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CHROMATOGRAPHY OF SOME QUATERNARY DRUGS ON COLUMNS OF SEPHADEX G-10

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SUMMARY

The chromatographic behaviour of some pyridinium oximes and related quaternary drugs on columns of Sephadex G-10 was studied. Comparable data for the elution of some of the drugs from a CM-cellulose column were also obtained. Hydrogen bonding between the acidic hydroxyl groups and the Sephadex gel seemed to be the major controlling factor in the chromatography. Interaction between the gel and aromatic nuclei in the drugs, and solute size, also played a part in determining the elution positions of samples. Quaternary compounds not possessing hydroxyl groups or aromatic rings tended to be eluted rapidly without separation. The system worked well, however, for the separation of a range of quaternary drugs and should be useful for analytical and small-scale preparative work.

INTRODUCTION

The tightly cross-linked dextran gel Sephadex G-10 has been found suitable for the chromatographic separation of amino acids and similar compounds[1]. Several authors have also reported on the properties of this and similar gels regarding the separation of inorganic salts[2,3], which has practical significance in the preparation of low-molecular-weight organic compounds free from inorganic salts[4]. These reports suggested that chromatography on Sephadex G-10 might be useful in the study of quaternary salts, many of which are employed as drugs for various purposes. This paper therefore describes some of the factors which influence the elution of quaternary drugs from Sephadex G-10 columns. Much of the work was performed on the cholinesterase-reactivating drug PAM (2-hydroxyiminomethyl-1-methylpyridinium chloride) and related compounds with ionizable acidic side groups. The columns were eluted with buffers of ionic strength similar to that of physiological saline solution to avoid some of the adsorption phenomena known to occur[5], and also to ensure that the drug cations would have the same degree of hydration as occurs in biological systems. This latter condition was necessary as the possibility of estimating the relative sizes of ions from the chromatographic data was considered.

EXPERIMENTAL

Drugs used and their estimation

PAM was recrystallized from a product of K and K Laboratories, U.S.A. It was estimated spectrophotometrically in column fractions by measurement of the optical density (O.D.) at 336 nm of the samples made alkaline with NaOH. TMB-4 [1,1'-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide)] was synthesized by Dr. M. JARVIS at these laboratories. It was assayed in the manner used for PAM, measurements being made at a wavelength of 347 nm. Edrophonium (N-ethyldimethyl-3-hydroxyanilinium chloride) from Hoffmann-La Roche, Switzerland, was estimated from the O.D. of alkaline solutions at 294 nm. Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) and diquat (1,1'-ethylene-2,2'-bipyridinium dibromide) were obtained from I.C.I. Ltd. Paraquat was assayed by measuring the O.D. at 604 nm after adding 0.2 ml of 1 % sodium dithionite in 0.1 *N* NaOH to 2.5 ml of the column fraction. The concentration of diquat was assayed from the O.D. of solutions at 310 nm. Other drugs, obtained from commercial sources, were assayed semi-quantitatively by a manual version of the bromocresol green method[6]. Samples containing alkali metal salts were quantitatively assayed by flame photometry.

Structures of some of the compounds are shown in Fig. 1.

Column chromatography

Sephadex G-10, particle size 40–120 μm, (AB Pharmacia, Uppsala, Sweden) from lot number 8062 was used as the stationary phase. The dextran was allowed to swell in 150 m*M* NaCl, 10 m*M* Tris–HCl, pH 7.4, and the supernatant was decanted off after the bulk of the solid had settled. The decantation was repeated twice to remove very fine particles. The gel was then packed into a glass column of 2.52 cm^2 cross-sectional area. The height of the gel bed was approximately 36 cm, the exact height

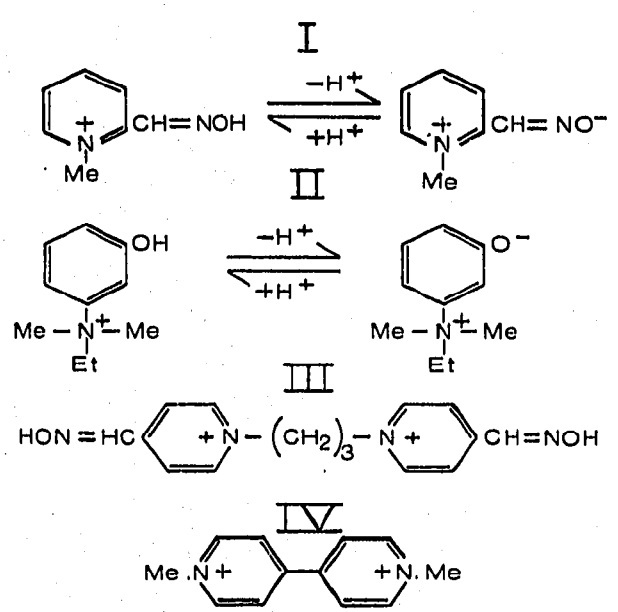

Fig. 1. The structure of some of the compounds discussed in the text. I, the two ionic forms of PAM (pK 8.0). II, the ionic forms of edrophonium (pK 8.25). III, the cationic form of TMB-4. IV, the paraquat cation.

being recorded during the experiments. The column was connected by polyethylene tubing to a peristaltic pump through which buffer was pumped at a rate of 5.0 ml/h. The freshly packed column was eluted with buffer for seven days before a sample was applied. Samples were invariably applied in a volume of 1 ml of the buffer used for elution. Concentrations of 5×10^{-4} *M* of pyridinium compounds, 1×10^{-3} *M* of edrophonium or 2×10^{-3} *M* of alkali metal chlorides were used in the samples. The pH and ionic strength of the samples were therefore nearly identical with those of the eluting buffers. Samples were applied after allowing the buffer to drain from the top of the column bed and were washed in with two 0.5-ml portions of buffer before reconnecting the pump system. The column was eluted at the rate of 5.0 ml/h and the effluent was collected in 30-min periods. The exact volume of the fractions was checked during each run by including some graduated tubes in the fraction collector. The composition of the buffers employed is given in the RESULTS section; when the sample was sodium chloride, the ionic strength was maintained by the use of potassium chloride. To avoid any interaction among samples on the column, a separate chromatographic run was performed for each sample. The temperature was 20 ± 2° throughout the experiments.

The chromatographic technique was later refined by employing a column with negligible dead space at top and bottom, and injecting the sample into the supply tubing through a two-way tap. The column (Wright Scientific Ltd., Great Britain) had a cross-sectional area of 4.00 cm^2. The bed height was 49.8 cm when packed at a flow rate of 14 ml/h. This apparatus was used to investigate the separation of mixtures of drugs.

The void volumes of the columns were determined at frequent intervals by measuring the elution position of 0.2 % Blue Dextran 2000 (Pharmacia). The pyridine-treated columns adsorbed Blue Dextran irreversibly; for these columns, the void volume was determined by the use of the polydextran Macrodex (Pharmacia) at a concentration of 0.01 %. The dextran was determined in the effluent by the anthrone reaction.

Calculation of results from column chromatography

The concentration of the sample substance in the collected fractions was plotted against the elution volume. The centre of the eluted peak was determined at a level of one third of the total peak height (Fig. 2).

After use for two months, the column was eluted with distilled water until the sodium chloride concentration in the effluent was less than 5×10^{-5} *M*. The packing was then removed and dehydrated in ethanol–water mixtures, dried at 70° and weighed.

The results are expressed in terms of the penetration of the sample solute into the solvent within the gel grains. This calculation gives a figure equivalent to the K_d value, but in these experiments the results were influenced by factors other than partition due to ionic size. Therefore the term K used here is apparently similar to K_d, but is not a simple partition coefficient. $K = (V_e - V_0)/V_t$ where V_e is the elution volume of the solute as calculated above, and V_0 is the void volume of the column. The internal volume, V_t, was estimated from the weight of dry gel, employing a value of 1.64 g/ml for its density[7]. $V_t = V_t - (V_0 + V_g)$, where V_t is the total bed volume and V_g is the volume occupied by the solid gel structure. From V_t, the water regain of

the gel could be calculated; from four column packings, a mean value of 0.95 ml/g dry gel was obtained. For each sample, two determinations of K were made at different times; mean values are quoted here.

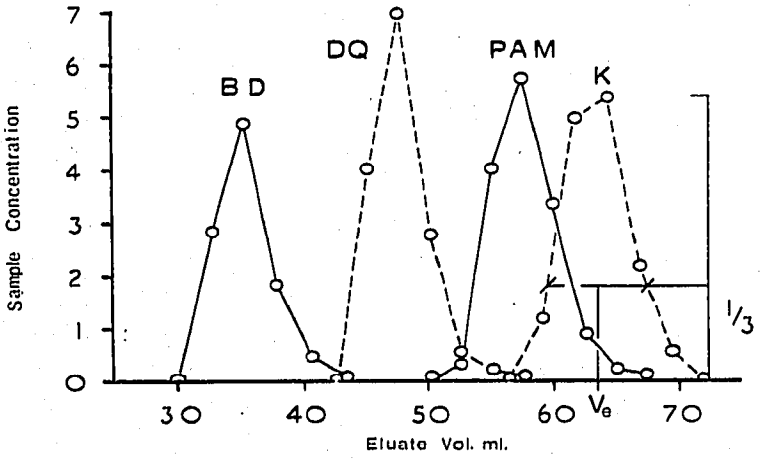

Fig. 2. Composite diagram to illustrate the chromatography of cations on Sephadex G-10. Four separate chromatographic runs are included. The cross-sectional area of the column bed was 2.52 cm^2 and the bed volume 91 ml. The vertical axis represents sample concentration in terms of optical density (see EXPERIMENTAL section) or molar concentration; the value of one unit on the scale is given below. BD = Blue Dextran 2000 (O.D. 0.05), DQ = diquat (O.D 0.25), PAM = 2-hydroxyiminomethyl-1-methylpyridinium chloride (O.D. 0.20), K = potassium chloride (one unit represents 5×10^{-5} M). The construction on the right of the diagram shows the method of finding the elution volume; that is at the centre of the peak at a height of one third of the maximum height.

Pyridine treatment of Sephadex G-10 columns

A column was packed with gel in 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, as described above. The eluting solution was then changed to 1.0 M pyridine, 0.03 M acetic acid, pH 6.7, and three bed volumes of this buffer were passed through the column at 5 ml/h. The buffer was then changed back to the original one and the column was washed for one week at the same rate. The column was then used for determining K values as described previously. After one set of figures was obtained, the pyridine treatment was repeated in a slightly different fashion. The column was eluted with 240 ml of 1 M pyridine, followed by 240 ml of 0.2 M acetic acid. Then it was washed with the original buffer, and a second set of K values was determined.

Chromatography on a column of CM-cellulose

Whatman carboxymethylcellulose (CM 11, W. and R. Balston Ltd., Great Britain) was stirred in a large volume of 0.1 N NaOH, then allowed to settle. The supernatant was decanted and replaced with 150 mM NaCl, 10 mM Tris–HCl, pH 7.4. After several washes with this buffer, the cellulose was packed into a column of cross-sectional area 2.08 cm^2 to a height of 16.5 cm. The column was washed with buffer using a pressure of 8 cm of water, which gave a flow rate of 0.5 ml/min. Samples were applied to the column and elution volumes determined essentially as described above for Sephadex G-10. Results were expressed as the elution volume (V_e) divided by the total bed volume (V_t), taking care to measure the latter at the same state of compression each time. Two determinations were made for each sample.

RESULTS AND DISCUSSION

The samples of organic cations and alkali metal ions were eluted from the G-10 columns as sharp, slightly asymmetrical peaks (Fig. 2). It was possible to determine the elution volumes with good reproducibility; duplicate measurements of K did not vary by more than 0.03 and were usually within 0.01. The sample recovery averaged 97 %. There was an indication that the recovery of Blue Dextran 2000 was lower on freshly packed columns, for which values of 62 %, 47 % and 71 % were obtained. The recovery of the Blue Dextran from the pyridine-treated column was nil.

The elution rate was normally 5.0 ml/h. However, an increase in rate to 20 ml/h did not affect the chromatography of PAM. No change in K for PAM was found when the sample load was increased from 1 ml of 5×10^{-5} M to 1 ml of 1×10^{-2} M (8.6 μg to 1.72 mg of the chloride).

When the effect of varying the buffer salt composition was studied, it was found that neither the substitution of potassium for sodium (Table I, results 1 and 2) nor the use of imidazole in place of Tris (Table II) affected the elution of PAM. In contrast, the total replacement of chloride with iodide greatly increased K for PAM and sodium (Table I, results 4 and 12). Partial replacement with iodide had no effect (result 3).

TABLE I

THE APPARENT PARTITION COEFFICIENTS FOR VARIOUS CATIONS ON SEPHADEX G-10 COLUMNS

The buffers used contained 10 mM Tris with additional salts as indicated, adjusted to the required pH with HCl.

Sample	*pH*	*Buffer salt composition*	*K*
1 PAM	7.4	150 mM NaCl	0.67
2 PAM	7.4	150 mM KCl	0.66
3 PAM	7.4	135 mM KCl, 15 mM KI	0.66
4 PAM	7.4	150 mM KI	1.25
5 Paraquat	7.4	150 mM NaCl	0.32
6 Paraquat	8.8	150 mM NaCl	0.325
7 Diquat	7.4	150 mM NaCl	0.34
8 Edrophonium	7.4	150 mM NaCl	1.03
9 Edrophonium	8.8	150 mM NaCl	0.735
10 Sodium	7.4	150 mM KCl	0.72
11 Sodium	7.4	10 mM KCl	0.93
12 Sodium	7.4	150 mM KI	1.445
13 Potassium	7.4	150 mM NaCl	0.845
14 Potassium	8.8	150 mM NaCl	0.82
15 Lithium	7.4	150 mM NaCl	0.665

When the ionic strength of the eluting buffer was reduced drastically, an increase in the K of PAM (Table III) and of sodium (Table I) was noted. This result was expected, since many other substances are known to adsorb to Sephadex gels at low ionic strength[5]. There was also a slight increase of K in 1 M NaCl, probably due to the salting out of the sample from the solution into bound water or more hydrophobic areas of the gel structure.

The pH of the eluting buffer had the greatest influence on the elution of the

TABLE II

THE EFFECT OF THE pH OF THE ELUTING BUFFER ON THE ELUTION POSITION OF PYRIDINIUM OXIMES FROM SEPHADEX G-10

The buffer composition was 10 m*M* Tris or imidazole with 150 m*M* NaCl, adjusted to the required pH with HCl. For some experiments, 0.5 *M* NaCl was used in the buffer.

Sample	*pH*	*Buffer*	*K*	*Percent ionization of oxime groups*
PAM	6.0	Imidazole	0.71	1
	6.4	Imidazole	0.68	2.5
	7.4	Imidazole	0.665	20
	7.4	Tris	0.67	20
	8.0	Imidazole	0.575	50
	8.8	Tris	0.535	86
PAM	7.4	Tris, 0.5 *M* NaCl	0.67	20
	8.8	Tris, 0.5 *M* NaCl	0.50	86
TMB-4	6.0	Imidazole	0.735	1
	7.4	Tris	0.695	10
	8.8	Tris	0.39	75.5

TABLE III

THE EFFECT OF SALT CONCENTRATION IN THE ELUTING BUFFER ON THE ELUTION OF PAM FROM SEPHADEX G-10

The buffers were composed of 10 m*M* Tris with NaCl in the concentration indicated, adjusted to the required pH with HCl.

NaCl concentration	*pH*	*K*
3.5×10^{-5} *M*[a]	6.8	$\geqslant 6$
10 m*M*	7.4	0.775
50 m*M*	7.4	0.63
150 m*M*	7.4	0.675
0.5 m*M*	7.4	0.67
1.0 *M*	7.4	0.72
150 m*M*	8.8	0.535
0.5 *M*	8.8	0.50

[a] This concentration was obtained by eluting the column exhaustively with distilled water. The sample was applied in distilled water.

pyridinium oximes (Table II). The effect occurred around the p*K* value of the PAM oxime group (8.0), and since there is no reason to suppose that the dextran gel itself was affected in this pH range, the phenomenon must be solely related to the ionization of the oximes[8]. For PAM as a cation, *K* was 0.71, but when the compound was nearly all in the zwitterionic form, *K* was 0.535. The change in *K* for TMB-4 was even greater, from 0.735 to less than 0.39. The effect persisted in 0.5 *M* NaCl. Edrophonium responded in the same way to change in pH (Table I), which was to be expected as the p*K* of its phenolic hydroxyl is 8.25. This drug demonstrated unequivocally a departure from simple partition due to size, since the *K* of 1.03 at pH 7.4 must have

been due to some form of adsorption or retention within the gel. As expected, the elution of neither potassium nor paraquat was affected by change in pH (Table I).

The Sephadex results were compared with similar chromatographic experiments on CM-cellulose (Table IV), on which material the ionic size of the sample would not have a direct effect on the elution. In fact, the results were consistent with purely cation-exchange effects, the elution volumes being in the order paraquat > potassium > sodium > PAM cation > PAM zwitterion. This was the same order as for elution from the Sephadex column, except for the anomalous position of paraquat. It is worth noting that the use of iodide in place of chloride did not alter the chromatography of

TABLE IV

THE ELUTION OF VARIOUS CATIONS FROM A COLUMN OF CM-CELLULOSE IN BUFFERS OF DIFFERENT COMPOSITIONS

Buffers were composed of 10 m*M* Tris or imidazole with additional salts as indicated and were adjusted to the required pH with HCl. The results are given as the elution volume expressed as a fraction of the total bed volume (V_e/V_t).

Sample	*pH*	*Buffer salt composition*	V_e/V_t
PAM	6.0	150 m*M* NaCl	1.165
PAM	7.4	150 m*M* NaCl	1.13
PAM	8.8	150 m*M* NaCl	0.95
PAM	7.4	150 m*M* KI	1.12
Paraquat	7.4	150 m*M* NaCl	1.83
Sodium	7.4	150 m*M* KCl	1.26
Potassium	6.0	150 m*M* NaCl	1.365
Potassium	7.4	150 m*M* NaCl	1.40

TABLE V

THE ELUTION OF A VARIETY OF QUATERNARY DRUGS FROM THE LARGE COLUMN OF SEPHADEX G-10

The eluting buffers were 150 m*M* NaCl, 10 m*M* imidazole (pH 6.0) or 150 m*M* NaCl, 10 m*M* Tris HCl (pH 8.8).

Sample	*pH*	*K*
PAM	6.0	0.74
Edrophonium	6.0	1.15
Edrophonium analogue[a]	6.0	1.12
Pyridostigmine	6.0	0.385
Paraquat	6.0	0.31
Succinylcholine	6.0	0.165, 0.32[b]
Pentolinium	6.0	0.21
Decamethonium	6.0	0.205
Hexamethonium	6.0	0.19
Gallamine triethiodide	6.0	0.11
PAM	8.8	0.50
TMB-4	8.8	0.365
Edrophonium analogue[a]	8.8	0.685

[a] The triethyl analogue of edrophonium, N-triethyl-3-hydroxyanilinium chloride.
[b] Succinyldicholine and the monocholine ester produced by hydrolysis.

PAM on CM-cellulose, so that the effect of iodide on the Sephadex column was not on ionic bonding.

A wider range of quaternary drugs was examined on the larger column (Table V). Comparison of K values with those from the small column showed no consistent differences. Little difference was to be expected because there was no great change in the ratio of column height to cross-sectional area. Examination of these results together with the previous ones allows some generalizations to be made about the elution behaviour of different compounds. Firstly, drugs without any form of acidic side group were eluted rapidly with K values around 0.1 to 0.2. The compounds with an ionizable hydroxyl group (oximes and edrophonium) were eluted slowly when the group was not ionized (*i.e.* at pH. 6.0). Ionization of the acidic group resulted in these latter drugs showing less affinity for the column and becoming eluted at intermediate K values (0.3 to 0.7).

Among the factors known to influence the behaviour of solutes on Sephadex columns are solute size, ionic interaction with carboxyl groups in the gel[1], and adsorption due to aromatic rings in the solute[5]. The first factor seemed to play little part in determining the present results, for many of the drugs (hexamethonium, decamethonium, succinylcholine, etc.) were eluted at nearly the same rate. Only in comparison of the alkali cations with the organic cations was there a suggestion of a size effect. Results obtained on the CM-cellulose column suggested that ion exchange played little part in elution from the Sephadex G-10, for the bisquaternary compound paraquat was strongly bonded to the CM-cellulose but not to the Sephadex. Further, the compounds with lowest K values on Sephadex (gallamine triethiodide, hexamethonium, etc.) were those with the greatest positive charges. Also, treatment with pyridine (Table VI) had little effect on the elution of quaternary compounds from the Sephadex. This treatment was reported by EAKER AND PORATH[1] to abolish ionic effects, so in this case we presume that none were present. That the pyridine washes had altered the nature of the column was shown by the irreversible adsorption of the Blue Dextran 2000. The "aromatic" adsorption effect of GELOTTE[5] did, however, seem to be present. The pyridinium or phenolic drugs (TMB-4, pyridostigmine, edrophonium, etc.) always had higher K values than non-aromatic compounds of

TABLE VI

THE EFFECT OF PYRIDINE TREATMENT OF A SEPHADEX G-10 COLUMN ON THE ELUTION OF VARIOUS CATIONS

The eluting buffer was 150 mM NaCl, 10 mM Tris–HCl at pH 7.4 or 8.8.

Sample	*pH*	*K*		
		Untreated	*1st treatment*	*2nd treatment*
PAM	7.4	0.67	0.66	0.645
PAM	8.8	0.535	0.515	0.515
TMB-4	7.4	0.695	0.66	0.63
TMB-4	8.8	0.39	0.415	0.38
Potassium	7.4	0.845	0.795	0.765
Paraquat	7.4	0.32	n.d.	0.295

n.d. = not determined.

comparable size. The difference was not great, but comparison between paraquat and pentolinium illustrates the point well. The only exception was that of gallamine triethiodide, which is a bulky, highly charged molecule perhaps not comparable to the others. It seems then that size and aromaticity may play some part in the chromatographic behaviour of these drugs, but these factors do not seem to be the determining ones.

Since major changes in elution pattern were associated with the acidic hydroxyl group, it seems probable that hydrogen bonding through this group to the Sephadex gel structure is the main factor which controls elution of the oximes and phenolic drugs, as suggested for other organic acids by BROOK AND HOUSLEY[8]. Thus PAM in acid media would form hydrogen bonds readily and be eluted slowly; on change of pH to the alkaline side, the proton would be lost from the oxime group and hydrogen bonding would cease. The elution of PAM zwitterion would then be controlled by "aromatic" adsorption to the gel, as would that of paraquat and other pyridinium compounds. Drugs with no possibility of hydrogen bonding or without aromatic character, such as hexamethonium, would be eluted faster still. These effects would be modified by ionic size, *e.g.* the alkali metal cations presumably penetrate the gel better than the organic cations and are therefore eluted more slowly. This theory of hydrogen bonding explains the present results well. However, in the absence of direct evidence, it cannot be regarded as conclusively proven.

Since iodide ion is believed to act as a structure breaker, the effect of this ion in increasing K values (Table I) may have been to decrease the amount of structural water around the gel, and thus increase penetration of the solute ions. The K value for PAM in iodide buffer would then be increased greatly, since greater penetration would afford the opportunity for adsorption effects to be increased.

These column elutions were all performed in buffers similar to physiological media, and therefore the compounds studied should have had the same hydrated ion size as they possess in biological fluids. If we accept the mechanism of chromatography postulated above, the organic cations must all have effective diameters greater than those of hydrated sodium and potassium ions. PAM cation has a lower K value than that of sodium ion (Tables I and III), which will become even lower when corrected for aromatic adsorption. Therefore we conclude that the PAM cation is much more

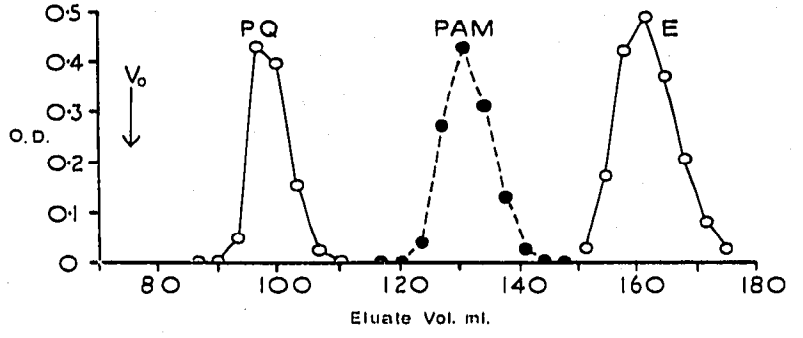

Fig. 3. The separation of a model mixture of three drugs on the larger column of Sephadex G-10 (4.00 cm² × 49.8 cm). A sample of 1 ml of buffer containing 5×10^{-4} *M* PAM, 5×10^{-4} *M* paraquat (PQ) and 2×10^{-3} *M* edrophonium (E) was applied to the column and eluted with 150 m*M* NaCl, 10 m*M* imidazole, pH 6.0. The drugs were detected by measuring the O.D. of the collected fractions at the appropriate wavelengths (see EXPERIMENTAL section). The O.D. scale for the PAM peak should be twice that shown.

bulky than the sodium ion. Such deductions are of interest in deciding how drugs pass through biological membranes[9].

Complete separation of three drugs in a model mixture was achieved (Fig. 3) on the larger column. Nearly complete separation of 10 mg each of PAM and TMB-4 was obtained on the same column run at pH 8.5. The presence of proteins in solution (0.5 ml of rabbit serum) did not alter the elution of PAM; the proteins were eluted cleanly at the void volume. Further practical use was made of the column technique to investigate the composition of a preparation of N-triethyl-3-hydroxyanilinium bromide (an analogue of edrophonium), which was suspected of having decomposed partially to N-diethyl-3-ethoxyaniline. UV spectrophotometry suggested that this decomposition had occurred, but a quantitative analysis was not possible. When chromatographed on the larger column of Sephadex G-10 at pH 6.0, 58 % of the compound was recovered as a peak at 157.5 ml, whereas at pH 8.8, 59.5 % was recovered at 125.4 ml. This demonstrated that 59 % of the sample behaved as a quaternary cation with an acidic group ionizing at about pH 8, and was therefore the authentic original compound. The non-quaternary products were eluted much later. The column technique was also used to compare suspected pyridostigmine from a sample mixture with the authentic compound and thus help to confirm its identity. The Sephadex G-10 chromatography therefore has much practical use for the separation of quaternary compounds in analytical or semi-preparative amounts. It is a useful alternative to chromatography on ion-exchange resins. The technique described here could be improved by the use of a flow detector system, either a UV monitor or a dye complex extraction system as described by McMARTIN *et al.*[6].

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