An Improved Method of Separating Amino Acids as N-2,4-Dinitrophenyl Derivatives

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The separation of DNP-amino acids is most frequently done by chromatography, either on a column or on paper. Column chromatography on wet silica gel with eluents such as chloroform, butanol in chloroform, propanol in cyclohexane, benzene etc., was introduced by Sanger (1945). The method was later modified by buffering the stationary phase (Blackburn, 1949a, b; Middlebrook, 1949) and by employing kieselguhr as the supporting medium (Bell et al. 1949; Perrone, 1951); Mills (1952) used dry kieselguhr containing traces of ammonium carbonate with a series of chloroform–ethyl methyl ketone–water mixtures. In such systems the bands of the DNP-amino acids are fairly broad and often overlap (Callow & Work, 1952), so that usually two or more columns are needed to isolate any one compound from a complicated mixture.

Two-dimensional paper chromatography will resolve most of the DNP-amino acids in a protein hydrolysate (Levy, 1954; Kubota, 1961), but losses due to irreversible adsorption on the paper occur. Further, $R_f$ values tend to depend on the load applied, and certain of the solvent mixtures readily change in composition with temperature.

Work in this Laboratory called for the determination of the amount and specific radioactivity of valine. This has led to a general method of separating the amino acids, as dinitrophenyl derivatives, which is an improvement on those currently used. A preliminary report has been given (Matheson, 1962).

MATERIALS AND METHODS

Chemicals. DNP-L-proline, bis(DNP)-L-lysine, DNP-L-valine, DNP-DL-serine, bis(DNP)-L-tyrosine, DNP-L-threonine and DNP-DL-glutamic acid were prepared by the method of Rao & Sober (1954). DNP-DL-alanine, DNP-DL-phenylalanine, DNP-glycine, DNP-DL-aspartic acid, DNP-DL-aminobutyric acid and DNP-γ-aminobutyric acid were given by Dr F. J. Bealing, and DNP-DL-methionine and DNP-DL-leucine by Dr R. L. M. Syngue. DNP-hydroxy-L-proline was from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; dinitrophenol was supplied by British Drug Houses Ltd. and ethyl acetate (analytical reagent grade) by Hopkin and Williams Ltd.

Kieselguhr. Hyflo Super-Cel (Johns–Manville Co.) (about 200 g.) was stirred for 0.5–1 hr with enough 3 N-HCl to give a freely flowing suspension. The greenish yellow liquid was filtered off and the residue was treated with more HCl. The washing procedure was repeated until no more colour was extracted. Four or five washes usually sufficed. The material was then washed with water until the pH of the washings rose to about that of the water. The resulting cake of Hyflo Super-Cel was broken up and dried thoroughly overnight at 105°. The powder was sieved mechanically and that passing through 100-mesh (B.S.410/1943) was collected.

Buffers. (a) 0.1 M-Tris–maleic acid buffer: 250 ml. of ‘tris acid maleate’ (24·2 g. of tris base plus 23·2 g. of maleic acid/L.) was mixed with 125 ml. of 0-2 M-tris base and made up to 500 ml.; to each 100 ml. of this mixture was added 3·5 g. of sodium chloride, which brought the pH to about 5·4.

(b) 0.1 M-Sodium phosphate–salt buffer: 26·3 ml. of 0-2 M-NaH₂PO₄ was mixed with 223·7 ml. of 0-2 M-Na₂HPO₄ and diluted to 500 ml.; to each 100 ml. was added 3·5 g. of sodium chloride, giving a final pH of 7·4.
(c) $0.1 \text{M}$-Tris–HCl–salt buffer: 250 ml. of $0.2 \text{M}$-tris base and 200 ml. of $0.1 \text{M}$-HCl were mixed and made up to 500 ml.; to each 100 ml. was added 3-5 g. of sodium chloride, which left the pH unchanged at 8.3.

(d) $0.1 \text{M}$ Sodium phosphate buffer: 50 ml. of $0.1 \text{M}$-Na$_2$HPO$_4$ was mixed with 500 ml. of $0.1 \text{M}$-Na$_2$PO$_4$; the pH of the resulting stationary phase is uncertain as carbon dioxide is absorbed during the packing of the column, but it is probably about 12.

Buffers (a), (b) and (c) are based on those of Gomori (1955).

Preparation of chromatographic columns. A glass tube (about 40 cm. x 1 cm. bore) was fused to a short piece of tubing (0.5 cm. bore) to form a spout and a shoulder. Ethyl acetate (200 ml.) and the appropriate buffer (40 ml.), each at room temperature, were shaken together and the resulting phases were separated. Hyflo Super-Cel (4 g.) was slurried in about 50 ml. of top phase, and 2.5 ml. of bottom phase was added dropwise. The mixture was shaken vigorously until free of lumps. The spout of the chromatographic tube was closed with a rubber stopper and enough top phase was poured in to cover the shoulder, on which was placed a 1 cm. perforated silver disk and a 1 cm. paper disk. About half of the suspension of Hyflo Super-Cel was poured into the tube, and a perforated packing tool (Howard & Martin, 1950) was inserted and plunged up and down rapidly to remove lumps and air bubbles. The column was then built up 1-2 mm. at a time and the process was repeated with further suspension. The height of the resulting column was 15-19 cm., depending on the exact internal diameter of the tube and the pressure used during preparation. Careful packing is essential if full use is to be made of the resolving power of the method.

Running of columns. All operations involving dinitrophenyl derivatives were carried out in artificial light. A 100 w tungsten-filament bulb was suitable even for quantitative work. Fluorescent lights were avoided because of their ultraviolet emission.

The sample was transferred to the top of the column in the minimum quantity of water-saturated ethyl acetate by means of a bent-tipped teat pipette, then allowed to run in. Two or three small (0.2 ml.) washes were given, and when these had entered the column the head-space was filled up with the appropriate top phase. The rate of flow was about 1 ml./min.

RESULTS

To isolate DNP-valine Syngue & Youngson (1961) used columns with a buffered stationary phase (pH 6.7) and ether or $2\%$ (v/v) butan-1-ol in chloroform as the basis of the mobile phase. They found that recoveries averaged $110\%$. The contaminant has since been shown to be bis(DNP)-lysine (N. A. Matheson, unpublished work). This compound runs very close to DNP-valine on the ether columns of Perrone (1951) and tails badly. On columns developed with butanol in chloroform the two R values were different but again bis(DNP)-lysine tailed into the DNP-valine band. No change in the pH of either column or in the composition of the butanol-chloroform mixture improved the separation or decreased the tailing.

At this point ethyl acetate was tried as a basis for the mobile phase with $0.25 \text{M}$-phosphate buffer, pH 7.0, as the stationary phase (see also Sanger, 1949; Bell et al. 1949). Such a column gave a moderately good separation of DNP-valine and bis(DNP)-lysine, though both had R values above 1. As the pH was increased the R values decreased and separation improved, but the decrease in the R values was much less than expected. However, the bands of the two DNP-amino acids were reasonably compact since tailing was much less than that in other solvents. This improvement was also noticed in the chromatographic behaviour of other DNP-amino acids; $0.1 \text{M}$-tris–hydrochloric acid buffer, pH 8.3 (Gomori, 1955), gave slightly better results than phosphate buffer. The effects of pH on the R values of several DNP-amino acids are shown in Fig. 1. Some of these compounds were far enough apart to allow them to be collected separately on chromatographing a mixture, but DNP-valine, DNP-methionine and dinitrophenol (an unavoidable by-product) ran very close together at all pH values tried.

It had been noted during synthetic work that, when dinitrophenyl derivatives were partitioned between organic and aqueous phases, the presence of Na$^+$ ions in the aqueous phase altered the partition in favour of the organic phase. Further, dinitrophenol seemed to be affected more strongly than DNP-valine. A $0.1 \text{M}$-tris–hydrochloric acid buffer,
pH 8-3, was made and portions were mixed with various amounts of sodium chloride. Columns were packed with each buffer–salt mixture as stationary phase and the $R$ values of a number of dinitrophenyl derivatives were measured on each column (Fig. 2). The effect of salt content was marked and different compounds were affected to different degrees. Dinitrophenol and DNP-methionine were more sensitive to salt than was DNP-valine; if one used columns prepared with a 0.1 m-tris–hydrochloric acid buffer, pH 8-3, to which 3.5 g. of sodium chloride/100 ml. of buffer had been added, one could isolate DNP-valine from a mixture of all three, on a single column. Salt had other effects too. It made the $R$ values of dinitrophenyl derivatives even less sensitive to pH, and in its presence the bands became still more compact and less inclined to tail.

The success of the tris–salt column with ethyl acetate as the mobile phase for the separation of DNP-valine led to attempts to devise columns for the isolation of any given ether-soluble DNP-amino acid from mixtures. Changes in the mobile phase by the addition of ethanol, butanol, chloroform or light petroleum led to loss of resolution, either by making several DNP-amino acids run together or by increasing band-width and tailing.

The most useful means of altering $R$ values was to change the pH by 1 or 2 units. The nature of the buffer did not seem to have much effect, provided that the concentration of alkali-metal cations was not appreciably different. However, the pH of several buffers alters on the addition of sodium chloride or on equilibration with an organic solvent.

Four columns, 1, 2, 3 and 4, were prepared as described in the Materials and Methods section with buffers (a), (b), (c) and (d) respectively. A mixture of 12 dinitrophenyl derivatives was placed on each column and washed in at zero time. The chromatogram was developed and photographs were taken (Plates 1a and 1b). The most striking features of these columns were the rate of development, the narrowness of the bands (0.5–2 cm.) and the very wide range in the $R$ values of the DNP-amino acids (Table 1). Large changes in pH altered the absolute $R$ values appreciably, though the order in which the DNP-amino acids ran on the columns was scarcely affected. The order was that listed in the description of the mixture, DNP-aspartic acid being slowest. By altering the pH, the $R$ values of most of the DNP-amino acids could be brought into a convenient range; the $R$ values became more pH-dependent as the pH was decreased. Column 1 resolved the four slowest amino acids, namely DNP-aspartic acid, DNP-glutamic acid, DNP-serine and DNP-threonine. On column 2, DNP-serine, DNP-threonine, DNP-glycine, DNP-proline and DNP-alanine were separated while a mixed DNP-aspartic acid and DNP-glutamic acid band remained at the top. Columns 3 and 4 gave increasingly better resolution of the faster DNP-amino acids, though on each the fastest band was a mixture of bis(DNP)-lysine and bis(DNP)-tyrosine, and both this band and the two following it tended to tail. Column 3 was designed for the isolation of DNP-valine and this band (at mid-column on Plate 1b) was well separated from the others.

DNP-methionine formed a mixed band with dinitrophenol on columns similar to 3 and 4. DNP-hydroxyproline ran between DNP-serine and DNP-glutamic acid on columns like 2 and 3. DNP-isoleucine could not be separated from DNP-leucine on any of the above columns. To ensure

**EXPLANATION OF PLATE 1**

On each photograph are four chromatographic columns (1, 2, 3 and 4 from left to right) prepared with (1) 0.1 m-tris–maleic acid–salt buffer, pH 5.4, (2) 0.1 m-sodium phosphate–salt buffer, pH 7.4, (3) 0.1 M-tris–HC1–salt buffer, pH 8.3, and (4) 0.1 m-sodium phosphate buffer, pH about 12. A solution containing DNP-dl-aspartic acid, DNP-dl-glutamic acid, DNP-dl-serine, DNP-dl-threonine, DNP-glucose, DNP-dl-proline, DNP-dl-alanine, DNP-dl-valine, dinitrophenol, DNP-dl-leucine, DNP-dl-phenylalanine, bis(DNP)-l-lysine and bis(DNP)-l-tyrosine in ethyl acetate saturated with water [2–3 mg. of each bis (dinitrophenyl) derivative and 1.5 mg. of each mono (dinitrophenyl) derivative/ml. of solution]. A portion (0.1 ml.) of this solution was chromatographed on each column. Photographs were taken (a) after 10 min. and (b) after 19 min. of development.
that none of the dinitrophenyl derivatives of the protein amino acids would interfere with the quantitative isolation of DNP-valine (Matheson, 1963), cystine, cysteine and histidine were each dinitrophenylated; the products were extracted as in the quantitative method (Matheson, 1963), then run on a column at pH 8.3 (column 3). Dinitrophenol occurred in each product. Cystine and cysteine each gave two compounds which were probably bis(DNP)-cystine and N-\(\text{S-}\)bis(DNP)-cysteine, since each amino acid is liable to contain a little of the other. Histidine gave only one compound [presumably \(N1(3)N\-\text{bis(DNP)}\)-histidine] which was extracted only slightly by ether from dilute aqueous acid but which was partitioned readily into ethyl acetate (Fraenkel-Conrat & Singer, 1956). The \(R\) values of all the DNP-amino derivatives discussed above are included in Table 1.

After the quantitative method for valine (Matheson, 1963) had been developed, it was found that, on columns at pH 8-3, DNP-\(\gamma\)-aminobutyric acid had almost the same \(R\) value as DNP-valine. Now \(\gamma\)-aminobutyric acid does occur in many plant extracts. The effect of pH on the \(R\) values of DNP-valine, DNP-\(\alpha\)-aminobutyric acid and DNP-\(\gamma\)-aminobutyric acid was studied in the hope of finding some means of obtaining DNP-valine alone. The compounds were run as a mixture and then individually, at pH 8-40, 8-64 and 9-10. DNP-valine and DNP-\(\alpha\)-aminobutyric acid were little affected by these changes in pH and were always easily separated from each other. The \(R\) value of DNP-\(\gamma\)-aminobutyric acid, on the other hand, was greatly decreased as the pH increased, so that though it had formed a mixed band with DNP-valine at pH 8-3 they could be distinguished at pH 8-40 and separated at pH 8-6 or above (Table 2). For the determination of valine in the presence of \(\gamma\)-aminobutyric acid, a column, buffered at pH 8-8 with 0.1 M-tris–hydrochloric acid–salt buffer (500 ml. of 0.2 M-tris base, 81 ml. of 0.2 M-hydrochloric acid and water to 1 l.; 3.5 g. of sodium chloride was added to each 100 ml. of buffer), was used. On this column the \(R\) values of DNP-valine or of the dinitrophenyl compounds that ran near it at pH 8-3 (other than DNP-\(\gamma\)-aminobutyric acid) were little altered, so that DNP-valine could still readily be isolated from mixtures.

**DISCUSSION**

In most work on the partition chromatography of DNP-amino acids it is assumed explicitly or implicitly that the compounds exist as an equilibrium mixture of ionized and non-ionized forms in the aqueous phase but that only the non-ionized form occurs in the organic phase. That is, only the non-ionized form is supposed to be partitioned between the phases and pH affects the gross partition coefficient by altering the position of the equilibrium in the aqueous layer in accordance with the equation:

\[
pH = pK + \log\left(\frac{[\text{Ionized form}]}{[\text{Non-ionized form}]}\right)
\]

where the \(pK\) is that of the DNP-amino acid. On these assumptions, a change of 0.3 pH unit will alter the ratio of the concentrations of ionized to

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**Table 1. \(R\) values of dinitrophenylamino acids on columns of different pH values**

Experimental details are given in the text. Compounds that cannot be separated on a particular column are linked by a brace.

<table>
<thead>
<tr>
<th>Column 1 (pH 5-4)</th>
<th>Column 2 (pH 7-4)</th>
<th>Column 3 (pH 8-3)</th>
<th>Column 4 (pH 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-aspartic acid</td>
<td>0.08 Slow</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-glutamic acid</td>
<td>0.27 Slow</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-hydroxyproline</td>
<td>0.27 0.047</td>
<td>0.015</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-serine</td>
<td>0.83 0.074</td>
<td>0.03</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-threonine</td>
<td>0.79 0.12</td>
<td>0.07</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-glycine</td>
<td>Fast 0.20</td>
<td>0.13</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-proline</td>
<td>Fast 0.26</td>
<td>0.15</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-alanine</td>
<td>Fast 0.33</td>
<td>0.20</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-valine</td>
<td>Fast Fast 0.50</td>
<td>0.83</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-methionine</td>
<td>Fast Fast 0.78</td>
<td>0.51</td>
<td>Slow</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>Fast Fast 0.78</td>
<td>0.55</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-leucine</td>
<td>Fast Fast 0.89</td>
<td>0.67</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-phenylalanine</td>
<td>Fast Fast 1.09</td>
<td>0.83</td>
<td>Slow</td>
</tr>
<tr>
<td>Bis(DNP)-lysine</td>
<td>Fast Fast 1.23</td>
<td>1.14</td>
<td>Slow</td>
</tr>
<tr>
<td>Bis(DNP)-tyrosine</td>
<td>Fast Fast 1.41</td>
<td>1.40</td>
<td>Slow</td>
</tr>
<tr>
<td>DNP-derivatives of cystine</td>
<td>—</td>
<td>1.41 (faint) and</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15 (strong)</td>
<td>—</td>
</tr>
<tr>
<td>DNP-derivatives of cysteine</td>
<td>—</td>
<td>1.33 (strong) and</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.09 (faint)</td>
<td>—</td>
</tr>
<tr>
<td>DNP-derivative of histidine</td>
<td>—</td>
<td>0.88</td>
<td>—</td>
</tr>
</tbody>
</table>
Table 2. Effect of pH on the R values of dinitrophenylvaline, \(\alpha\)-dinitrophenylaminobutyric acid and \(\gamma\)-dinitrophenylaminobutyric acid

Three 0-1 M tris–hydrochloric acid buffers were made up as described by Gomori (1955) to give pH values of approx. 9-0, 8-6 and 8-4; 3-5 g. of NaCl was added/100 ml. of each buffer and the pH values were measured accurately and found to be 9-10, 8-64 and 8-40. A column was packed with each buffer as stationary phase as described in the text and a mixture of about 150 \(\mu\)g. of each DNP-amino acid was run on each column. The identity of the bands was confirmed by running each of the dinitrophenyl derivatives of the aminobutyric acids alone.

<table>
<thead>
<tr>
<th></th>
<th>pH 8-40</th>
<th>pH 8-64</th>
<th>pH 9-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-valine</td>
<td>0.52</td>
<td>0.52</td>
<td>0.47</td>
</tr>
<tr>
<td>DNP-(\alpha)-aminobutyric acid</td>
<td>0.35</td>
<td>0.33</td>
<td>0.34</td>
</tr>
<tr>
<td>DNP-(\gamma)-aminobutyric acid</td>
<td>0.43</td>
<td>0.43</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>pH 8-40</th>
<th>pH 8-64</th>
<th>pH 9-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-(\alpha)-aminobutyric acid</td>
<td>0.38</td>
<td>0.33</td>
<td>0.32</td>
</tr>
<tr>
<td>DNP-(\gamma)-aminobutyric acid</td>
<td>0.49</td>
<td>0.36</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Non-ionized forms by a factor of 2, and when the ratio is large the concentration of non-ionized molecules will also change by a factor of approximately 2. Ramachandran & Sastry (1962) have shown that many DNP-\(\alpha\)-amino acids have a pK of about 3. The ratio of the concentrations of ionized to non-ionized molecules in the whole pH range covered by the columns (5-5–12) will therefore be large. If the gross partition coefficient depends only on the proportion of non-ionized molecules present, it, too should alter by a factor of 2 for a change of 0-3 pH unit in the buffer, with a corresponding change in the R value. In studies of counter-current distribution Khokhlov & Chi (1960) did find this effect when a number of DNP-amino acids were partitioned between butyl acetate and phosphate buffer, pH 6–8. When butanol was used instead of butyl acetate, however, the DNP-amino acids could not be extracted from the organic phase even with 0-1N-sodium hydroxide. In partition chromatography, the R values of DNP-amino acids are very dependent on pH when ether (Perrone, 1951) or chloroform (Blackburn, 1949b) forms the mobile phase. In the present work, however, when ethyl acetate was used to form the organic phase the pH-dependence of the R values was greatly diminished, especially when the aqueous phase contained Na\(^+\) ions (Fig. 1). Even at pH values 6 or 7 units above their pK values, the R values of many DNP-amino acids were large, though the proportion of non-ionized molecules must have been very small. Further, dinitrophenol, which is almost colourless unless ionized, is quite yellow in the ethyl acetate phase at pH 8-3. In the ether phase at pH 5-5 it is almost colourless, however, though the aqueous phase is strongly yellow. One must assume that ionized DNP-compounds can dissolve to an appreciable extent in water-saturated ethyl acetate and that partition of these ions is occurring on the columns described above. The pH-dependence of the R values increases as the pH decreases in the range pH 12 to pH 5-5 (see also Portugal, Green & Sutherland, 1963), so that it seems possible that both ionized and non-ionized molecules are being partitioned between the phases with finite and different partition coefficients.

Almost any one of the ether-soluble DNP-amino acids can be separated from a complicated mixture on a single column of suitable pH, salt content, solvent etc. The columns illustrated in Plate 1 were selected to cover a wide range of DNP-amino acids. They are not necessarily the best ones for any specific purpose. A series of columns like those in Plate 1 can be used for the complete resolution of a complicated mixture of ether-soluble DNP-derivatives into its components in a few hours. The mixture is loaded on to the column of lowest pH which resolves the slowest amino acids. A mixture of the faster-moving compounds is eluted quickly and is concentrated and applied to the column with the next lowest pH. The process is repeated until the complete mixture is resolved. This method is essentially different from group separation as the slower-moving DNP-amino acids are not only separated from the faster-moving ones but each is itself isolated. As a result only a few columns are needed for the complete resolution of a protein hydrolysate.

After a preliminary communication on this work had been made (Matheson, 1962), it was learnt that Kesner, Muntwyler, Griffin & Abrams (1962, 1963, and personal communication) had developed a method of separating and determining the DNP-amino acids on a 100 cm. silica-gel column. They used silica gel containing 0-5N-sulphuric acid as the stationary phase and eluted with a concentration gradient made from heptane,
ethyl methyl ketone and 2-methylbutan-2-ol. The system they describe requires special apparatus but it can resolve most of the common DNP-amino acids on one column. In its operation and applications it resembles more closely that for automatic analysis of amino acids on ion-exchange resins than it does the method described in the present paper.

The advantages of separating the amino acids as their dinitrophenyl derivatives are considerable. Dinitrophenylation is a simple non-destructive reaction giving high yields of known products. The DNP-amino acids are strongly coloured, which helps in their separation and determination.

The dinitrophenyl derivative of any one of aspartic acid, glutamic acid, hydroxyproline, serine, threonine, glycine, proline, alanine and valine can be isolated from a dinitrophenylated protein hydrolysate on a single 16 cm. partition column of the type described. Any given column will isolate several pure DNP-amino acids from a mixture. If only one is needed, however, it is advisable to choose a pH that will allow fairly rapid elution while still giving a complete separation; a shorter column is often useful here. The DNP-amino acids that travel faster than DNP-valine are not as well resolved as the slower-moving ones, and changes in pH above 9 have only a small effect on R value and on resolution. The columns described will not separate DNP-methionine from dinitrophenol nor bis(DNP)-lysine from bis(DNP)-tyrosine. Though DNP-leucine and DNP-phenylalanine can both be obtained fairly pure from synthetic mixtures, the effects of the dinitrophenyl derivatives of cystine, cysteine and histidine have not been fully investigated.

Matheson (1963) has shown that valine may be accurately determined in a protein hydrolysate by dinitrophenylation and chromatographic separation of the DNP-amino acid. Portugal et al. (1963) have shown that aspartic acid and glutamic acid may be determined by a modification of the above procedure with good results. If the quantitative method is generally applicable it is potentially of considerable value, for one could assay one or two amino acids in a large number of samples rapidly, without expensive apparatus. There are many fields in which such a method would be useful, notably in end-group analysis, in studies of limiting amino acids in nutrition and in studies of the incorporation of radioactive amino acids into proteins, peptides etc. where specific activity has also to be determined.

**SUMMARY**

1. An improved method of separating ether-soluble DNP-amino acids by partition chromatography on short kieselguhr columns is described. DNP-amino acids are partitioned, largely as ions, between aqueous buffers and ethyl acetate; they form unusually narrow bands with a wide range of R values which are much less dependent on pH than in purely non-ionic partition.

2. Columns of this type allow the isolation of almost any one of the common ether-soluble DNP-amino acids from a dinitrophenylated mixture within an hour or two.

3. The R values of many of the common DNP-amino acids on columns at different pH values are listed.

The author thanks Dr R. L. M. Syng for introducing him to this field and for valuable discussion during the work. He is also indebted to Dr Syng and to Dr F. J. Bealing for gifts of DNP-amino acids. The technical assistance of Mr D. Allan is gratefully acknowledged. Thanks are also due to Mr A. V. Portugal, Mr R. Green and Dr T. M. Sutherland, who made their results on aspartic acid and glutamic acid determinations available before publication.

**REFERENCES**


