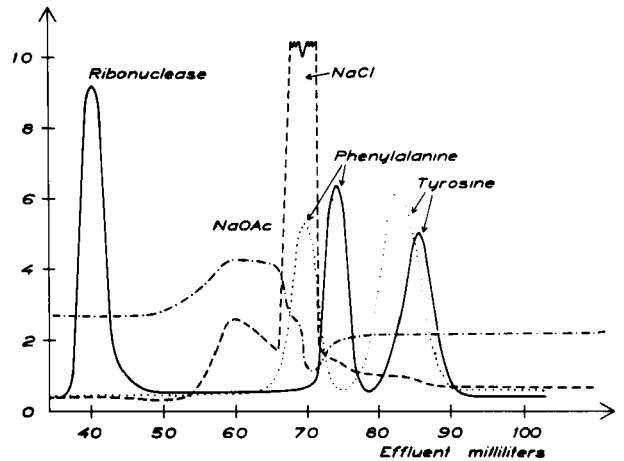

FIG. 5. Composite patterns showing drastic alterations in environmental conditions caused by the presence of a large amount of salt in the sample applied to a 1 × 142-cm column of Sephadex G-10 eluted with 0.2 *M* acetic acid at 10.0 ml/hr. The sample consisted of 110 mg of NaCl, 2 mg of phenylalanine, 1 mg of tyrosine, and 1.5 mg of ribonuclease dissolved in 1 ml of 0.2 *M* acetic acid. The transmission at three different wavelengths, the pH, and the conductivity of the effluent were monitored continuously with a Beckman Spectrochrome. ——, transmission at 230 mμ (ribonuclease), 260 mμ (phenylalanine), and 280 mμ (tyrosine). Ordinate: ----, conductivity in ohm^{-1} × 10^{-3}; -.-.-, pH. In the case of the transmission curves, 0 and 10 on the ordinate scale represent 100 and 0% transmission, respectively. The dotted curve (...) was obtained with phenylalanine and tyrosine in a separate run performed without including salt in the sample.

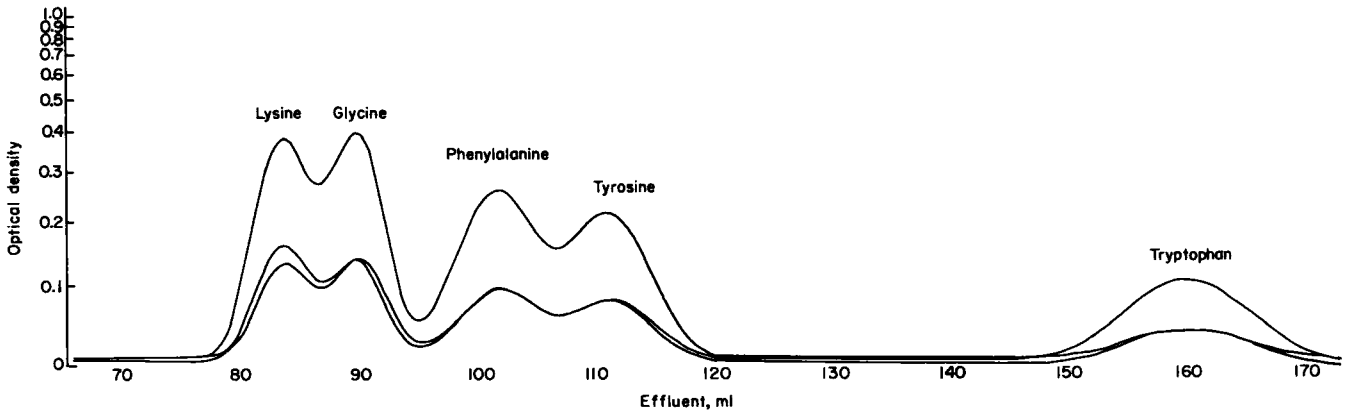

FIG. 6. Separation of five amino acids (1 μmole of each) on a 1 × 42-cm column of Sephadex G-25 in 0.2 *M* acetic acid. Flow rate: 10.0 ml/hr. Ordinate: ninhydrin color.

Identification of Peaks

This preliminary survey was not undertaken to solve any particular separation problem and our approach has not been particularly systematic. Rather, our intention was simply to determine the extent to which the elution behavior of various types of amino acids (e.g., acidic, basic, aliphatic, aromatic, etc.) on highly cross-linked dextran gels was influenced by the composition of the solvent environment. Although Table 1 has many gaps, it contains 132 K_D values. Since the amino acids and other substances studied behaved independently of each other at the low concentration (0.5–2 m*M*) involved in the runs performed with the amino acid analyzer, it was not necessary to determine all these values separately. In fact, all the samples run contained at least two solutes. Often four or more were present.

The quantitative nature of the strip-chart records provided a simple means of identifying the peaks obtained with samples containing several solutes when the relative amounts of each were chosen properly. Since the ninhydrin color values of ammonia and all the amino acids studied except proline differed by less than 20% under the conditions of our experiments, peaks representing 0.5, 1.0, and 2.0 μmoles, respectively, of any three of these substances could be identified easily and unequivocally on the basis of the differences in height or area.

The essentially simultaneous records of ninhydrin color at 440 and 570 mμ provided by the amino acid analyzer proved very useful for identifying peaks corresponding to lysine and glutamic acid. Of course, the 440 trace was almost essential for detecting proline, which gives very little ninhydrin color at 570 mμ. With all the other substances studied the ninhydrin color at 570 mμ is 2.5–3 times that at 440 mμ. Therefore, the 570 N curve is uppermost by a considerable margin in all the peaks which appear in the chromatograms shown, none of which contains proline. However, an inspection of the chromatograms reveals that the two lower curves, corresponding to the 440 N and the suppressed 570 mμ (570 S) channels, run very close together in all peaks except those of glutamic acid and lysine, where the 440 N trace runs about 20% above the 570 S curve. This characteristic permitted unambiquous identification of either of these substances when both were not present.

With these qualitative and quantitative means for peak recogni-

tion in mind, samples were usually prepared in such a way that any and all of the peaks obtained could be identified without reference to any other run. Most of the chromatograms shown were run for illustration purposes and all of them are exceptions to the general practice just mentioned. However, we must emphasize that all the illustrations are quite typical with regard to the sharpness and symmetry of the peaks and the base-line stability.

Column Performance

The elution patterns shown in Figs. 2, 3, and 4 and the K_D values listed in the table indicate that G-10 is considerably selective toward amino acids. By comparing these patterns with the K_D values, one can obtain a rough idea of the differences in K_D required for various degrees of separation on the particular column used.

The efficiency of the column can be rated in a more absolute way by calculating H, the bed height equivalent to a theoretical plate. According to Glueckauf (*24*),

$$H = (L/8)\,(\beta/V_e)^2$$

where L is the total length of the column below the center of the original starting zone, V_e the elution volume of the zone, and β the width of the elution curve at the height corresponding to the maximum height divided by 2.71 (e.g., c_{max}/e).

Theoretically, the lower limit of H that might be achieved through perfect packing and ideal flow conditions is approximately equal to the average diameter, $\bar{d}_p$, of the particles used to pack the column (*25*). This lower limit of H seems to be a realistic goal, since values only two or three times d_p are observed routinely with the very fine and very uniform spherical resins now used for high-speed amino acid analysis.

As calculated directly from the elution curves of the various nonaromatic substances chromatographed, the average value of H for the G-10 column used here is about 600 μ, which is about 15 times the average diameter (ca. 40 μ) of the *swollen* gel particles. For the aromatic amino acids, which exhibit more band spreading, H was about 1000–1200 μ. Since for any particular value of V_e, β is proportional to the square root of H, the β volumes of all the elution curves obtained are at least three to four times the theoretical minimum values. However, the apparently excessive width of the elution

curves observed on the recorder charts is not due entirely to zone broadening that occurs in the gel bed itself. Since the β volumes involved here are only a few times greater than the sample volume, the width of the starting zone cannot be neglected. In true elution chromatography, where the sample is applied to the column in the medium with which the column is equilibrated and is eluted with the same medium, β can never be less than the sample volume. In our experiments, the sample volume was always 1 ml, and some dilution occurred during application to the gel bed due to uneven flow through the porous membrane. Some additional zone broadening occurred at the outlet end of the column and in the analytical system. We attempted to estimate this "nonchromatographic" zone broadening using a 1 × 1-cm column of the same gel fraction. The short column was fitted with the same plungers as were used in the long column. When 1-ml samples containing 1 μmole of glycine were passed through the short column and the analytical system, the β volume of the recorder peak was about 2 ml.

With the long column, the β volume for glycine is 3.1 ml, indicating an H of 596 μ. If it is valid to correct this β volume for "nonchromatographic" broadening simply by subtracting the 1-ml spreading increment observed with the very short bed, then H is only 274 μ. As calculated from the β volumes of unretarded ribonuclease peaks, similarly corrected, H was 218–284 μ, or about five to seven times $\bar{d}_p$.

It is interesting to consider what lower values of H would mean in terms of separation efficiency. For elution curves that approximate normal Gaussian curves of error, the β volumes correspond to $2\sqrt{2}\sigma$, or 2.83σ, where σ is the standard deviation. Assuming that the individual elution profiles have the form of the standard normal distribution, a mixture containing equal amounts of two substances whose respective elution volumes are V_{e_1} and V_{e_2} would be more than 99% separated if $V_{e_2} - V_{e_1} = 0.82(\beta_1 + \beta_2)$.

If $\beta_1 \sim \beta_2$, the two substances would be separated adequately for many purposes when $V_{e_2} - V_{e_1} = 0.5(\beta_1 + \beta_2)$. Elution curves obtained with mixtures of two substances which satisfy the latter condition should show two maxima if the mixture contains at least 25% of either substance. At higher ratios, the component present in lesser amount would fade from a distinct shoulder to a slight skew on the elution curve of the major component as the ratio became more disproportionate. If the two substances were present

in equal amounts, the elution curve would show a distinct minimum between two approximately equal maxima, and the total material on either side of the minimum would be a mixture containing 92% of one substance and 8% of the other. At any rate, the degree of separation afforded by $V_{e_2} - V_{e_1} = 0.5(\beta_1 + \beta_2)$ is probably sufficient for many applications in qualitative analytical work.

When $V_{e_2} - V_{e_1}$ is less than about $0.4(\beta_1 + \beta_2)$, the elution curve will be unimodal (no minimum), even when the substances are present in equal amounts.

In our experiments with the 1 × 142-cm G-10 column, all the elution curves obtained with substances other than the aromatic amino acids, urea, and bacitracin showed β volumes between 2.5 and 3.5 ml, as read directly from the recorder chart. Since the aromatic amino acids studied (phenylalanine, tyrosine, and tryptophan) could be separated completely from each other and from all other amino acids in most of the solvent media tested, their greatest β volumes present no practical difficulty.

By definition, $V_{e_2} - V_{e_1} = V_i(K_{D_2} - K_{D_1})$ for any pair of substances in the particular chromatographic system for which the K_D values apply. For the 1 × 142-cm G-10 column used in our experiments, $V_i = 43$ ml. Since with this column $V_{e_2} - V_{e_1} = 5.25$ ml when $K_{D_2} - K_{D_1} = 0.122$, a mixture containing approximately equal amounts of two substances whose individual β volumes are 3.2 ml or less [i.e., $0.82 \times (3.2 + 3.2) = 5.25$ ml] will be at least 99% separated on the column if the difference in K_D is greater than 0.12. Thus any pair of nonaromatic amino acids can be separated almost completely on this column in any medium where the difference between their K_D values is greater than 0.12. With regard to the chromatograms shown in Figs. 2, 3, and 4, we probably should point out that when tall peaks overlap near the bottom, the degree of overlap is exaggerated greatly by the logarithmic ordinate scale.

The differences in K_D required for essentially complete separation of the pairs phenylalanine-tyrosine, phenylalanine-urea, and tyrosine-urea depends on the solvent medium. However, any of these pairs was always separated where the difference in K_d is greater than 0.25. In fact, tyrosine and phenylalanine were well separated in all media except G.

The difference of 0.12 in K_D required for 99% separation of pairs of nonaromatic amino acids on this column is associated with an operational H of about 600 μ. If H could somehow be reduced ten-

fold, complete separations might be accomplished on the basis of K_D differences less than 0.03. Theoretically, at least, this ought to be possible with gel particles of the size used here. For each of a series of solutes whose elution behavior on a 1 × 142-cm column is associated with an H of 62 μ, $\beta = 0.02V_e$. For substances eluting with K_D's between 0 and 1, the mean difference in K_D required for 99% separation would be about 0.028. If our column had this efficiency, all the amino acids for which K_D values are listed in column C of Table 1 would be well separated in medium C except the pairs glycine–aspartic acid and glutamic acid–arginine.

Methodological Accuracy and Precision

Most of the K_d values listed in columns B and I of Table 1 and many of those listed elsewhere in the table are calculated from the average of two or more determinations of elution volume. Since ribonuclease was eluted from the column at the same position as Blue Dextran 2000 in all the media listed and gave a much greater recorder response, the enzyme was used routinely as a V_0 reference. Unless a sample contained some other substance that was expected to elute from the column with a K_d less than 0.10, ribonuclease was usually included.

Ammonium chloride was included in most runs, and since it was virtually impossible to prepare ammonia-free samples a small ammonia peak with a height corresponding to 0.01–0.02 optical density unit on the chart was always present even when ammonium chloride was not added. Ammonia overlapped considerably (still some trough present) with lysine, histidine, or ornithine in medium A′; with isoleucine in media B and C; with glycine in medium F; and with phenylalanine in medium G. It merged (no trough present) with tetraglycine or arginine in medium A′; with leucine or glycine in medium E; with tyrosine in medium G; and with glycine in medium H. Ammonia was not added to the samples used for determinations of the elution volumes of these substances in the media indicated.

Tyrosine overlaps or merges with phenylalanine and ammonia in medium G; with urea in media A′, D, E, F, and H; and with glycine in medium I. Phenylalanine merges with urea in medium B. Otherwise, the aromatic amino acids and urea were always well separated from each other and from all other substances present.

Therefore, phenylalanine and tyrosine or urea were included in many of the samples run. Sometimes all three of these substances could be included.

When duplicate or multiple determinations of elution volume were made with these or any other substances in any particular series of runs, the precision was better than ±1% in all cases where the elution volume was less than about 100 ml. For example, glycine was included in three of the seven samples run in series A and ammonium ion was present in all of them. The average elution volumes of glycine and ammonia under these conditions were 61.9 ± 0.2 ml and 66.9 ± 0.1 ml. For three determinations each with glutamic acid, tyrosine, and phenylalanine in medium I, the average elution volumes were 47.3 ± 0.3 ml, 58.3 ± 0.2 ml; and 68.3 ± 0.4 ml, respectively.

The positions of peaks which were retarded beyond 100 ml (e.g., tyrosine in B and C, phenylalanine in C, and tryptophan in A′ and I) seemed to vary somewhat more than those of peaks which eluted earlier. Since the very retarded zones were quite broad and rather flat at the top, part of the variation is probably due to uncertainties in locating the exact position of the peak. For the five cases just mentioned, the variation was 2–3 ml.

The elution volumes were read directly from the recorder chart. The standard chart paper used contained 10 vertical rulings per inch. Since the chart speed was 3 in./hr and the flow rate through the column was 10 ml/hr, the distance between adjacent vertical rulings corresponded to 0.33 ml of column effluent. Peak positions were read to the nearest half division. Therefore, the precision indicated above corresponds approximately to the accuracy with which the chart was read. In fact, any pair of chromatograms obtained in any one of the experimental series listed are virtually superimposible with respect to any and all of the peaks that they might contain in common.

The average rate of flow through the column was 10.0 ± 0.1 ml in all experiments. This value was obtained by collecting the effluent delivered through the column over periods of 1 to 10 hr. The same value was obtained by collecting the total effluent from the analyzer during a run and subtracting the volume of ninhydrin reagent present. These determinations showed that the flow rate through the column was not affected by the additional backpressure produced in the reaction coil by the ninhydrin pump. The average

backpressure supported by the peristaltic pump during a run was about 15 psi.

When the outlet flow meter of the analyzer was used to determine the flow rate through the column, an average value of 10.0 ± 0.25 was obtained. The time interval of 196 ± 5 sec involved in the latter measurements is somewhat less than the time required for one complete revolution of the roller wheel of the peristaltic pump. Since slight relaxations of backpressure, or "backlashes," occurred at certain positions of the roller wheel, the flow rate pulsated slightly during each pump cycle. Therefore, the "instantaneous" flow rate measured with the flow meter shows somewhat more variation than that determined from the output of the pump over intervals involving 15 or more cycles of the roller wheel. The minor fluctuations in flow rate produced no visible wobble in the recorder base line, as evidenced by Figs. 2–4 and 6.

Further evidence for the uniformity of the flow rate and for the over-all stability of the analytical system is the excellent quantitative agreement observed upon comparing the areas of recorder chart peaks corresponding the same amount of the same amino acid among chromatograms obtained in the same solvent medium. Significant differences in ninhydrin color yield were observed among runs performed in different solvent media.

To make quantitative comparisons, the recorder peaks were integrated by the usual graphical $H \times W$ method, wherein W is determined by dot counting.

Since the individual amino acid stock solutions used for the preparation of column samples were not prepared with analytical accuracy, none of the $H \times W$ values obtained can be considered as absolute calibration values. However, all the column samples were prepared very accurately from the same stock solutions and were applied carefully to the column. Therefore, any absolute errors in the concentrations of the stock solutions need not be considered in estimating the quantitative precision.

At any rate, the average deviations in peak area were within 3% in all cases where quantitative comparisons were made. For example, the average area of the glutamic acid peak in three runs performed in 0.01 N NaOH (medium I) with samples containing the same amount of glutamic acid (ca. 1 μmole) was 36.5 ± 0.3 $H \times W$ units. Here, the average deviation is less than 1%. In the same medium, the $H \times W$ values of peaks representing 0.5, 1, and

2 μmoles of phenylalanine were 15.3, 31.4 ± 0.2 (average from two runs), and 60.1, respectively, indicating a linear relationship between peak area and the amount of amino acid present.

The quantitative precision attained with the experimental arrangement employed here compares favorably with that provided by the amino acid analyzer in routine amino acid analyses involving the use of standard cation-exchange columns driven by piston pumps.

The elution data are tabulated in the form of K_D values. Since the elution behavior of the substances studied will be compared and discussed in terms of these values, we ought to provide an estimate of their precision. About one-third of the values given in Table 1 are based on single determinations, and for these no direct measure of precision is available. However, on the basis of the many cases where duplicate or multiple determinations were performed we have estimated some average ranges of probable error which ought to be valid for all the values listed in Table 1. For all K_D values less than 0.5, the average error is about ±0.01 K_D unit. For all higher K_D values, the average error is within ±2%.

Since all the runs represented by Table 1 were performed on the same column, the same value of V_i (43 ml) was used in the calculation of all the K_D values listed. Therefore, the ranges of error given for the K_D values correspond to the maximum experimental variation observed among duplicate determinations of $V_e - V_0$. When comparing K_D values obtained with the same solute in different series of runs, differences which fall outside the limits given above should be indicative of real differences in elution behavior. That differences greater than 0.01 K_D unit among K_D values obtained with different substances in the same solvent medium are real and significant was demonstrated experimentally with several appropriate pairs of substances. For example, the difference between the K_D values obtained separately with 1-μmole samples of isoleucine and norleucine in medium A′ is 0.02. When a mixture containing 0.5 μmole of each was run under the same conditions, the compound peak was flatter and about 1 ml broader than those obtained with 1 μmole of each substance separately.

The accuracy of the K_D values in Table 1 as indicators of relative elution positions depends only on the accuracy of the measurements of $V_e - V_0$, since V_i is simply a constant scale factor. However, if the K_D values are to be regarded as true molal distribution

coefficients, V_i must be determined accurately. Whether the K_D values reported here are accurate in this absolute sense depends on the validity of the indirect method used to estimate V_i (see Methods). The value of 1.55 ml/g found for v_g, the specific volume of the swollen G-10 beads, is slightly less than the sum (1.57) of 0.606 and 0.96, which are the manufacturer's figures for the specific volume of dry Sephadex and for the water regain of the G-10 used, respectively. Since v_g ought to be somewhat lower than the latter sum, our value is probably fairly accurate. If so, V_i should be accurate as well. At any rate, the error is not likely to be greater than about 3%. When exact values are desired, one probably should determine V_i in a more direct fashion by the use of tritiated water, as recommended by Marsden (9).

Factors Affecting Reproducibility

Condition of Gel. The experimental precision was uniformly high in each series of experiments represented by the data in Table 1. However, all these runs were done on the same column and all the runs in each series were done consecutively in a reasonable period of time (e.g., a few days to several weeks). Although we naturally assumed initially that the K_d values given in Table 1 would apply with equal accuracy for other G-10 volumns, an investigation of this point was prompted by the disturbing observation that the K_d values obtained in at least one of the buffers used seemed to depend on how the gel had been treated previously. As a result of the investigations described below, we are now confident that all the K_d values given in Table 1 except those listed in column A are valid in general for sample concentrations up to 0.005 *M*, and probably much higher, provided the G-10 is washed with 1 *M* aqueous pyridine before it is used. The observations upon which this conclusion is based are given below.

The pattern shown in Fig. 2A and the K_d values listed in column A of Table 1 were obtained in 0.2 *M* acetic acid in the first series of experiments performed after packing the column. The eight series of experiments represented by columns B–I were then performed in the order indicated. The upper pattern in Fig. 3 and the K_d values listed in column A′ of Table 1 were obtained upon returning of 0.2 *M* acetic acid as eluant after completing the runs in 0.01 *M*

NaOH (series I), in other words, after the column had been exposed to all the other buffers used.

Although no drift in K_d values was observed within any series of runs, some of which extended over periods of 2 or more weeks, a comparison of the results obtained in series A′ with those obtained in 0.02 *M* acetic acid initially (series A) shows clearly that the properties of the stationary phase in the column had changed at some time between the two series of runs. The uppermost patterns in Figs. 2 and 3 were obtained in 0.2 *M* acetic acid under the same conditions, but they are not at all compatible as regards the elution positions of the substances that they contain in common. The change that occurred in the column between series A and A′ seems to be permanent, for when lysine, glutamic acid, aspartic acid, tyrosine, phenylalanine, and urea were run on the same column in 0.2 *M* acetic acid more than 1 year after Table 1 was compiled, the K_d values observed were in every case within 3% of those listed in column A′. During the intervening year, the column had been used repeatedly in many different buffers.

The nature of the change that had occurred was inferred from the following observations. First, if the K_d values given in columns A and A′ are compared on a relative basis, the change is seen to be most pronounced with the substances that bear the highest positive charge. Thus the average value of $K_{d(A')}/K_{d(A)}$ for the basic substances lysine, histidine, and ammonia is 0.23; and for the "neutral" amino acids glycine, valine, leucine, tyrosine, and phenylalanine (which are, of course, positively charged at pH 2.7) the average value is 0.73. For glutamic acid (isoelectric point ea. pH 3.2) and for the uncharged substance, urea, the above ratio is 0.93 and 0.97, respectively. The elution behavior of glutamic acid is thus affected very little by the change in the column and in the case of urea the difference is barely significant.

The second important finding was that the apparent change in the properties of the gel could not be demonstrated in any of the buffers used except 0.2 *M* acetic acid. The gross discrepancies between the results obtained in series A′ and those obtained initially prompted us to check the data obtained in the other buffers as well. Most of the K_d values obtained in buffers B and C were redetermined and the results agreed excatly with those obtained the first time, indicating that the structure of the gel matrix had not

been altered significantly. Therefore, only a few K_d's were checked in the other buffers. Since lysine and ammonium ion seemed to be the most sensitive indicators of the change when 0.2 *M* acetic acid was used as eluant, the behavior of these, and in some cases, a few other substances was reinvestigated in buffers D–H. The K_d of glutamic acid was checked in 0.01 *M* NaOH. In every case, the K_d values observed agreed almost exactly with those obtained initially.

On the basis of these observations we concluded that the change that had occurred was a reduction in the cation-exchange capacity of the column. Our hypothesis was that the column had initially contained a small but significant amount of some anionic substance that was either adsorbed to the gel matrix or attached to it by some sort of labile linkage and that most of this substance had been eluted from the column by one of the media to which the gel had been exposed subsequent to the initial series of experiments in 0.2 *M* acetic acid.

Proceeding on the assumption that this explanation was correct, we first had to determine whether the hypothetical anionic substance had been present in the G-10 to begin with or whether it had been introduced inadvertently by us at some stage before the first chromatographic experiments were done. Therefore, duplicate columns (1 × 101 cm) were packed with gel from another elutriated fraction (30–60 μ) of the same batch of G-10 from which the gel used for the original column (<40-μ fraction) was obtained. The portion of gel used for one of the columns was washed with 50% acetic acid and the column was packed in 0.2 *M* acetic acid containing 1% NaCl, exactly as was done with the original column. The gel used for the other column was not washed with 50% acetic acid and the packing was done in 0.2 *M* acetic acid in the absence of salt. The properties of the two columns were then compared by chromatographing a few representative substances on each using 0.2 *M* acetic acid as eluant. No significant differences were observed between the two columns with regard to the elution behavior of any of the substances studied. The K_d values obtained with one of the columns are listed under 2 in Table 2. These values are seen to be similar to those obtained in the first series of runs (series A in Table 1) performed in 0.2 *M* acetic acid with the original column.

After the runs of series I had been completed with the original

TABLE 2

K_d Values Observed in Dilute Alkali (1) and in Dilute Acetic Acid Before (2) and After (3) Exposing the Gel to Alkali[a]

	Eluant		
	0.01 *M* NaOH	0.2 *M* HOAc	
Substance	1	2	3
BSA	0	0	0
Glutamic acid	0.03	—	—
Lysine	—	0.18	—
Glycine	—	0.39	0.39
Phenylalanine	—	0.81	0.82
Tyrosine	—	1.17	—
Urea	1.06	—	1.06
Ammonia	0.78	0.43	0.44

[a] Column: 1 × 101 cm; 30–60-μ beads, not washed with 1 *M* pyridine. Flow rate: 10.0 ml/hr; temp. 26°C.

column, the gel bed stood in 0.01 *M* NaOH during the interval of 5 weeks that elapsed before the second series of runs in 0.2 *M* acetic acid (series A′) was begun. This was ostensibly the harshest treatment that the gel had received, for among the several media to which the gel had been exposed 0.01 *M* NaOH is the only one that might be expected to promote or produce chemical changes in the gel matrix itself (*26*). The glycosidic linkages of the dextran chains and the ether linkages to the glyceryl bridges are both very resistant to hydrolysis by alkali but are susceptible to oxidation by oxygen in strongly alkaline media (*26*). However, it seemed very unlikely that the relatively mild alkaline treatment described above could do more than catalyze oxidation of terminal reducing groups of dextran chains and perhaps some very small fraction of the secondary alcohol functions of the glyceryl cross-linkages. Since any of these oxidative reactions would increase the carboxyl content and hence the cation-exchange capacity of the gel, such reactions certainly could not be the basis of the apparent reduction in cation-exchange capacity observed.

To determine whether dilute alkali might, however, be able to effect the release of our hypothetical bound anion, one of the pair of identical columns described above was treated with 0.01 *M*

NaOH for 27 days at 26°. Freshly prepared alkali was pumped through the column for 1 or 2 hr each day to elute any substance that might have been released. The K_d values obtained in a run done on the day 27 are listed in column 1 of Table 2. The values listed in column 3 were obtained after returning to 0.2 *M* acetic acid as eluant and are nearly identical to those that were obtained before the column had been treated with alkali. The fact that the properties of the stationary phase of this new column were not altered perceptibly by the long exposure to NaOH strengthened our belief that the change that had occurred in the original column was due to the elimination of some adsorbed anion rather than to chemical changes in the structure of the gel matrix itself.

The column was then washed for several days with 0.2 *M* acetic acid containing 0.2 *M* NaCl (medium C) to test whether our hypothetical anion might be displaced by very high concentrations of salt. The K_d values observed for lysine, ammonium ion, and phenylalanine in this medium were identical to those given in Table 1 for the original column, and after returning again to 0.2 *M* acetic acid as eluant the column still gave the same K_d values as listed in columns 2 and 3 of Table 2.

Since neither dilute alkali nor the concentrated salt solution had any effect on the column—or at least none that could be detected using 0.2 *M* acetic acid as eluant—we began to suspect that the adsorbed anion might be an organic substance that was not soluble in ordinary aqueous buffers.

Of the several media to which the original column had been exposed, buffer F (1 *M* pyridine–0.03 *M* acetic acid) was the one that would be most likely to solubilize a hydrophobic molecule and displace it from the gel. Therefore, we proceeded to wash the new column with about 200 ml of buffer F. When the column was returned to 0.2 *M* acetic acid after this treatment, all the substances tested eluted almost exactly in accordance with the K_d values listed in column A′ of Table 1. This result established almost conclusively that buffer F had caused the change that had occurred in the original column, and indicated that our explanation of the change was probably correct.

All the experiments described so far had been performed with columns prepared from the same batch of gel. Although we had already established to our satisfaction that the organic anion which we had postulated as the basis of the change just described had not

been introduced into the gel by us, it was important to determine whether this substance might be present in any batch of G-10 other than the one that we had used in our initial studies. Unfortunately, the container in which the original batch of gel had arrived had been discarded without first recording the lot number, and since the sample had been a gift from the manufacturer, the lot number could not be traced subsequently. We are certain, however, that it was not lot No. 9493, which did not exist at the time when the original batch of gel was received. Since lot No. 9493 had been distributed widely and would undoubtedly be used by many other investigators, it seemed suitable for testing the general relavancy of the findings described above. We have not attempted to investigate samples of gel from all the existing lots of Sephadex G-10.

The experiments described below were performed with a fairly uniform fraction (particle diameter, 60–80 μ) of gel obtained by elutriation from a 5-kg sample of Sephadex G-10, lot No. 9493, $W_r = 1.0$ g/g. Several identical columns (bed dimensions 1.4×90 cm) were packed in 1% NaCl after the gel had been allowed to swell in that medium for several hours, usually overnight. The initial condition of each column was established by chromatographing a few representative substances in 0.2 *M* acetic acid. The sample volume was always 1.0 ml and the solute concentrations were the same as before: 0.5–2.0 m*M* for the amino acids and ammonium ion, and 1–2 mg in the case of bovine serum albumin (BSA). The K_d values obtained in these preliminary calibration runs varied significantly among the different columns tested but were in every case rather close to those listed in column A of Table 1. A typical set of values is given in column 1 of Table 3. Apparently, this lot of gel, like that studied originally, contains significant amounts of anionic groups. The BSA preparation used gave a double peak: a small peak, probably corresponding to dimer or higher polymers, appeared at the void volume and was followed closely by a much larger peak of monomer. This separation is almost certainly due to cation exchange. The cation-exchange capacity of these columns could be practically eliminated by pumping 6–7 void volumes of buffer F, or just 1 *M* aqueous pyridine, through the packed gel bed, as was the case with the original batch of gel. This is illustrated by the data presented in Table 3, which were obtained in the following sequence of operations.

The data listed in column 1 was obtained using 0.2 *M* acetic acid

TABLE 3

K_d Values Observed in Dilute Acetic Acid and in Distilled Water Before (1,3) and After (2,4) Washing Gel with 1 *M* Pyridine[a]

	Eluant			
	0.2 *M* HOAc		dist. H_2O	
Substance	1	2	3	4
BSA dimer	0	0	—	—
BSA monomer	0.04	0	—	—
Lysine	0.24	0.04	>3	0.03
Histidine	—	—	—	0.21
Valine	0.42	0.29	0.39	0.39
Phenylalanine	0.86	0.63	0.7–0.8	0.85
Tyrosine	1.2	0.86	—	1.03
Ammonium acetate	0.49	0.04	—	—
Ammonium chloride	—	—	>3	0.1

[a] Column parameters: 1.4 × 90-cm bed, bead size 60–80 μ; $V_t = 141$ ml, $V_0 = 48.0$ ml, $V_g = 93.0$ ml, $V_i = 57.5$ ml. Flow rate: 20.0 ml/hr; temp. 26–28°C.

as eluant before the gel had been exposed to any other medium except the 1% NaCl solution in which the column had been packed. The column was then washed with deionized water until the pH of the effluent leveled off at about 5.5. The data given in column 3 were then obtained using deionized water as eluant. The neutral amino acids valine and phenylalanine gave broad peaks and the elution volume of the latter substance varied considerably in consecutive runs. Furthermore, the basic substances lysine (monohydrochloride) and ammonia (ammonium chloride) had not yet appeared in the effluent when elution was discontinued at 200 ml. The retention of these cationic substances can only be ascribed to the presence of anionic groups in the stationary phase of the column.

The column was then washed with 1 *M* pyridine for 19 hr at a flow rate of 20 ml/hr. The pyridine was washed away with 0.2 *M* acetic acid and the K_d values given in column 3 were determined. All these values are considerably lower than those listed in column 1 and are even significantly lower than the corresponding values given in column A′ of Table 1, indicating that the cation-exchange capacity of the column has been reduced even more than before.

In this series of runs, BSA gave only a single peak at the void volume.

The extent to which the cation-exchange capacity had been reduced by the pyridine treatment is best illustrated by the data presented in column 4, which were obtained using deionized water as eluant after washing away the acetic acid as before. These data presumably apply for ammonium chloride, the monochloride salts of lysine and histidine, and the isoionic forms of valine, phenylalanine, and tyrosine. The K_d values listed in column A show that after the gel has been washed with pyridine the basic amino acids and ammonium chloride eluted earlier than the neutral amino acids even when deionized water was used as eluant. In fact, lysine (monochloride salt) was almost completely excluded from the gel. Furthermore, the peaks obtained with the amino acids in this series of runs were as sharp and symmetrical as those obtained in 0.2 *M* acetic acid and other buffers of higher ionic strength. The only indication that some small fraction of the original cation exchange capacity had survived the pyridine wash was the fact that the peaks obtained with ammonium chloride in deionized water showed some "trailing."

Although the cation-exchange capacity of Sephadex G-10 can thus be reduced to almost negligible levels by washing a packed bed of the gel with a few void volumes of 1 *M* pyridine, we discovered in the course of our investigations that 1 *M* pyridine is not itself a useful medium for the chromatography of amino acids on this gel. When 1 *M* pyridine was used as eluant, ammonium chloride (0.5–2 m*M*) and urea (4 m*M*) gave symmetrical peaks with K_d's of 0.34 and 0.95, respectively. However, all the amino acids tested (lysine, glycine, phenylalanine, and tyrosine) eluted in very broad "smears" with K_d's greater than 1. A possible explanation of this is that in the absence of any other cations or counterions, the amino acids are adsorbed to the gel in the form of pyridinium salts. At any rate, this undesirable effect can be eliminated by including salt (medium G) or even very small amounts of acetic acid (medium F) in the 1 *M* pyridine, as is evidenced by the K_d values given in Table 1 and the pattern shown in Fig. 2B.

We should also mention here that pyridine (the free base) adsorbs strongly to the gel and cannot be eluted completely with deionized water. However, pyridinium ion is readily removed from the

column by dilute acetic acid. For this reason, the four series of runs represented by the data given in Table 3 were performed in the order 1, 3, 2, 4. Our conclusion that all traces of bound pyridine can be removed from a column of Sephadex G-10 by passing a few void volumes of 0.2 *M* acetic acid through it is based on the following experiment. A 3.2 × 36.5-cm bed of Sephadex G-10 was packed in deionized water. A 5-ml sample of 0.001 *M* pyridine was then applied and 200 ml of deionized water was pumped through the column at a rate of 45 ml/hr. No pyridine appeared in the effluent. Then 0.2 *M* acetic acid was pumped into the column at the same flow rate. The pyridine emerged from the column in a sharp peak at the breakthrough of the acetic acid. The recovery was 103%, as determined by absorbance measurements at 260 mμ. When 5 ml of 0.001 *M* pyridine was chromatographed on the same column using 0.2 *M* acetic acid as eluant throughout, spectrophotometric analysis of the effluent at 260 mμ showed a single sharp, symmetrical peak with a K_d of 0.36. Again, the recovery was quantitative. We have not chromatographed pyridine under these conditions on a column that had been washed previously with 1 *M* pyridine, but presumably the K_d would be less than 0.36 in such a case.

The experiments just described show that the reduction in cation-exchange capacity that occurs upon washing the gel with 1 *M* pyridine is not due to the retention of pyridine. Our hypothesis that aqueous pyridine effects this reduction in cation-exchange capacity by eluting a hydrophobic anion from the gel is supported by the fact that no significant reduction in cation-exchange capacity was observed when an untreated column was washed with 6 void volumes of medium G (1 *M* pyridine containing 0.5 *M* NaCl). Here the solubilizing influence of the pyridine is apparently cancelled by a salting-out effect. The K_d values given in columns A′, B, and C of Table 1 show that salt increases the retention of most of the substances studied. The amino acids isoleucine, leucine, and norleucine, which have relatively large hydrophobic aliphatic side chains, are caused to adsorb to the gel by high concentrations of salt. The aromatic amino acids, which tend to adsorb even in the absence of salt, are adsorbed much more strongly when salt is included in the medium. In the presence of 2 *M* NaCl, tryptophan has a K_d of about 9.

The various observations described above provide the basis for

our hypothesis that Sephadex G-10, as supplied by the manufacturer, contains small amounts of some hydrophobic anion adsorbed to the gel matrix, and that most of this substance is eluted when the gel is washed with aqueous pyridine. We have not yet attempted to test this hypothesis directly by examining the pyridine washings for the presence of some such anion. However, if the gel does contain such an anion, then we can offer a reasonable guess as to what it is and how it comes to be there.

An emulsifying agent is used to stabilize the gel suspension during the preparation of Sephadex G-10 and G-15. Probably some of the ester linkages of the emulsifying agent are hydrolyzed by the strongly alkaline medium that is present in the gel beads during the cross-linking reaction. Most of the sodium oleate that would arise from this reaction would be expected to remain in the aqueous phase around and within the gel beads, since the salt is not very soluble in the organic solvent used as the suspending phase. Sodium dodecyl sulfate is known to adsorb very strongly to Sephadex G-10, and one would expect sodium oleate to behave similarly. Furthermore, any oleate ions that might remain free in the aqueous phase under strongly alkaline conditions would certainly adsorb to the gel as oleic acid when the medium is acidified at the end of the cross-linking process. We therefore propose that most, if not all, of the small cation-exchange capacity exhibited by Sephadex G-10 is due to the presence of adsorbed oleic acid and that most of the oleic acid is eluted when the gel is washed with 1 *M* pyridine. The effects of detergents on Sephadex gels have not yet been investigated thoroughly, but several observations suggest that such substances reduce the ability of the gels to swell in water. Flodin has observed that beds of swollen gel can be caused to collapse suddenly by passing detergent solutions through them.* Apparently the detergents adsorb to the gels and make them hydrophobic, thereby reducing their natural tendency to "dissolve" by imbibing water.

In general, one should take care to avoid inadvertently contaminating Sephadex gels with detergents and other sparingly soluble substances containing sizable hydrophobic moieties (e.g., long-chain fatty acids), particularly those which also contain charged or ionizable groups. However, as we mentioned earlier, the K_d

* P. Flodin, personal communication.

values obtained in the original series of experiments (series A in Table 1), performed in 0.2 *M* acetic acid before the gel had been exposed to the pyridine buffer, were as reproducible and the elution patterns were as sharp and symmetrical (Fig. 2A) as any obtained subsequently. Furthermore, these K_d values reflect a greater selectivity than those obtained in the same medium (series A') when the gel presumably no longer contained bound oleic acid. If the oleic acid hypothesis is correct, then it might be feasible to prepare useful weak cation and anion exchangers from Sephadex by deliberately adsorbing appropriate anions or cations to the gel. By the use of a contacting procedure that would provide a uniform distribution of the adsorbed ion among the gel beads, the capacity of the gel could be controlled to an exquisite degree. Small amounts of charged groups introduced in this way might often enhance the selectivity of the tightly cross-linked gels toward small charged solutes, and could therefore be useful for micro-scale separation work in media of low ionic strength.

If weak cation-exchange effects are not desired, we recommend that existing batches of Sephadex G-10 and G-15 be washed with 1 *M* pyridine before they are used. The washing can be done most efficiently by passing at least six void volumes of the aqueous pyridine through the packed gel bed. The pyridine concentration is not critical, but concentrations much lower than 1 *M* (80.6 ml/liter) are less efficient and higher concentrations are more likely to damage any plastic components (e.g., Perspex columns, and the flexible tubing in the peristaltic pump) that might be present in the chromatography apparatus used.

The cation-exchange capacity of a Sephadex column can be checked conveniently by chromatographing a small sample of dilute ammonium chloride (<0.005 *M*) using distilled water as eluant. The two lots of Sephadex G-10 that we have tested give K_d values less than 0.1 for this substances after being washed with pyridine as described above.

The Composition of the Sample. For reasons that will be discussed below, the behavior of small charged solutes on tightly cross-linked gels such as Sephadex G-10 depends very much on the ionic strength and ion composition of the solvent medium, even when immobile ionic groups are absent. If the solute is one whose charge changes with pH (e.g., a weak acid or base, or an ampholyte such as the amino acids studied here), its elution behavior will generally

vary with pH in any interval where the charge changes with pH. To obtain reproducible results with such solutes, the sample must always be applied in exactly the same medium and, in general, the sample medium should not differ from that which is to be used for elution. If the sample solution contains buffer salts that are not present in the eluting medium, pH variations can occur in the column even if the sample solution and the eluting medium have the same pH initially.

Figure 5 shows the pH and conductivity changes that were observed in the effluent when 110 mg of NaCl was chromatographed on a column equilibrated with 0.2 *M* acetic acid. The sample was applied in the same medium. The pH and conductivity curves show that the NaCl–acetic acid sample solution has separated into sodium acetate, NaCl, and HCl; and if the complete 230-mμ trace were shown, one would observe a broad negative peak at about 86 ml, which is the elution position of acetic acid. The acetic acid that was present in the sample solution would have eluted there if it had not migrated as sodium acetate instead. The tall conductivity peak labeled NaCl is actually a double peak. The front half consists of NaCl in equilibrium with 0.2 *M* acetic acid. The back half of the double peak contains HCl, as is evidenced by the fact that the pH drops below 1 in this region. When 35 mg of NaCl was run under the same conditions, the sodium acetate peak and the negative peak in the acetic acid position were nearly identical to those obtained in the run with the larger sample. However, the front part of the NaCl-HCl doublet was absent, leaving only a tall sharp peak of HCl. In this case, all the sodium ion had migrated as sodium acetate. When 110 mg of NaCl was applied in distilled water instead of 0.2 *M* acetic acid, the NaCl eluted in a single peak, and no distinct peak of sodium acetate was present. However, a zone of increasing conductivity at the leading edge of the NaCl peak and a slight dip in pH at the back revealed, respectively, the sodium acetate and HCl that had formed continuously on the column during the run. Large samples of ammonium chloride gave pH and conductivity patterns very similar to those shown for sodium chloride.

Figure 5 also shows how the behavior of tyrosine and phenylalanine is altered by the inclusion of large amounts of salt in the sample solution.

Such effects obviously complicate the use of Sephadex G-10 for

desalting molecules that are small enough to penetrate the gel. In cases where it is necessary to apply the sample in a medium different from that with which the column is equilibrated, much confusion can be avoided by monitoring the pH, conductivity, or some other appropriate property that could reveal any changes in conditions that might be produced by the "foreign" constituents of the sample medium.

It should be both fascinating and rewarding to investigate the behavior of mixed ion systems of Sephadex G-10.

DISCUSSION

The various technical and practical aspects of the chromatography experiments described above have already been discussed in considerable detail and require no further comment. The contribution of cation-exchange effects to the K_d values listed in column A of Table 1 has also been discussed and these data will not be considered further, since electrostatic interactions obscure other more interesting effects. However, we doubt that the data given in columns A'–I of Table 1 contain any significant contribution of electrostatic solute-gel interactions, and these latter data shown some definite trends that permit a few reasonable generalizations regarding the nature of the interactions that determine the behavior of small solutes on tightly cross-linked xerogels.

Fundamental Importance of Effective Molecular Size

With Sephadex G-10, as with gels of higher water regain, the behavior of a solute is fundamentally determined, or perhaps it is more accurate to say *restricted,* by its *effective* size. Beyond that, the range of behavior that a given molecule might display on a gel that it is able to penetrate depends on whether it contains structures that are susceptible to adsorption, and whether its effective size can be altered by changing the solvent environment. If a molecule contains an exposed group, such as an aromatic ring, that is susceptible to adsorption, adsorption will occur if this group is able to come into intimate contact with the gel matrix. The amount of adsorption that will occur with a molecule that contains adsorbable structures thus depends on the amount of gel that is accessible to the solute: the greater the penetration, the greater the adsorption.

This is illustrated nicely by comparing the behavior of tryptophan

and the tripeptide, H-Val-Try-Arg-OH, on Sephadex G-25 and Sephadex G-10 in 0.2 *M* acetic acid. The indole group of tryptophan interacts rather strongly with the dextran matrix, and tryptophan consequently adsorbs to all of the dextran gels. Tryptophan penetrates G-25 almost completely; that is, it has access to most of the available water present in the gel grains and can therefore come into contact with a large fraction of the gel matrix. In 0.2 *M* acetic acid, the K_d of tryptophan on G-25 is 1.85, indicating appreciable adsorption. The adsorptive retardation is even greater with Sephadex G-10, where tryptophan elutes with a K_d of 2.74 under the same conditions. Tryptophan probably has access to less than half of the available water present in the G-10 grains, but apparently the smaller extent of penetration is more than compensated by the greater density of the G-10 matrix, with the result that the absolute amount of matrix material available to the indole group is greater in the G-10 gel.

The tripeptide, H-Val-Try-Arg-OH, also penetrates Sephadex G-25 enough to allow substantial adsorption, as indicated by the K_d of 1.18. On G-10, however, its K_d is only 0.42. The relatively bulky tripeptide does not penetrate G-10 sufficiently to allow much adsorption. The retardation of the peptide on G-10 could undoubtedly be increased considerably by including high concentrations of salt in the eluting medium.

Effect of Salt on Sterical Parameters of Solutes and Gel

A comparison of the K_d values listed in columns A′, B, and C of Table 1 shows that salt increases the retention of nearly all the solutes studied. This can be explained in the following way.

The effective size of an ion in a sterically hindered diffusion process must include the electrical double layer and the hydration layer surrounding the ions. The thickness of these layers and hence the effective size of the ion can be decreased by increasing the ionic strength of the medium and, in the case of the electrical double layer, by using smaller counter- and coions. All the substances studied except glucose, urea, and aspartic acid are cationic at pH 2.7, the pH of 0.2 *M* acetic acid, and it is these substances that are least effected by salt. When sodium chloride is added to the medium, the effective size of the cationic solutes is decreased in two ways.

First, the chloride ion is smaller than the acetate ion, so the chloride salts can achieve greater penetration than the acetate salts. The importance of the counterion is illustrated by some elution data obtained with ammonium chloride, sodium chloride, ammonium acetate, sodium acetate, HCl, and acetic acid on the long G-10 column. The sample volume was 1 ml and the sample concentration was 1 *M* in all cases. The acetate runs were done in 0.2 *M* acetic acid and the chloride experiments were run in 0.001 *M* HCl. Ammonium acetate and sodium acetate both showed a K_d of 0.47, while ammonium chloride and sodium chloride had a K_d of 0.66. HCl eluted with a K_d of 0.70. Acetic acid adsorbs slightly and elutes with a K_d of 1.0. The corresponding sodium and ammonium salts elute in the same position because the hydrated sodium and ammonium ions happen to have very nearly the same size. The chloride salts elute later than the acetates because of the smaller size of the chloride ion as compared to the acetate ion.

However, the elution position of a given salt, such as ammonium chloride, also depends on the ionic strength of the medium, and consequently in media of very low ionic strength the elution position of the salt will vary with the concentration of the sample itself. When a very dilute sample ($< 0.005\ M$) of ammonium chloride is chromatographed on a pyridine-washed G-10 column in distilled water or in 0.001 *M* HCl, the K_d is less than 0.1. But a sample of 1 *M* ammonium chloride eluted with a K_d of 0.66 in the latter medium, as was mentioned above. In the presence of 0.5 *M* and 2.0 *M* NaCl, 0.005 *M* ammonium ion eluted with K_d's of 0.68 and 0.79, respectively. Apparently the effective sizes of the individual ions of the salt decrease with increasing ionic strength, probably as a result of decreases in the extent of hydration.

At any rate, in media of low ionic strength the effective size of a small molecule that contains charged groups is considerably greater on a relative basis than the nominal size implied by the molecular weight. The magnitude of the contribution that a charged group makes to the effective size of a small molecule is indicated by comparing the K_d's of glucose and glucosamine in 0.2 *M* acetic acid, where the amino group of the latter substance is ionized. In this medium glucose elutes with a K_d of 0.40, but glucosamine has a K_d of 0.07, indicating almost complete exclusion. Upon the addition of salt, however, the effective size of the glucosamine ion

is reduced to the extent that it elutes in the same position as glucose. This is partly due to the smaller size of the chloride ion as compared to the acetate ion, but the main effect is probably a decrease in the hydration of the amino group.

It is somewhat more difficult to account for the fact that the K_d of glucose is also increased in the presence of salt. However, it might be that salt also decreases the hydration of the glucose molecule. Another possibility is that relatively high concentrations of salt decrease the extent of the hydration layer in the dextran gel, thereby increasing the amount of solvent in which penetrant species can diffuse freely. It seems very probable that in the absence of salt the gel phase contains pockets of highly organized water. Although there is no adequate quantative theory for the effect of electrolytes and polar solutes on water structure, there is little doubt that such substances reduce the ability of water to form organized domains (27). We suggest that at relatively high concentrations small ions and other small but highly polar molecules are able to penetrate and disrupt these pockets of organized water. The structure-breaking effect of concentrated salt would thus increase the amount of space available to a dilute sample of glucose. Probably high concentrations of glucose itself would accomplish the same thing. This is only one of the many points that requires further investigation.

At any rate, it seems safe to conclude that high concentrations of small electrolytes increase the degree to which small polar molecules, particularly those bearing charged groups, are able to penetrate the gel. At low ionic strength, highly charged molecules tend to be excluded if they are run at low concentration. In 0.2 *M* acetic acid, all the basic amino acids elute with K 's less than 0.1, and at alkaline pH (medium I) glutamic acid and aspartic acid elute earlier than all other amino acids. Tyrosine also tends to be excluded at high pH, where the phenolic group is ionized and the molecule consequently has a double negative charge.

If a charged molecule contains some structure that is susceptible to adsorption (e.g., an aromatic ring), adsorption can increase enormously when the excluding effect of the charge is overcome and more gel becomes accessible to the molecule. At acid pH, the aromatic amino acids tyrosine and phenylalanine show moderate adsorption and tryptophan adsorbs rather strongly in the absence

of salt, but the K_d increase threefold in the presence of 2 *M* NaCl. In medium C, tryptophan has a K_d of 8–9.

Urea also adsorbs to the gel in all simple aqueous media, but as it has no charge, the degree of penetration, and hence the amount of adsorption do not increase significantly in the presence of salt. The guanidino group has a coplanar π-electron system similar to that of urea, and the behavior of arginine relative to the other basic amino acids in media B and C indicates that high concentrations of salt allow arginine to adsorb to the gel. The fact that tetraglycine is affected more by salt than is glycine suggests that the peptide bond is also susceptible to adsorption.

The adsorption of the aromatic acids and urea is decreased in the presence of high concentrations of pyridine, as is evidenced by the K_d values obtained in media F and G. It is not easy to explain why tyrosine and phenylalanine show lower K_d's in the latter medium than they do in the former. However, at pH 8.5, the amino acids are negatively charged and in the presence of salt they are probably forced to migrate in association with sodium ions, while the adsorption capacity of the gel is largely satisfied by the neutral pyridine molecule.

An inspection of the data in Table 1 shows clearly that in media of low ionic strength and in the absence of specific "displacers," such as pyridine, the amino acids are most retarded at pH's where they have the smallest net charge, i.e., at their isoelectric points. At pH's far removed from their isoelectric points, the excluding effect of the charge can be reduced or eliminated by increasing the ionic strength of the medium. If the role of salt in increasing the retention is indeed passive, then salt should have little effect on the behavior of an amino acid at its isoelectric point. This seems to be the case with the hydrophilic amino acids. For example, aspartic acid is nearly isoelectric at pH 2.7, and salt has no effect on its elution behavior at the pH.

"Hydrophobic" Adsorption

When the solute contains a sizable hydrophobic moiety, however, salt seems to actively promote adsorption by a "salting-out" effect. The solubility of hydrocarbons in water depends on the ability of water to form organized domains, as evidenced by their negative entropies of solution, and the solubility of nonpolar molecules in

water is thus reduced considerably by "structure-breaking" substances such as salt. The aliphatic amino acids isoleucine, leucine, and norlecucine adsorb to the gel in medium C because their hydrocarbon side chains prefer regions within the gel where there is no salt, or, as Marsden put it (9), regions where they are partly not surrounded by water. Part of the increase in adsorption that occurs with the aromatic amino acids in the presence of salt might also be due to a salting-out effect.

Practical Applications

By manipulating the pH and composition of the eluting medium, one ought to be able to separate almost any pair of small molecules, except perhaps such substances as isomeric neutral hexoses and pentoses, etc., on Sephadex G-10. The gel should be very suitable for isolating small water-soluble organic substances from reaction mixtures, and might therefore be particularly useful in synthetic work.

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REFERENCES

1. B. Gelotte and J. Porath, in *Chromatography* (E. Heftman, ed.), 2nd ed., Reinhold, New York, in press.
2. J. Porath and P. Flodin, *Nature*, **183**, 1652 (1959).
3. P. G. Squire, *Arch. Biochem. Biophys.*, **99**, 466 (1962).
4. J. Porath, *J. Appl. Chem.*, **6**, 233, (1963).
5. T. C. Laurent and J. Killander, *J. Chromatog.*, **14**, 317 (1964).
6. G. K. Ackers, *Biochemistry*, **3**, 723, (1964).
7. L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta*, **112**, 346 (1966).
8. K. A. Granath and P. Flodin, *Makromol. Chem.*, **48**, 160 (1961).
9. N. V. B. Marsden, *Ann. N.Y. Acad. Sci.*, **125**, 428 (1965).
10. J. S. Fawcett and C. J. O. R. Morris, *Separation Sci.*, **1**, 9 (1966).
11. H. Determann and W. Michel, *J. Chromatog.*, **25**, 303 (1966).
12. R. L. M. Synge and M. A. Youngson, *Biochem. J.*, **78**, 31P (1961).
13. M. Bagdasarian, N. A. Matheson, R. L. M. Synge, and M. A. Youngson, *Biochem. J.*, **91**, 91 (1964).
14. P. R. Carnegie, *Biochem. J.*, **95**, 9P (1965).

15. V. Stepanov, D. Handshuh, and F. A. Anderer, *Z. Naturforsch.*, **16b**, 626 (1961).
16. G. H. Lathe and C. R. J. Ruthven, *Biochem. J.*, **62**, 665 (1956).
17. J. Porath, *Biochim. Biophys. Acta*, **39**, 193 (1960).
18. B. Gelotte, *J. Chromatog.*, **3**, 330 (1960).
19. F. Miranda, H. Rochat, and S. Lissitzky, *J. Chromatog.*, **7**, 142 (1962).
20. H. J. Cruft, *Biochim. Biophys. Acta*, **54**, 611 (1961).
21. M. A. Ruttenberg, T. P. King, and L. C. Craig, *Biochemistry*, **4**, 11 (1965).
22. J. Porath and H. Bennich, *Arch. Biochem. Biophys. Suppl.*, **1**, 152 (1962).
23. A. M. Crestfield, *Anal. Chem.*, **35**, 1762 (1963).
24. E. Glueckauf, in *Ion Exchange and Its Applications*, Society of Chemical Industry, London, 1955.
25. H. Walton, in E. Heftman (eds.), *Chromatography*, Reinhold, New York, 1961, p. 277.
26. P. Flodin, *Dextran Gels and their Application in Gel Filtration*, AB Pharmacia, Uppsala, Sweden, 1962.
27. H. A. Scheraga, *Ann. N.Y. Acad. Sci.*, **125**, 253 (1965).

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