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The Fractionation of Phosphate Esters on Ion-Exchange Resin by a new System of pH-Gradient Elution

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During a study of division lag in bacterial cultures an attempt was made to compare the acid-soluble phosphates from resting cells of *Escherichia coli* with those from rapidly dividing cells and also to compare the processes of ribonucleic acid degradation. A scheme of analysis on filter paper was devised for this purpose (Wade & Morgan, 1955) but, upon applying it to acid-soluble extracts, the constituents were found to be too numerous to fractionate and isolate in this way. Ion-exchange techniques were considered as an alternative method of achieving this.

The technique most commonly used is essentially that applied by Cohn (1950) to the fractionation of ribonucleoside 2′(3′)-phosphates. It consists of passing a solution of the phosphates through an anion-exchange resin at a pH which strongly favours adsorption and then to elute them, in order of increasing affinity for the resin, with a solution (eluent) of lower pH. It is usual to employ several eluents with progressively higher salt concentration and lower pH (Cohn & Volkin, 1953). The separation of very dissimilar phosphates is greatly simplified by changing the composition of the eluent continuously, e.g. Hurlbert, Schmitz, Brumm & Potter, 1954; Pontis & Blumson, 1958. A disadvantage of such systems is that the useful range of conditions is usually very limited and sometimes necessitates a discontinuous change in the elution programme (Hurlbert *et al.* 1954).

The system of pH-gradient elution described in this paper avoids these limitations by providing a uniform fall in eluent pH between wide limits of pH and rate of pH change. This has enabled the effects of changing the rate of pH change and the eluent salt concentration upon the fractionation of a standard mixture of ribonucleoside 2′(3′)-phosphates to be examined. The practical information gained in this way has been summarized and used to select the best conditions for the fractionations of nucleoside polyphosphates and nucleoside 2′:3′-cyclic phosphates.

MATERIALS AND METHODS

Symbols. The symbols G, S and V have been used for the rate of pH change (pH units/l. of eluent), eluent salt concentration (molarity) and resin volume (l.) respectively.

Preparation of resin column. Dowex-1 resin (Dow Chemical Co., Mich., U.S.A.) with 2% divinyl cross-linkage and 200–400 mesh was used in the chloride or formate form. It was regenerated by passing 5 vol. (resin volume) of 10% (w/v) NaCl, 2 vol. of 2% (w/v) NaOH, 2 vol. of N-HCl or N-formic acid, 2 vol. of water and 10 vol. of 1 mN-HCl or 5·3 mN-formic acid successively through it. The regenerated resin was stored in this final solution.

The resin column was prepared in the following way. A volume (10 or 20 ml.) of the resin, measured by allowing it to sediment in a measuring cylinder, was transferred to a glass column (1 cm. diam.), HCl or formic acid at pH 3 being used to wash it in. At this pH no difficulty is experienced with resin adhering to the sides of the column or failing to form a flat surface. The resin was allowed to settle in the column, the free liquid above it was drained away and a volume (1 ml.) of dry, acid-washed glass beads (no. 18, English Glass Co., Leicester) was delivered onto its surface. The resin was washed finally with 5 vol. of water (resin chloride) or 0·01 M-sodium formate (resin formate).

A much poorer resolution was obtained when the diameter of the resin column was reduced to 0·7 cm., owing to the resin settling unevenly along the length of the column and causing the eluent to channel. Another practical feature which discouraged the use of narrow columns is the high resistance of the resin to the flow of eluent.

The solutes were applied in 1 vol. (resin volume) of water (resin chloride) or 0·01 M-sodium formate (resin formate) at 0·2 ml./min.

pH *control of eluent.* Salt solution, at a progressively decreasing pH, was passed through the resin column with the system illustrated in Fig. 1.

Salt solutions passes from vessel A to vessel B (300 ml. capacity), where it is mixed, by means of the magnetic stirrer D, with acid from vessel F. The flow of acid is maintained by a Sigmamotor pump X, type T6 (Sigmamotor Inc., Middleport, N.Y., U.S.A.) and is stopped when the pH in B falls to a level determined by a control system which comprises an E.I.L. pH meter, type 23A (Electronic Instruments Ltd., Richmond, Surrey) and a Transistrol Indicating Temperature Controller, type 994B (Ether Ltd., Birmingham). The controller functions in the following way. The current from the pH meter (100 μA/pH unit) is indicated on a calibrated scale by the pointer of a galvanometer to which is attached a small vane. The vane is free to pass between a phototransistor and a small filament lamp which are both mounted on a control arm. A pointer attached to the latter indicates the required pH on the galvanometer scale, and its movement, i.e. the programme of pH change, is controlled by the shape of a celluloid cam and the speed of a synchronous motor which turns it. The motor is easily exchanged for another of different speed and a set of spur-gear wheels of different diameters allows a further adjustment of the turning speed.

A second Sigmamotor Y pumps the pH-adjusted salt solution (eluent) at 1 ml./min. from B, through the resin column, and a rotameter flow meter (0·2–2·0 ml./min. range; Rotameter Manufacturing Co. Ltd., Croydon, Surrey), which is not shown, to a time-controlled fraction collector. The speed of each pump is controlled by a Zeromax infinitely variable speed box, type 143 (Revco Inc., Minn., U.S.A.).

To ensure that data from a small-scale fractionation is sufficiently reliable to be used for the design of a large-scale preparative fractionation, an accurate synchronization of the rates of eluent flow and of pH change is essential. A constant rate of flow is not obtained with a conventional gravity-dependent system since, in the course of pH-gradient elution, the volume of the resin, and therefore its resistance to flow, changes considerably. The positive control afforded by the Sigmamotor pump is insensitive to these changes and is therefore an essential feature of the scheme.

Source of phosphates. The characteristics of pH-gradient elution were studied with the eight nucleotides produced by an alkali hydrolysis of ribonucleic acid, i.e. the ribonucleoside 2′- and 3′-phosphates of adenine, guanine, cytosine and uracil. Sodium ribonucleate from yeast (British Drug Houses Ltd., London) was hydrolysed in 0·3 N-NaOH at 37° for 18 hr. and then brought to the required pH with HCl. A small deposit which remained was filtered off and the hydrolysate was stored over chloroform at 0°.

Ribonucleoside 5′-phosphates, ribonucleoside 5′-diphosphates and ribonucleoside 5′-triphosphates were purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A.

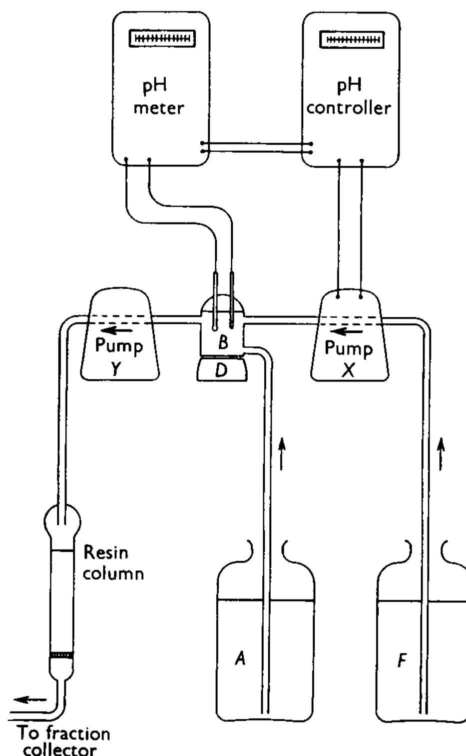


Fig. 1. The system of pH-gradient elution. A, Salt solution: B, mixing vessel; D, magnetic stirrer; F, acid solution; X and Y, Sigmamotor pumps.

Ribonucleoside 2′:3′-cyclic phosphates were kindly prepared by Mr S. Lovett by the method of Smith, Moffatt & Khorana (1958).

The quantity of material fractionated is indicated on each figure by the total quantity of phosphorus applied to the resin.

Identification of fractions in the eluate. The positions of bases and nucleotides in the eluate were determined by measuring the extinction at 260 mμ in 1 cm. cells of a Unicam spectrophotometer, type SP. 500 (Unicam Instruments Ltd., Cambridge) after suitable dilutions. The nucleotides were identified from the ratio of extinctions at 260 and 280 mμ and, when necessary, by paper ionophoresis (Wade & Morgan, 1955). The distinction between ribonucleoside 2′-phosphate and ribonucleoside 3′-phosphate was made on the assumption that the least strongly adsorbed isomer is the nucleoside 2′-phosphate (Cohn, 1950).

RESULTS AND DISCUSSION

Fractionation on 10 *ml. of resin chloride with solute displacement by hydrochloric acid starting at* pH 4. The upper useful working pH is limited to pH 4 by

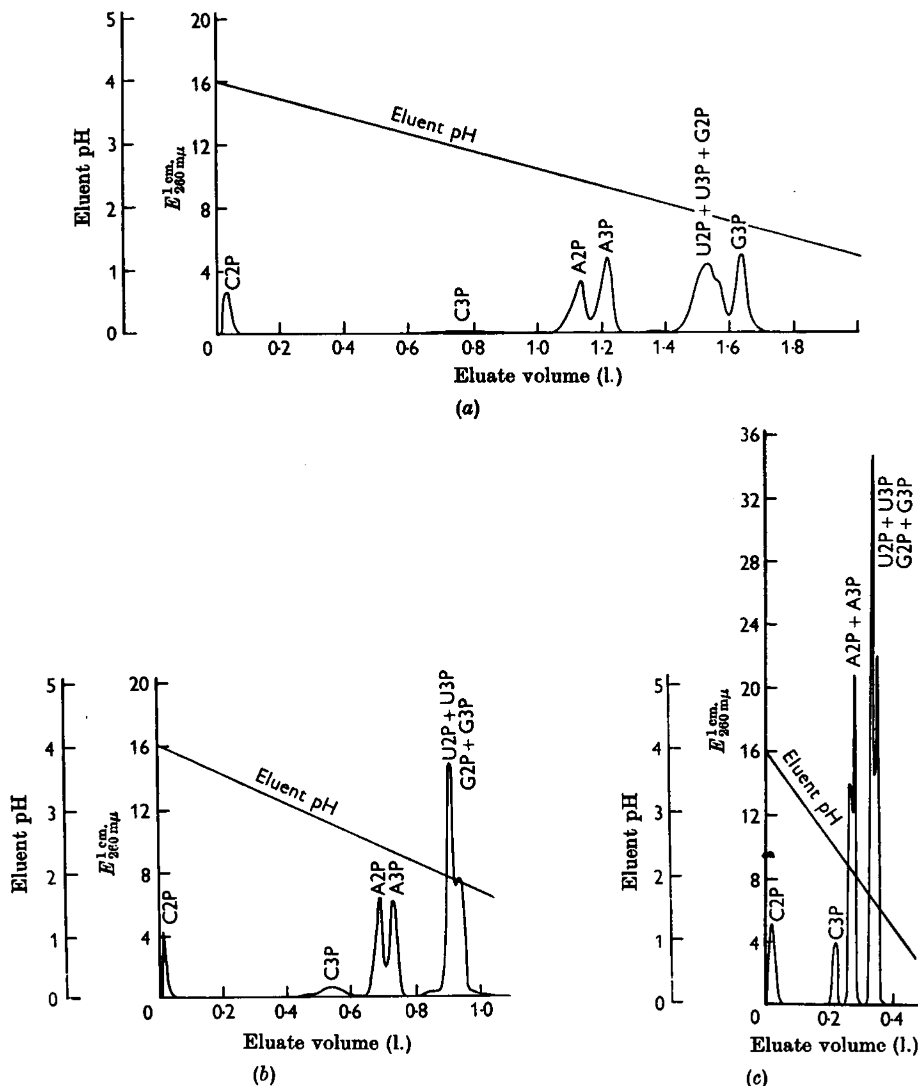


Fig. 2. Fractionation of ribonucleoside 2′(3′)-phosphates on resin chloride with hydrochloric acid solution. The nucleotides (3·5 mg. of P) were adsorbed on to 10 ml. of resin at pH 4 and eluted with hydrochloric acid solution adjusted to a pH gradient of (*a*) 1·38 pH/l., (*b*) 2·31 pH/l. or (*c*) 6·93 pH/l. C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate.

the changing capacity of the resin above this pH and the difficulty of controlling the pH in an unbuffered system.

The effect of varying the rate of pH change upon the distribution and the resolution of the fractions can be seen in Fig. 2. As the rate of pH change is increased from 1·38 pH/l. of eluent to 6·95 pH/l. of eluent the relative distribution of the fractions remains unchanged and the point of elution of any one fraction is associated with a particular eluent pH. This association is more exact than is suggested by Fig. 2, for there is a time lapse of 40 min. (equivalent to 40 ml. of eluent) between the records of eluent pH and of eluate extinction. The most prominent change which occurs is an increase in the sharpness of the solute concentration peaks, but this is not accompanied, however, by an improvement in the resolution. The behaviour of the two adenine isomers illustrates this point (Fig. 2).

Fractionation on 10 *ml. of resin chloride with solute displacement by sodium chloride–hydrochloric acid solution starting at* pH 4. The effects of including sodium chloride in the eluent at the pH gradient used in Fig. 2 (*b*) can be seen in Figs. 3 (*a*), 3 (*b*) and 3 (*c*). Increasing the salt concentration from

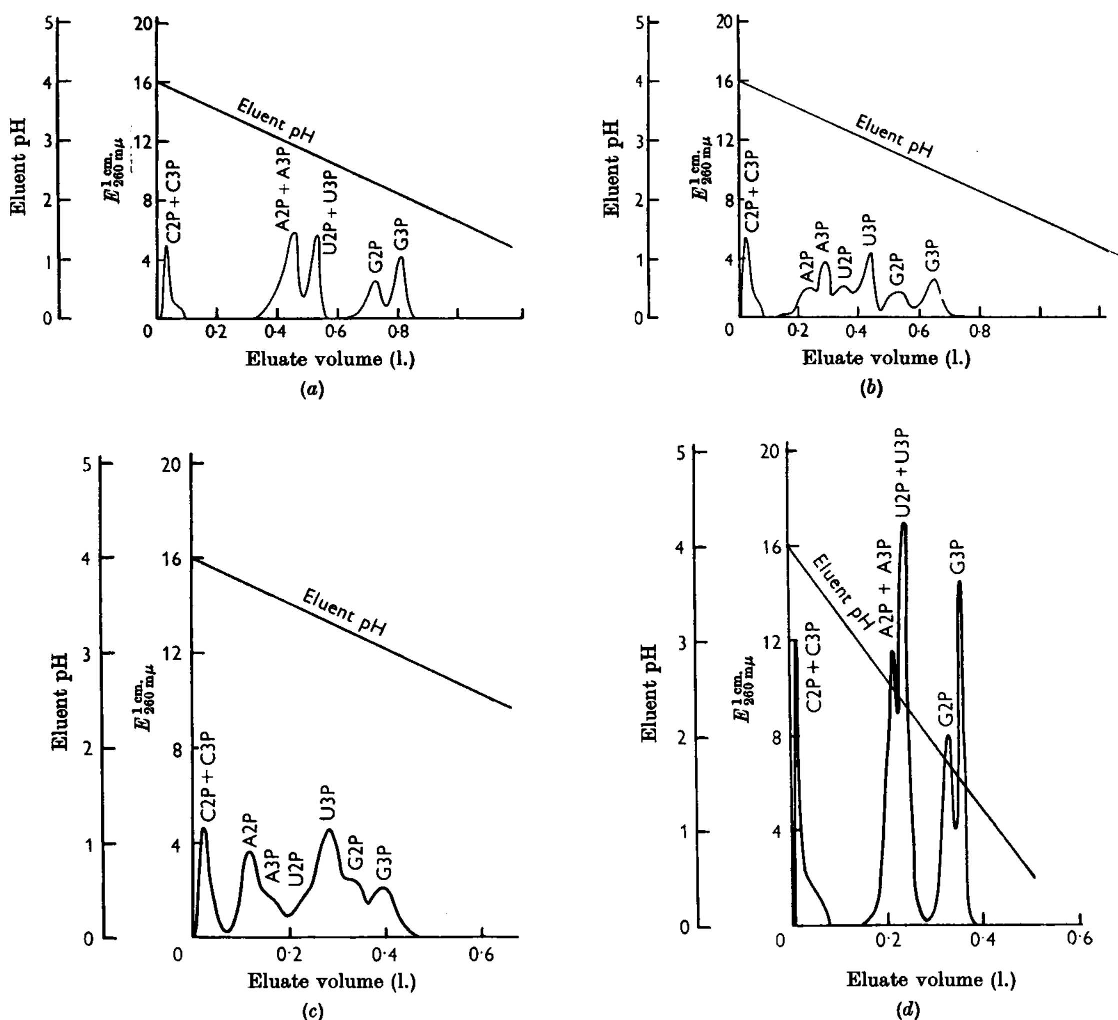


Fig. 3. Fractionation of ribonucleoside 2′(3′)-phosphates on resin chloride with sodium chloride solution adjusted with hydrochloric acid solution. The nucleotides (3·5 mg. of P) were adsorbed on to 10 ml. of resin at pH 4 and eluted with a pH gradient either of 2·31 pH/l. with (*a*) 0·01 M-sodium chloride, (*b*) 0·02 M-sodium chloride or (*c*) 0·05 M-sodium chloride solution, or of (*d*) 6·95 pH/l., with 0·02 M-sodium chloride solution. C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate.

0·01 to 0·05 M alters the relative distributions of the fractions but has very little influence upon the sharpness of the peaks. A comparison between Fig. 2 (c) and Fig. 3 (c), in both of which the fractionation is complete with 500 ml. of eluate, illustrates the better resolution obtained by increasing the rate of pH change rather than the eluent salt concentration.

When 0·02 M-sodium chloride is used and the pH gradient is increased from 2·31 (Fig. 3*b*) to 6·95 pH/l. (Fig. 3*d*), the sharpness of the peaks is increased, as expected, but, unlike a similar change in the absence of salt from the eluent (Figs. 2*b* and 2*c*), the distribution is altered. The resulting distribution (Fig. 3*d*) resembles neither that produced by a similar pH gradient in the absence of salt (Fig. 2*c*) nor that produced by a similar salt concentration with a different pH gradient (Fig. 3*b*). It resembles more closely the distribution produced by 0·01 M-sodium chloride with a pH gradient of 2·31 pH/l. (Fig. 3*a*). The only other feature which distinguishes these fractionations (Figs. 3*a* and 3*d*) from the others is the ratio of the pH gradient to the eluent salt concentration. Although these ratios are not identical in Figs. 3 (*a*) and 3 (*d*), they are of the same order of magnitude. The interdependence of pH gradient and eluent salt concentration suggested by these results is examined more fully later with a buffered system.

Difficulties of pH *control with a resin chloride system above* pH 4. A number of mixtures such as the nucleoside di- or tri-phosphates can be fractionated successfully by pH-gradient elution on a resin chloride with an unbuffered eluent starting at pH 4. For the separation of less strongly acidic phosphates, however, a higher starting pH is necessary and certain difficulties, less serious at lower pH values, become more acute.

The adsorption of phosphates on to a resin chloride in solutions of weak buffering capacity below pH 4 results in wide variations in eluate pH which are reflected in the behaviour of the less acidic solutes. When solutes are applied to the resin at pH 4 and the elution is started at this pH, the eluate pH falls rapidly to about pH 2·8 and then slowly rises to about the same level as the eluent pH. The probable explanation is that chloride ions on the resin are displaced by less than an equivalent of phosphate ions. When nucleoside 2′(3′)-phosphates are fractionated, the practical outcome is that cytidine 2′-phosphate is not adsorbed on to the resin. A similar result is obtained when the mixture is applied at pH 5 or pH 6. At pH 8 there is sufficient increase in resin capacity between pH 8 and pH 4 to avoid a pH fall below pH 4, with the result that the cytidine 2′-phosphate becomes adsorbed and is later eluted as a distinct solute peak like its isomer.

Another practical difficulty, when the elution is started above pH 4, is that the eluate pH falls initially at a much slower rate than the eluent pH and then falls rapidly when the increasing capacity of the resin is finally overcome. This results in the close grouping of the solutes in the eluate during the latter period. A similar situation arises in a buffered system when the rate of pH change, relative to the eluent salt concentration, is too high (Fig. 6*b*).

Ideally, the difference in pH between the eluent and eluate should remain nearly constant. When a resin chloride is used an improvement to this end is obtained by using one or more buffers in the eluent. The separation of orthophosphate, pyrophosphate, adenosine 5′-monophosphate, adenosine 5′-diphosphate and adenosine 5′-triphosphate, for example, can be achieved by including formate in the eluent. An improvement is not invariably obtained by including a buffer in the eluent, however, for the latter sometimes becomes adsorbed on to the resin at a high pH and later resists a pH fall at a lower value. A better control of pH is obtained by using a weak acid on the resin and in the eluent. This enables a uniform fall in eluate pH to be obtained at relatively low salt concentrations.

Fractionation on 20 ml. of resin formate with solute displacement by ammonium formate–hydrochloric acid solution starting at pH 5. The better pH control afforded by a buffered eluent allows the solutes to be delivered on to the resin at pH 6 and the elution to be started at pH 5 or 6. In order to avoid unnecessary delay in the elution of the first fraction which would otherwise occur, the elution of the standard mixture of nucleoside 2′(3′)-phosphates was started at pH 5.

It has already been observed in an unbuffered system that the rate of pH change and the eluent salt concentration are somehow interdependent with respect to their influences upon the relative distribution of the fractions in the eluate. The results of a large number of fractions carried out with a formate-buffered system showed that the distribution is largely determined by the ratio (*R*) of the rate of pH change to the eluent salt concentration:

$$R = \frac{GV}{S}, \tag{1}$$

where G is the rate of pH change (pH units/l. of eluent), V is the wet volume of the resin (l.) and S is the eluent salt concentration (molarity).

Fig. 4 illustrates the relative constancy of the distribution when R is kept constant. The marked influence of R upon the point of elution of individual fractions and upon the distribution is illustrated in Fig. 5.

The reason for the sudden deterioration in the resolution above R 1·6 (Fig. 5) can be seen in Fig. 6, in which the results of fractionating at R 1·38 and R 2·76 are compared in detail. At the higher value

(Fig. 6*b*) the eluent is unable to accommodate the increasing capacity of the resin and the association of the liberated formic acid, without itself undergoing a marked change in pH. The sudden drop in pH of the eluate when the changing capacity of the resin is finally overcome, causes the close grouping of the fractions and a very poor resolution of the mixture.

The interdependence of G and S with respect to their effects upon the eluate pH and solute distribution can be explained in the following way. In the period taken for a given fall in eluent pH, a certain equivalent of anion is required by the resin to satisfy the increase in its capacity created by the increased dissociation of its basic groups and the association of the formate ions attached to it. If the eluent is unable to supply this equivalent without suffering a serious change in pH then one of two remedies is available. The anion concentration supplied can be increased to a satisfactory level either by increasing the quantity of anion in the volume of eluent passing through the resin during this period, i.e. by increasing S, or by retaining the existing value of S and increasing the volume, i.e. by decreasing G. The same end is achieved by increasing S or decreasing G by a common factor (equation 1).

The small changes in the distribution of solutes which are observed when S is varied and R is kept constant (Fig. 4) are probably due to changes in the capacity of the resin brought about by changes in the ionic strength of the eluent. If this supposition is correct then increasing the eluent salt concentration from 0·02 M to 0·08 M should be equivalent to increasing V or R (equation 1). Conversely, decreasing the concentration from 0·02 M to 0·01 M should be equivalent to decreasing R. A comparison of Figs. 4 and 5 shows that this does in fact happen with the adenosine and guanosine phosphate; increasing the salt concentration in Fig. 4 shows to a smaller degree the same effect as increasing R in Fig. 5. The absence of any similar effect with cytidine and uridine phosphates can be attributed to the over-riding influence of the starting pH, which has been found to influence the elution of the less-strongly adsorbed solutes in a mixture.

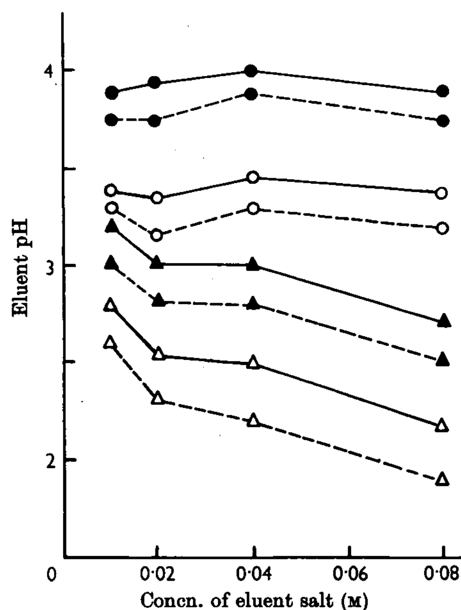


Fig. 4. Effect of varying the eluent salt concentration at R 1·38 upon the relative distribution of the fractions in the eluate. Ribonucleoside 2′(3′)-phosphates (1·75 mg. of P) were adsorbed on to 20 ml. of resin formate at pH 6 and eluted with solutions containing different concentrations of ammonium formate adjusted with hydrochloric acid. The rate of pH change was adjusted, at each level of salt concentration, to maintain R at 1·38. ●—●, Cytidine 2′-phosphate; ●- - -●, cytidine 3′-phosphate; ○—○, uridine 2′-phosphate; ○- - -○, uridine 3′-phosphate; ▲—▲, adenosine 2′-phosphate; ▲- - -▲, adenosine 3′-phosphate; △—△, guanosine 2′-phosphate; △- - -△, guanosine 3′-phosphate.

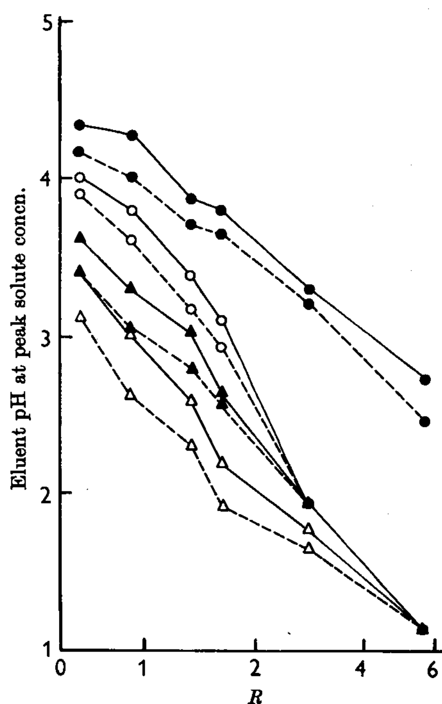
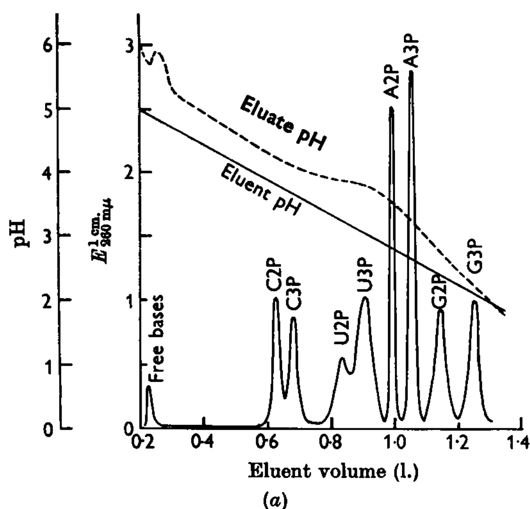


Fig. 5. Effect of varying R upon the relative distribution of the fractions. Ribonucleoside 2′(3′)-phosphates (1·75 mg. of P) were adsorbed on to 20 ml. of resin formate at pH 6 and eluted with 0·02 M-ammonium formate adjusted with hydrochloric acid to different rates of pH change. ●—●, Cytidine 2′-phosphate; ●- - -●, cytidine 3′-phosphate; ○—○, uridine 2′-phosphate; ○- - -○, uridine 3′-phosphate; ▲—▲, adenosine 2′-phosphate; ▲- - -▲, adenosine 3′-phosphate; △—△, guanosine 2′-phosphate; △- - -△, guanosine 3′-phosphate.

Fractionation of a mixture of ribonucleoside 5′-mono, di- and tri-phosphates. With the information gained from the fractionation of nucleoside 2′(3′)-phosphates, an attempt was made to separate a mixture of ribonucleoside 5′-monophosphates and ribonucleoside 5′-polyphosphates. The upper useful limit of R (R 1·6) imposed by changes in the anion capacity of the resin was further restricted by the acid-lability of the nucleoside polyphosphates. At high values of R there is a danger that these phosphates will be hydrolysed by the low pH values required to elute them.

The influence of R upon the relative distribution of fractions in the eluate was first examined at the levels 0·138, 0·276, 0·55 and 1·38. A plot of the distributions obtained (Fig. 7) suggested that a value of R 0·43 would provide an adequate resolution of the mixture and this was confirmed when the fractionation was carried out (Fig. 8).

The separation of some of these nucleotides has been described by Hurlbert *et al.* (1954) in a discontinuous form of gradient elution and also by Pontis & Blumson (1958).



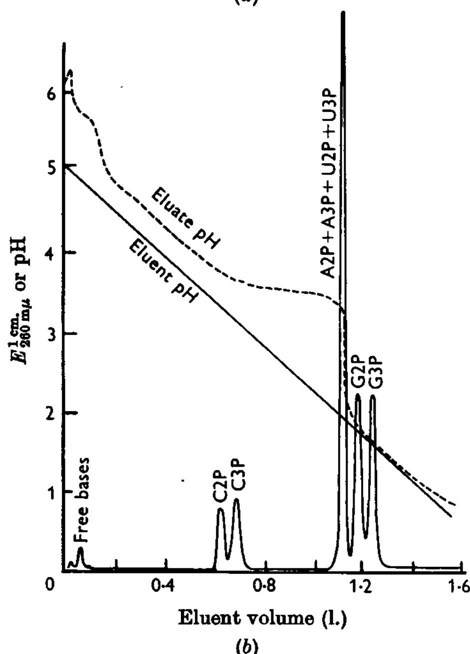


Fig. 6. Deterioration in resolution as R increases. Ribonucleoside 2′(3′)-phosphates (1·75 mg. of P) were adsorbed on to 20 ml. of resin formate at pH 6 and eluted with 0·02 M-ammonium formate adjusted with hydrochloric acid to a pH gradient of (*a*) 1·38 pH/l. (R 1·38) and (*b*) 2·76 pH/l. (R 2·76). C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate.

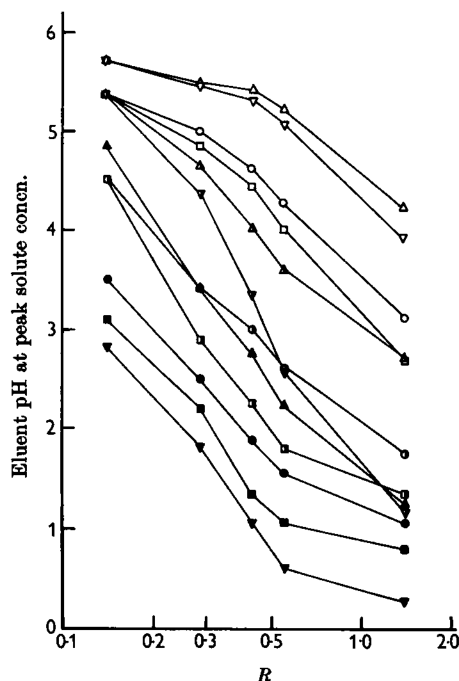


Fig. 7. Effect of varying R upon the relative distribution of ribonucleoside 5′-phosphates. The nucleotides (about 0·8 mg. of P) were adsorbed on to 20 ml. of resin formate and eluted with ammonium formate solution adjusted with hydrochloric acid to a pH gradient of 2·76 pH/l. The value of R was varied by varying the concentration of the eluent salt solution. △, Cytidine 5′-phosphate; ▲, cytidine 5′-diphosphate; ▲, cytidine 5′-triphosphate; ○, adenosine 5′-phosphate; ◑, adenosine 5′-diphosphate; ●, adenosine 5′-triphosphate; ▽, uridine 5′-phosphate; ▼, uridine 5′-diphosphate; ▼, uridine 5′-triphosphate; □, guanosine 5′-phosphate; ◧, guanosine 5′-diphosphate; ■, guanosine 5′-triphosphate.

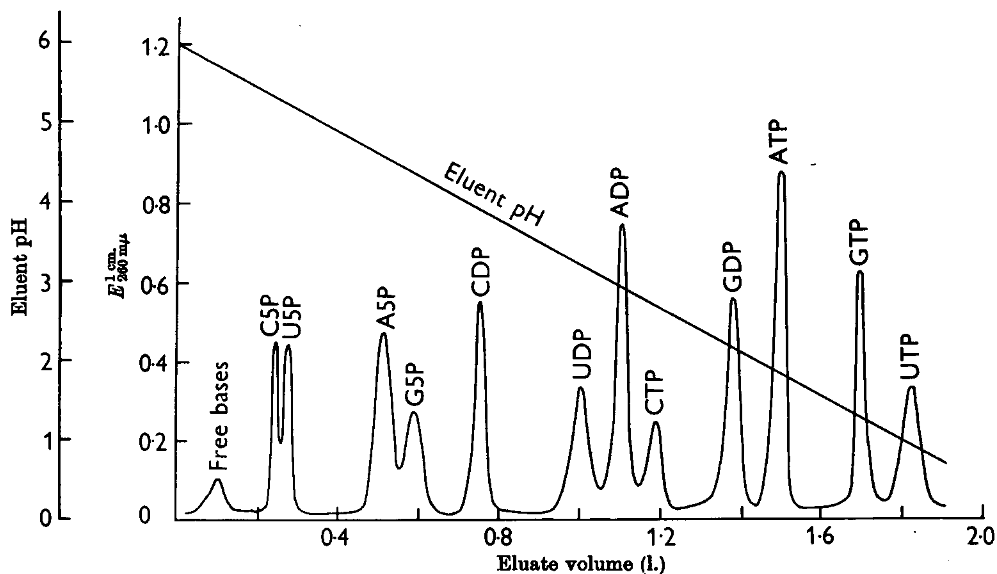


Fig. 8. Fractionation of ribonucleoside 5′-phosphates on resin formate. Ribonucleoside 5′-monophosphates (0·1 mg. of P), ribonucleoside 5′-diphosphates (0·5 mg. of P) and ribonucleoside 5′-triphosphates (1 mg. of P) were adsorbed on to 20 ml. of resin and eluted with 0·13M-ammonium formate solution adjusted with hydrochloric acid to a pH gradient of 2·76 pH/l. (*R* 0·43). C5P, Cytidine 5′-phosphate; CDP, cytidine 5′-diphosphate; CTP, cytidine 5′-triphosphate; A5P, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; U5P, uridine 5′-phosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; G5P, guanosine 5′-phosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate.

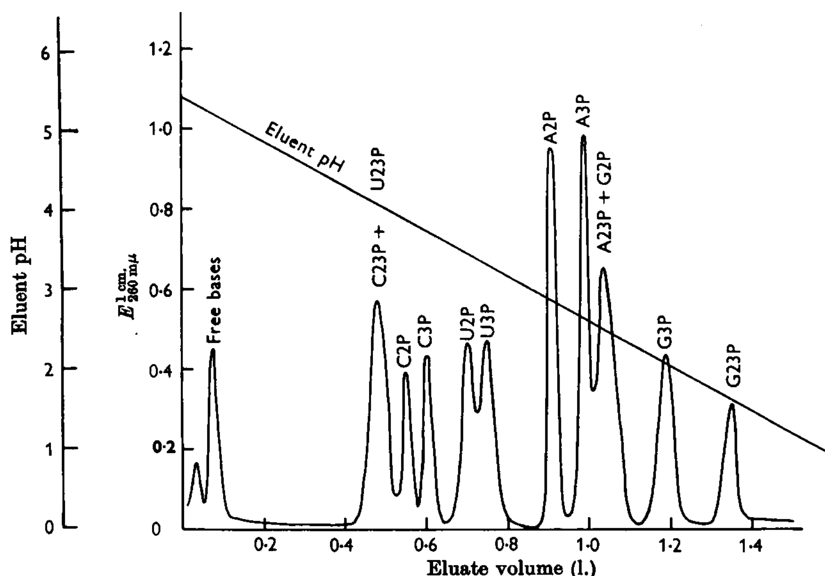


Fig. 9. Fractionation of ribonucleoside 2′:3′-cyclic phosphates and ribonucleoside 2′(3′)-phosphates on resin formate. The nucleotides (1 mg. of P) were adsorbed on to 20 ml. of resin and eluted with 0·04M-ammonium formate solution adjusted with hydrochloric acid to a pH gradient of 2·76 pH/l. (*R* 1·38). C2P, Cytidine 2′-phosphate; C3P, cytidine 3′-phosphate; C23P, cytidine 2′:3′-cyclic phosphate; U2P, uridine 2′-phosphate; U3P, uridine 3′-phosphate; U23P, uridine 2′:3′-cyclic phosphate; A2P, adenosine 2′-phosphate; A3P, adenosine 3′-phosphate; A23P, adenosine 2′:3′-cyclic phosphate; G2P, guanosine 2′-phosphate; G3P, guanosine 3′-phosphate; G23P, guanosine 2′:3′-cyclic phosphate.

Fractionation of a mixture of nucleoside 2′(3′)-phosphates and nucleoside 2′:3′-cyclic phosphates. The results illustrated in Fig. 5 suggest that when the elution programme is started at pH 5 the most satisfactory value of R for the separation of nucleoside 2′-phosphates and nucleoside 3′-phosphates is about 1·38 (Fig. 6*a*). The nucleoside 2′:3′-cyclic phosphates can also be separated from one another under these conditions. The separation of an individual cyclic nucleotide from the corresponding nucleoside dihydrogen phosphates, however, cannot be obtained in this way for there is a coincidence between cytidine 2′-phosphate and cytidine 2′:3′-cyclic phosphate and also between cytidine 3′-phosphate and uridine 2′:3′-cyclic phosphate. It had been observed previously that the relative distribution of the less strongly absorbed fractions is influenced by the starting pH of the elution. By starting the fractionation at pH 5·5 these separations could be achieved (Fig. 9).

The separation of nucleoside 2′(3′)-phosphates in an alkali hydrolysate of RNA with a system of four eluents has been described by Cohn & Volkin (1951). The separation of purine 2′(3′)-phosphates by a gradient system has been described by Hurlbert *et al.* (1954). The separation of cytidine, uridine and guanosine 2′:3′-cyclic phosphates from their respective 2′-phosphates and 3′-phosphates with single eluent systems has been described by Brown, Dekker & Todd (1952).

Features of practical significance in the pH-*gradient elution of phosphates.* The following generalizations have been drawn from the results of a large number of fractionations and are presented as a practical guide.

Unless the fractionation is to be carried out below pH 3, a weak acid form of the resin should be used in conjunction with an eluent containing the salt of the same acid.

Above pH 3, where

$$R = \frac{GV}{S}$$

and G is the rate of pH change, V is the resin volume and S is the eluent salt concentration, the upper useful limit of R should be determined and not exceeded.

The variation of R below this limit affords a means of varying the relative distribution of the fractions in the eluate.

Within certain limits, the time taken to carry out a fractionation can be varied, without seriously altering the relative distribution of the fractions, by changing G and S while keeping R constant and retaining the same starting pH.

At any value of R the distribution of the less strongly adsorbed solutes can be altered by changing the starting pH.

The sharpness of a concentration peak in the eluate is increased by increasing G and is not influenced by S.

At a given value of R the degree of resolution of a mixture increases as G and S decrease.

SUMMARY

1. A system of pH-gradient elution is described for the fractionation of naturally occurring phosphate esters on ion-exchange resin.
2. The method uses a direct form of pH control and offers a wide choice in conditions of elution.
3. The effects of varying the rate of pH change and the eluent salt concentration upon the relative distribution of the fractions and upon the sharpness of their concentration peaks have been examined with a standard mixture of nucleoside 2′(3′)-phosphates.
4. The relative distribution of the solutes in the eluate is largely determined by the ratio (R) of the rate of pH change to eluent salt concentration [$R = (GV)/S$, where G is the rate of pH change in pH units/l. of eluent, V is the wet volume of resin and S is the eluent salt concentration]. The time taken for a fractionation can be varied, without radically changing the distribution, by varying these values without changing R.
5. The sharpness of the solute concentration peak is determined by the rate of pH change and is not influenced by the eluent salt concentration.
6. On a standard column the resolution of a mixture at any given level of R is improved by decreasing the rate of pH change and the eluent salt concentration.
7. In addition to the fractionation of ribonucleoside 2′(3′)-phosphates, the fractionations of ribonucleoside 5′-mono-, di- and tri-phosphates and of ribonucleoside 2′:3′-cyclic phosphates are described.
8. From the results of a large number of fractionations, several generalizations have been made and are presented as a practical guide.

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