A METHOD FOR THE PRODUCTION OF A DESIRED BUFFER GRADIENT AND ITS USE FOR THE CHROMATOGRAPHIC SEPARATION OF ARGININO-SUCCINATE

J. A. BURNS*, C. F. CURTIS** AND H. KACSER
Institute of Animal Genetics, University of Edinburgh (Great Britain)
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INTRODUCTION

SPACKMAN, MOORE AND STEIN[1] separated the amino acids obtained from protein hydrolysates by column chromatography using a succession of buffers with different pH and salt concentrations. In physiological solutions many more ninhydrin positive substances than the usual 20 protein amino acids may be present and HAMILTON[2] using a similar system to SPACKMAN *et al.* established the time of elution of 140 ninhydrin-positive substances. A number of these substances were unresolved from each other.

An alternative method of elution is to use a continuous gradient of pH and salt concentration. Multichambered devices (Varigrads) provide a convenient method for the production of such gradients. The theory and practice of these devices has been considered by PETERSON AND SOBER[3]. A system using such a gradient has been developed by the Technicon Co. but this leaves many substances unseparated from each other.

In some circumstances it may be necessary to improve the separation in regions containing substances of interest without losing resolution in other regions. It seemed reasonable to approach this problem by decreasing the slope of the gradient immediately preceding the elution of such regions without seriously interfering with the rest of the gradient.

Hence we have the problem of altering the initial concentration in the chambers in order to achieve the "desired" gradient. The direct algebraic solution, using data from PETERSON AND SOBER[3] was attempted but was found to be laborious and often to lead to negative values for some of the initial concentrations.

Another method was to make a judicious guess at the initial concentrations, calculate the gradient which would result and compare it with the one desired. A series of such guesses and comparisons should enable us to reach the desired curve and only positive concentrations will be tried. However, since several chambers contribute at any one time an accurate fit by this process will be laborious. If we could remove the labour from the calculation stage of the guessing method it would be much more useful.

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* M. R. C. Epigenetics Research Group.
** D. S. I. R. Research Student.

Accordingly we decided to simulate the behaviour of the "varigrad" using an analogue computer. Once this is done the initial concentrations are represented by 9 potentiometer settings and the machine will plot out the gradient resulting from these settings. We can then do a series of plots and adjustments until the desired curve is approximated as well as is possible.

SIMULATION OF SYSTEM

Consider a nine chambered system. Each chamber is connected to its two adjacent chambers by narrow tubes which prevent appreciable diffusion between chambers but allow flow under a pressure difference.

The first chamber is connected to a constant rate pump which removes F ml/sec from it. The flux F out of the apparatus is sufficiently slow that, at any moment the depth of liquid, and hence the volume, is effectively the same in all chambers. This is shown diagrammatically in Fig. 1. All the chambers are well stirred.

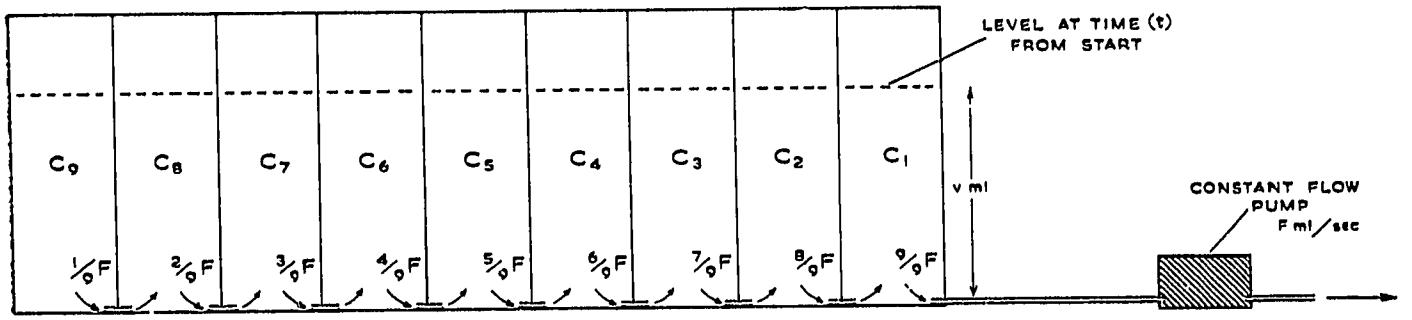

Fig. 1. Diagram of varigrad.

Let $C_1, C_2 \cdots C_9$ be the concentrations of a substance in the respective chambers at time t from the start of the run, and V be the volume in any chamber at time t, C_1°, C_2°, $\cdots C_9^\circ$ and V_0 the initial concentrations and volume.

Let C_1 become $(C_1 + dC_1)$, C_2 become $(C_2 + dC_2)$, $\cdots$ etc. at $(t + dt)$.

If we consider inflow and outflow of the substance for chamber (1) we get:

[increase in substance in chamber (1) in time dt] = [amount entering from chamber (2) in time dt] — [amount leaving to outlet in time dt]

(volume) (concn.) at $(t + dt)$ — (volume) (concn.) at t = [volume flow from (2)] × time × [average concn. in (2)] — [volume flow out of (1)] × time × [average concn. in (1)]

$$(V + dV)(C_1 + dC_1) - VC_1 = \frac{8}{9} F.\, dt.\left(\frac{C_2 + (C_2 + dC_2)}{2}\right) - \frac{9}{9} F.\, dt.\left(\frac{C_1 + (C_1 + dC_1)}{2}\right).$$

Dropping 2nd order terms and dividing by dt we get:

$$V\frac{dC_1}{dt} + C_1\frac{dV}{dt} = \frac{8}{9}FC_2 - \frac{9}{9}.F.C_1$$

and since the net volume outflow for any chamber is 1/9 F ml/sec, therefore:

$$\frac{d\,V}{d\,t} = -\frac{1}{9}F$$

Hence:

$$\frac{d\,C_1}{d\,t} = \frac{8}{9}\frac{F}{V}(C_2 - C_1)$$

If we apply the same reasoning to each of the other chambers we get:

$$\begin{aligned}
\frac{d\,C_2}{d\,t} &= \frac{7}{9}\frac{F}{V}(C_3 - C_2)\\
\frac{d\,C_3}{d\,t} &= \frac{6}{9}\frac{F}{V}(C_4 - C_3)\\
&\vdots \qquad\quad \vdots\\
\frac{d\,C_8}{d\,t} &= \frac{1}{9}\frac{F}{V}(C_9 - C_8)\\
\frac{d\,C_9}{d\,t} &= 0
\end{aligned} \tag{A}$$

and:

$$\frac{d\,V}{d\,t} = -\frac{1}{9}F.$$

The set of differential equations (A), together with the initial conditions $C_1°$, $C_2°$, $\cdots$ $C_9°$ (specifying the concentrations placed in the chambers at the start) can now be integrated with respect to time on the analogue computer and C_1 (concentration leaving the apparatus) plotted against time. The curve thus produced will be the gradient resulting from the initial concentrations $C_1°$, $\cdots$ $C_9°$.

However, the equations in the form (A) are not suited to accurate solution on an analogue computer and it is advantageous to introduce a new variable x with respect to which the integration can be performed.

The new variable x is defined by $dx = (1/V)\,dt$, $x = 0$, when $t = 0$. Introducing this into (A) as the independent variable we get:

$$\begin{aligned}
\frac{d\,C_1}{d\,x} &= \frac{8}{9}F(C_2 - C_1)\\
\frac{d\,C_2}{d\,x} &= \frac{7}{9}F(C_3 - C_2)\\
\frac{d\,C_3}{d\,x} &= \frac{6}{9}F(C_4 - C_3)\\
&\vdots \qquad\quad \vdots\\
\frac{d\,C_9}{d\,x} &= 0
\end{aligned} \tag{B}$$

$$\frac{d V}{d x} = -\frac{1}{9} F.V$$

and, of course:

$$\frac{d t}{d x} = V$$

This is now a set of 10 linear differential equations and can be integrated on a small analogue computer with good accuracy. The variables are now "scaled" so that:

(i) The highest initial chamber concentration which might be expected is represented as 1 machine unit (say 100 volts).

(ii) The initial volume V_0 is 1 machine unit (M.U.).

(iii) The time t is scaled so that $t = 1.00$ when $V = 0$ (all the chambers are empty).

(iv) The rate of solution of the problem is such that after 10 sec of computing the varigrad chambers are half full, after 20 sec a quarter full, after 30 sec one eighth full and so on. Only after an infinite computing time would we actually reach the end of a run, *i.e.*, $t = 1.00$ and $V = 0$, but in practice one minute of computing is sufficient to define the gradient.

The set of equations with their initial conditions (I.C.), when $x = 0$, is now:

	I.C.
$\frac{d C_1}{d x} = 0.5568\ (C_2 - C_1)$	C_1^0
$\frac{d C_2}{d x} = 0.4872\ (C_3 - C_2)$	C_2^0
$\frac{d C_3}{d x} = 0.4176\ (C_4 - C_3)$	C_3^0
$\frac{d C_4}{d x} = 0.3480\ (C_5 - C_4)$	C_4^0
$\frac{d C_5}{d x} = 0.2784\ (C_6 - C_5)$	C_5^0
$\frac{d C_6}{d x} = 0.2088\ (C_7 - C_6)$	C_6^0
$\frac{d C_7}{d x} = 0.1392\ (C_8 - C_7)$	C_7^0
$\frac{d C_8}{d x} = 0.0696\ (C_9 - C_8)$	C_8^0
$\frac{d C_9}{d x} = 0$	C_9^0

The values for the set $C_1^0, C_2^0, \cdots C_9^0$, representing the initial chamber concentrations, are multiplied by a common scaling factor such that none of them will be greater than 1 M.U.:

$$\frac{d\,V}{d\,x} = -0.0696\,V \qquad V_0 = 1.00$$

$$\frac{d\,t}{d\,x} = 0.0696\,V \qquad t = 0$$

The analogue computer circuit which will solve these equations is shown in Fig. 2.

As a check on the computer model simple initial conditions such as $C_5^0 = 1.00$

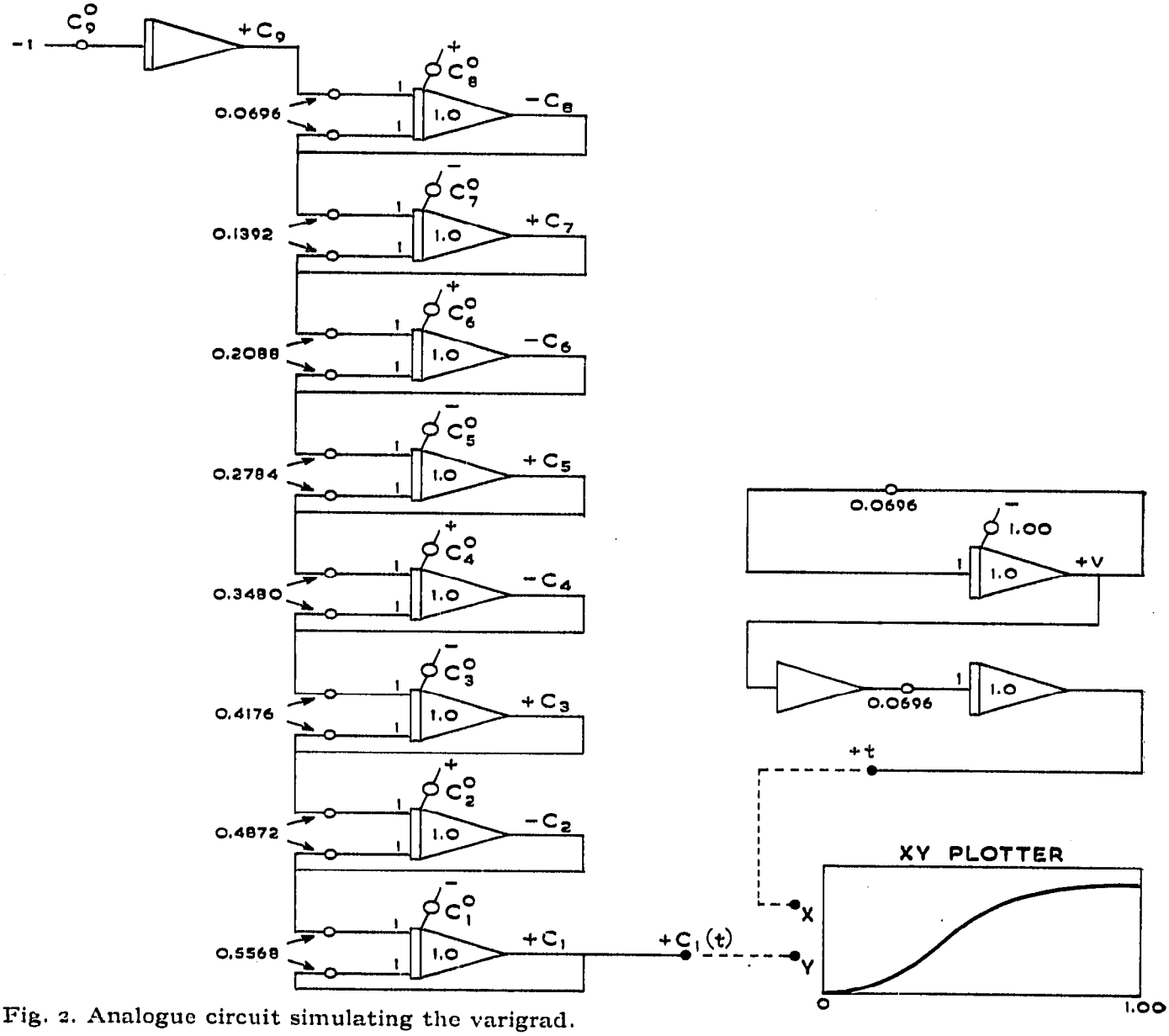

Fig. 2. Analogue circuit simulating the varigrad.

and all others = 0 were set up. The results agreed with those listed by PETERSON AND SOBER[3] to a good accuracy.

CONSTRUCTION OF A DESIRED GRADIENT

As has been explained, the desired gradient will usually be a modification of some existing gradient. The initial concentrations for the old gradient are first set on the I.C. potentiometers of the analogue computer and the computer is made to draw out the old gradient. The new gradient is then sketched in on the same graph paper and a series of guesses made as to how to adjust the I.C. in order to achieve the new gradient. Each time the trial gradient is compared with the desired one until near identity is achieved. About 10 trials of 1 min each generally suffice. If the computer used has a "repetitive operation mode" this process will be much facilitated.

A further useful extension of this approach is that the desired gradient can often be reasonably approximated using rather less than the maximum of nine different chamber compositions. This results in a considerable saving of labour in the making up of the buffer solutions.

APPLICATION OF THE METHOD

This technique was applied to the problem of resolving arginino-succinate (ASA), the immediate precursor in the biosynthesis of arginine, from deproteinised extracts of *Neurospora crassa* using a Technicon amino acid autoanalyzer.

ASA forms two anhydrides at low pH and high temperature[4] and since the initial pH on the column is 2.875 and the temperature 60° usually 90–95 % conversion of ASA to the anhydrides occurs on the column. A pre-treatment of boiling the sample with 0.1 *N* HCl for 1 h ensures that more than 95 % is in the anhydride form. Although the boiling itself results in no destruction of ASA or other amino acids, some loss of ASA on passage through the column occurs.

The recovery of a known quantity of boiled ASA from the autoanalyzer column was found to be only 68 % relative to an internal standard of norleucine. The same % recovery was obtained over at least a five-fold range of ASA concentrations so that it seems reasonable to apply this correction factor. The cause of the loss is not known.

Neither the uncyclised ASA nor the anhydrides are completely resolved from other substances using the Technicon system. In view of the greater than 95 % conversion to anhydrides the problem reduces to one of resolving the two anhydrides.

Unless otherwise stated all details of autoanalyzer technique are as specified by the Technicon Co. The buffers used to produce the gradient are sodium citrate–HCl and, over the range used, the pH of mixtures of buffers is simply calculated from the mean of the pH's of the buffers which are mixed weighted for their respective volumes.

The initial concentrations in the 9 chambered varigrad, suggested by the Technicon Co. are shown in Table I. The calculated pH and salt gradients which result, and times of elution of certain amino acids when the gradient is used, are shown in Fig. 3. The predominant anhydride of ASA has a readily recognisable product with ninhydrin since the ratio O.D.570 : O.D.440 is 2.1 compared to values for the other amino acids (except the prolines) of about 5. The two anhydrides elute close together just after γ-aminobutyrate and when there are small quantities of the anhydrides they are not satisfactorily resolved.

TABLE I

INITIAL CONDITIONS IN THE CHAMBERS OF THE VARIGRAD TO PRODUCE THE FOUR GRADIENTS

All solutions contain 0.2 g ions Na^+ per l contributed by trisodium citrate and NaOH. Greater Na^+ concentrations are produced by adding sodium chloride. (For ease of operation it was found that the modified pH gradient combined with Technicon salt gradient could be simplified by using 0.22 g ions Na^+/l in both chambers 4 and 5 and 0.74 in chambers 6 and 7, without any effect on resolution.)

Chamber	*Technicon pH*	*Modified pH*	*Technicon salt (g ions Na^+/l)*	*Modified salt (g ions Na^+/l)*
1	2.875	2.875	0.20	0.20
2	2.875	2.875	0.20	0.20
3	2.875	2.875	0.20	0.20
4	2.875	5.0	0.20	0.20
5	3.89	5.0	0.24	0.23
6	4.70	4.16	0.68	1.16
7	5.0	4.16	0.80	0.218
8	5.0	5.0	0.80	0.33
9	5.0	5.0	0.80	1.67

In the time shortly before the anhydrides elute, both the pH and salt concentration are changing appreciably and it was therefore thought desirable to test both salt and pH gradients whose slope had been reduced in this area. The initial conditions required to produce such gradients were derived on the analogue computer and the gradients and initial chamber compositions are shown in Fig. 3 and Table I.

A flattened salt gradient having the same final salt concentration combined with the Technicon pH gradient caused all substances between isoleucine and histidine to elute earlier than before. This gradient, however, prevented arginine from being eluted and hence, as an additional modification, a considerable increase in the final salt concentration was made. This resulted in the modified gradient in Fig. 3. There was, however, no improvement in the resolution of the ASA anhydrides.

The modified pH gradient, on the other hand, combined with the Technicon salt gradient caused more marked changes in the pattern. The spaces between all the neutral amino acids from proline to phenylalanine were reduced, while from there to γ-aminobutyrate the space was greatly increased. As can be seen in Fig. 3 these contractions and the expansion relative to the conventional pattern correspond quite well to the times during which the new gradient has been increased and decreased in slope. The anhydrides of ASA now appear before γ-aminobutyrate and because of the long gap between phenylalanine and γ-aminobutyrate the anhydrides resolve satisfactorily from all other ninhydrin-positive material which occurs in *Neurospora*. The reduction in the spacing between the early amino acids, however, sometimes led to poor resolution in the proline region. This was solved by starting the run with the pH 2.875 solution for one hour before the varigrad was switched in. With this further modification none of the major substances which resolved with Technicon gradient failed to resolve with the new one.

The width of peaks produced by a given quantity of an amino acid is somewhat different with different gradients, usually being reduced where the gradient is steeper and vice versa. This effect was in all cases proportionately less than the effect of the change of gradient on the space between the maxima of neighbouring peaks.

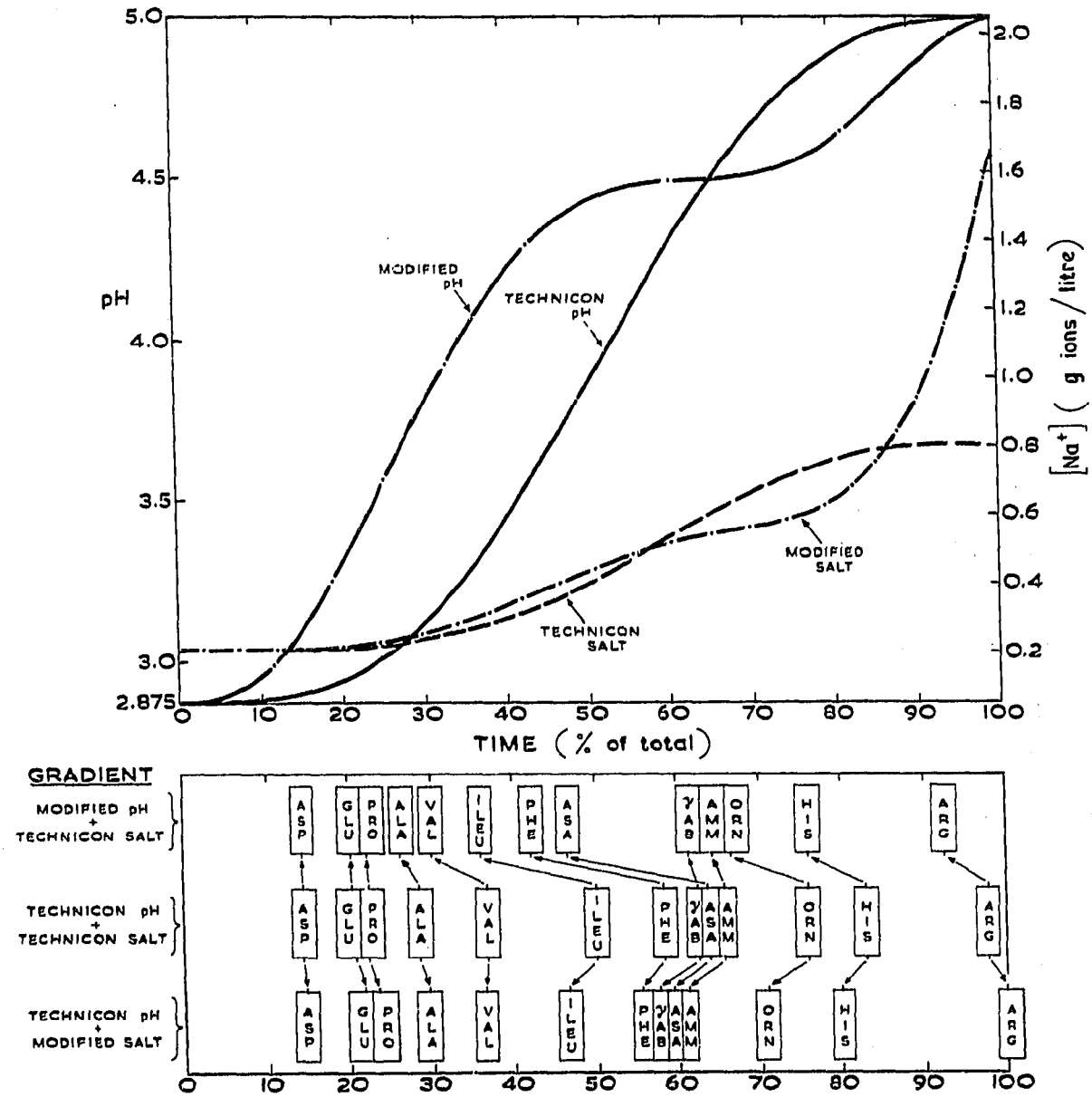

Fig. 3. pH and salt gradients and the resulting times of elution of certain amino acids. The times of elution are measured from the time at which a frontally eluted substance (cysteic acid) is recorded, so that the zero time on the figure corresponds to the time at which the beginning of the gradient has just reached the end of the analytical system. Abbreviations, apart from the conventional ones: γAB = γ-aminobutyrate; ASA = the predominant anhydride of ASA; AMM = ammonia; ORN = ornithine.

DISCUSSION

The value of an exact method of fitting a particular gradient depends on the extent to which changes in the slope of a gradient cause predictable changes in the elution pattern of substances from a column.

A simple assumption regarding the action of the column is that the velocity of movement down the column of each amino acid depends continuously on the conditions local to itself at the time and increases rapidly over a certain range of pH

and/or salt concentration. This range will be different for different substances. From this assumption it follows that flattening the pH or salt gradient over a time interval will cause an increase in the spacing of substances having a large difference in velocity during that interval. The problem is therefore the identification of that interval for a given set of substances. If the time between this interval and the time of elution is not great, flattening of the gradient immediately prior to elution should be successful. It was with this in mind that both gradient modifications were made. The fact that the neutral amino acids eluted at about the same pH value with the Technicon and the modified pH gradient suggests that the time between the large increase in velocity and the elution of these substances is indeed not very great. The inversion of the order of elution of γ-aminobutyrate and ASA suggests that their curves of velocity against pH intersect, which would be expected to occur in a number of cases. Only where considerable changes in the slope of a gradient have been made would such intersections result in inversions.

Modifying the salt gradient at about 60 % of the way through the run strongly affects the elution of arginine at the end of the run (*i.e.* about 7 h later). This indicates that, in this case, the delay between the acceleration of arginine movement down the column due to rising salt and elution of arginine from the column is relatively much larger than for the neutral amino acids.

Since the width of peaks tend to increase, when the space between their maxima is increased, the number of cases in which separately measurable peaks can be obtained, will be restricted, but, as stated, the increase in width is always proportionately less than the change in spacing and this indicates that some improvement may always be hoped for. The fact that with the modified pH gradient, not only ASA but also two other previously unobserved small peaks were separated in the gap between phenylalanine and γ-aminobutyrate indicates that the method described has considerably improved the resolution of amino acids near to ASA.

SUMMARY

A general method is suggested for making changes in the shape of an existing chromatographic gradient which will improve resolution in a region of interest whilst keeping the overall resolution virtually unaltered.

The varigrad concentrations necessary to produce this new and more complicated gradient are extremely difficult to calculate using the standard algebraic approach but are easily found by a new method using a small analogue computer and an X–Y plotter.

These ideas have been applied in an amino acid system to improve the resolution in the region between phenylalanine and ornithine in order to separate argininosuccinate.

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