The Isolation of Amino Acids from Mixtures as their *N*-2,4-Dinitrophenyl Derivatives

METHIONINE, LEUCINE, PHENYLALANINE, ORNITHINE, LYSINE AND TYROSINE

By N. A. MATHESON

Rowett Research Institute, Bucksburn, Aberdeen

(Received 28 July 1964)

1. The separation of the dinitrophenyl derivatives of methionine, leucine (together with isoleucine), phenylalanine, ornithine, lysine and tyrosine from each other, and from the dinitrophenyl derivatives of the other protein amino acids, by partition chromatography on columns is described. 2. The harmful effect on resolving power of using too close-fitting a packing tool in preparing these partition columns is indicated and the dimensions needed to obtain good resolution are given.

In previous papers (Matheson, 1962, 1963*a,b*) an improved method of isolating many of the amino acids from mixtures, as their DNP derivatives, was described. Separation of the mixture of ether-soluble DNP-amino acids was carried out by partition chromatography on columns. In the partition chromatography of an organic acid with an organic and an aqueous phase, it is usually assumed that the ionized molecules will dissolve only in the aqueous phase whereas the non-ionized molecules will dissolve in both (see Martin, 1949). In such a system the *R* value of the acid will fall as the pH rises, tending to zero as the number of molecules remaining un-ionized falls to zero. When DNP-amino acids were partitioned between ethyl acetate and an aqueous buffer, however, different results were obtained (Matheson, 1963*a*). The *R* values did fall as the pH rose, but the rate of decrease itself fell off steadily; at pH 9 the *R* values of some DNP-amino acids were still greater than 1, but further increases in pH had only a small effect. The interpretation placed on these observations was that both ionized and non-ionized molecules were soluble in either phase, but that the partition of the ionized form in favour of the organic phase was smaller though still appreciable. The ionization of the carboxyl groups was virtually complete at about pH 8–9, and movement of the DNP-amino acids on a column at higher pH was then due to the partition of the ionized form between the phases, which is not greatly affected by pH. By this method aspartic acid, glutamic acid, hydroxyproline, serine, threonine, glycine, proline, alanine or valine could be separated from each other, and

from the remaining common amino acids, as DNP derivatives. These remaining DNP-amino acids moved too quickly to allow them to be well separated from each other even at pH 9. The present paper describes columns capable of resolving them. A preliminary account of this work has been published (Matheson, 1964).

MATERIALS AND METHODS

The materials and methods used in the present work were the same as those described by Matheson (1963*a,b*), except where noted.

Chemicals. DNP-L-tryptophan was the preparation of James & Synge (1951), DNP-L-isoleucine was bought from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., *NO*-bis-(DNP)-L-tyrosine (90–95% pure), *NS*-bis-(DNP)-L-cysteine (95% pure) and *NN*^{lm}-bis-(DNP)-L-histidine (95–100% pure) were bought from Sigma Chemical Co., St Louis, Mo., U.S.A. Many commercial samples of DNP-amino acids claimed to be chromatographically homogeneous have in fact lower purities than those stated for the last three samples above. Hopkin and Williams Ltd., Chadwell Heath, Essex, supplied the 2,4-dinitroaniline, and L. Light and Co. Ltd., Colnbrook, Bucks., the DL-3,4-dihydroxyphenylalanine. Methyl acetate, B.P. grade, contained enough acid to swamp the buffer; it was purified by first washing it with two 0.1 vol. of saturated NaCl solution saturated also with NaHCO₃, then redistilling it and collecting the fraction with b.p. 55–57°. Urea and glycine were of analytical-reagent grade.

Buffers. The buffers employed were tris-HCl, 2-amino-2-methylpropane-1,3-diol-HCl and glycine-NaOH (see Gomori, 1955). An equimolar mixture of tris and glycine was also used; this had pH about 9.1 but it could be adjusted with HCl or NaOH where necessary. The buffer concentrations ranged from 0.01 to 1.0 M.

RESULTS

During much of this work difficulties were experienced in preparing reproducible columns by the method described by Matheson (1963*a*). The resolving power of any given type of column varied from day to day and several repetitions of each experiment were needed to give unequivocal answers. It was eventually found that the resolving power of the column increased rapidly with its internal diameter, there being a limiting diameter above which further changes had no effect. Experiments showed that the resolution was determined by the clearance between the tube and packing tool, and not by the column diameter itself. DNP-amino acids that could be separated completely when the difference in diameter was greater than 0.06 cm. were not resolved at all with a difference in diameter of 0.01 cm. In all subsequent work the diameter of glass tube used was 0.1 cm. or more greater than the diameter of the packing tool.

To improve the resolution of the faster DNP-amino acids it was first necessary to decrease their *R* values. Increases in pH in the range pH 4–8 had been shown to decrease the *R* values of most DNP-amino acids (Matheson, 1963*a*), but further increases had only slight effects. The rapid movement of certain DNP-amino acids observed at pH values greater than 9 was due almost entirely to the fully-ionized molecule, which was soluble in both aqueous and organic (ethyl acetate) phases. The decrease in *R* value on a column packed with phosphate buffer, pH 12, compared with one packed with tris-hydrochloric acid-sodium chloride buffer, pH 8.3 (Matheson, 1963*a*), was in fact due to the absence of sodium chloride in the former, not to high pH. By omitting the sodium chloride one can obtain lower *R* values, which allow the separation of DNP-methionine, DNP-leucine and perhaps DNP-phenylalanine from mixtures, but the bands formed by other DNP-amino acids are still rather diffuse. Reversed-phase columns on kieselguhr treated with dichlorosilane were tried and they gave some promise of useful separations, but there were great packing difficulties and the rate of flow was excessively slow and so they were not tested further.

An obvious means of decreasing the *R* values of ionized DNP-amino acids would be to decrease the solubility of their ions in the organic phase by the addition of another solvent to it. Chloroform and light petroleum were tested but, though the *R* values fell, the DNP-amino acids gave broad tailing bands. The replacement of ethyl acetate by isopropyl acetate or isopentyl acetate had a similar effect, though the tailing was less pronounced.

An alternative approach was to increase the

partition in favour of the aqueous phase by increasing the solubility of the DNP-amino acids in it. It had already been found that sodium chloride increased the partition in favour of the organic phase (Matheson, 1963*a*); other tests showed that sucrose had no effect even in high concentration, but that large amounts of urea altered the partition markedly in favour of the aqueous phase. Further, the addition of urea intensified the colour of an aqueous solution of the DNP derivatives.

Columns were prepared that had a stationary phase buffered at pH 8.4–9.6 (tris-hydrochloric acid, 2-amino-2-methylpropane-1,3-diol-hydrochloric acid or glycine-sodium hydroxide buffer) and containing urea (0–8 M). On these columns the DNP derivatives of interest had a suitable range of *R* values that could be varied by varying the urea concentration. The bands of the DNP-amino acids were still rather broad, however, and tended to have a sharp front and diffuse back ('tailing'). The addition of sodium chloride to the buffer improved the compactness of the bands, but the effect was small.

When methyl acetate was used instead of ethyl acetate to form the organic phase for columns, the back of the bands had a sharp edge but the front was diffuse ('bearding'). The *R* values were too high and too close together. Bearding suggested that, with dilution, the partition of the DNP-amino acids changed in favour of the mobile methyl acetate phase, whereas the tailing found with ethyl acetate usually results from a partition coefficient that alters with dilution in favour of the stationary aqueous phase. A suitable mixture of methyl acetate and ethyl acetate therefore seemed likely to give compact bands of DNP-amino acids that would neither beard nor tail. Trials on columns [stationary phase 1 M-glycine-sodium hydroxide buffer, pH 9.1, containing sodium chloride (0.02 M) and urea (2 or 4 M)] with such mixtures forming the mobile phase confirmed this idea. The most compact bands were obtained with methyl acetate-ethyl acetate (2:3, v/v) mixture.

During the elaboration of the chromatographic system several buffers with a pH in the range 8.4–9.6 were used, e.g. tris-hydrochloric acid, 2-amino-2-methylpropane-1,3-diol-hydrochloric acid and glycine-sodium hydroxide. There was little to choose between them. A buffer containing tris (0.2 M) and glycine (0.2 M), approx. pH 9.1, was finally selected. It contained no inorganic cations or anions, both of which affect the *R* values of DNP-amino acids, so that the concentration of these ions could be varied as required. It also gave a linear curve for pH against volume (ml.) of acid or alkali added over the range pH 7.5–10.2. Increasing the concentration of the buffer increased the *R* values of all the DNP derivatives but, unlike

Table 1. *Effect of urea concentration on the R values of DNP-amino acids*

The columns were packed as described in the text. The *R* values given are means of two to four determinations. Compounds that cannot be clearly separated are linked by a brace.

Compound	<i>R</i> value			
	Concn. of urea (M).....0	2	4	8
Dinitroaniline	Fast	Fast	Fast	Fast
DNP derivative* of				
3,4-dihydroxyphenylalanine	Fast	Fast	Fast	1.14
Bis-(DNP)-tyrosine	Fast	1.26	1.06	0.57
Bis-(DNP)-cysteine	Fast	0.98	0.67	0.23
Bis-(DNP)-lysine	Fast	0.96	0.61	0.19
Bis-(DNP)-ornithine	1.07	0.65	0.39	0.10
DNP-tryptophan	0.84	0.42	0.25	Slow
DNP-phenylalanine	0.74	0.40	0.24	Slow
Bis-(DNP)-histidine	0.74	0.39	0.16	Slow
DNP-leucine	0.55	0.29	0.19	Slow
DNP-methionine	0.30	0.22	0.12	Slow
DNP-valine	0.26	0.14	0.10	Slow
Dinitrophenol	0.21	0.14	0.09	Slow

* Prepared by method of Rao & Sober (1954) but not crystallized: it is probably an *NO*-bis-(DNP)-DL-3,4-dihydroxy-phenylalanine, but may be a tris-(DNP) derivative.

increases in the sodium chloride content (see Matheson, 1963a), this did not speed up dinitrophenol and one or two DNP-amino acids relative to the others.

Recommended chromatographic columns. The buffer contains tris (0.2 M), glycine (0.2 M), sodium chloride (0.02 M) and urea (0–8 M, depending on which DNP-amino acids are required). The organic solvent consists of methyl acetate–ethyl acetate (2:3, v/v). This solvent and the buffer (5:1, v/v) are shaken together to equilibrate, and separated when both phases are within 1° of room temperature. The columns are prepared and packed as described by Matheson (1963a), except that the glass tubes should have an internal diameter at least 0.1 cm. greater than that of the packing tool; convenient sizes are a column with an internal diameter of 1.10–1.25 cm. and a packing tool disk with a diameter of 0.95–1.00 cm.

Columns of this type will resolve mixtures of the faster DNP-amino acids into discrete narrow bands as effectively as those illustrated in Plate 1 of Matheson (1963a) will separate the slower ones. The order in which the compounds travel is not affected by the urea concentration, but the absolute *R* values fall as the urea concentrations increase. The *R* values of a number of DNP derivatives are given in Table 1. Columns with 0–4 M-urea will be suitable for the isolation of DNP-methionine, DNP-leucine (mixed with DNP-isoleucine) and DNP-phenylalanine from protein hydrolysates, and 4–8 M-urea will isolate bis-(DNP)-ornithine, bis-(DNP)-lysine and bis-(DNP)-tyrosine. No method has yet been found to separate DNP-leucine and

DNP-isoleucine, and it seems unlikely that they will be separated completely on such short columns.

Certain of the DNP-amino acids tend to run very close to others at all urea concentrations. Bis-(DNP)-histidine runs with DNP-phenylalanine at 0–2 M-urea and close to DNP-leucine at 4 M-urea. It can be separated from a mixture of ether-soluble DNP-amino acids before chromatography, however, by partition between ether and aqueous acid of sufficiently low pH, when it is found almost entirely in the aqueous phase (Lucas, Shaw & Smith, 1963). Tryptophan gives a DNP derivative running close to or along with DNP-phenylalanine. It is normally destroyed during acid hydrolysis (but see Hotchkiss, 1941; Synge, 1950; Moore & Stein, 1963). Cysteine, whose *NS*-bis-(DNP) derivative runs fairly close to bis-(DNP)-lysine at 0–8 M-urea, is generally absent from a sample hydrolysed in air, but if present may be oxidized to cysteic acid (Moore, 1963) before dinitrophenylation.

DISCUSSION

Martin (1949), who designed the packing tool used in this work, stated that it should fit the tube closely. The experimental evidence presented above shows that, in the system used, a difference between the internal diameter of the tube and the disk of the packing tool of at least 0.06 cm. is desirable. The tubes used in the earlier stages of these experiments had an internal diameter ranging from 1.00 to 1.10 cm., and the disk of the packing tool a diameter of 0.99 cm.; with these

tubes and packing tool, resolution achieved on apparently identical columns varied greatly from day to day. When all tubes with an internal diameter of less than 1.06 cm. were discarded, reproducible columns of excellent resolving power could easily be packed. With the tool of 0.99 cm. diameter, columns were packed in tubes of up to 1.25 cm. with satisfactory results.

The reason why too small a clearance between the disk and the tube results in a poor column is uncertain. Microscopically, the kieselguhr recovered from columns packed with large or small clearance is similar, so that shattering of the structure of the Hyflo Super-Cel is not a likely explanation. It may be inherently more difficult to pack a column uniformly when the packing tool disk fits closely; certainly, bands on such columns become skew more frequently than on those packed in a tube where the disk is a looser fit. Though the underlying cause has not been established, the effect has been clearly shown. It may be a general phenomenon where this type of packing tool is employed. Unless coloured materials are being separated and many identical columns are being used, the effect is likely to be dismissed as being due to an unsuitable chromatographic system or as random variation in materials or packing.

The problem of decreasing the R values of the faster DNP-amino acids without decreasing the sharpness of the bands was solved by the addition of urea to the aqueous phase. This altered the partition in favour of this phase not only for DNP-amino acids but also for dinitrophenol. The mechanism has not been investigated, but it may depend on electrostatic attraction between the dipoles of the nitro groups of DNP derivatives and those of the urea molecule or on the ability of urea to increase the solubility of hydrocarbons in an aqueous medium (Wetlaufer, Malik, Stoller, & Coffin, 1964). On columns with urea the colours of the bands of DNP-amino acids were considerably more intense than on those with no urea. In the aqueous phase, the presence of 4M-urea produces a shift in the absorption spectrum of bis-(DNP)-lysine of about $10\text{ m}\mu$ towards longer wavelengths, so that the light-absorption in the visible region increases. The spectrum of bis-(DNP)-lysine is also shifted about $10\text{ m}\mu$ in the organic phase compared with the spectrum in an aqueous phase in the absence of urea, but this shift is away from the visible region. A substance moving with a high R value will have a large proportion of its molecules in the organic phase and these will give much less absorption in the visible region; hence the band produced will be less intense than that for a substance of low R value, even when the latter has travelled the same distance and spread to the same extent. Both these shifts would help to

explain the intensification of DNP-amino acid bands on columns containing urea.

The recommended chromatographic columns allow one to isolate the DNP derivatives of methionine, leucine (mixed with isoleucine), phenylalanine or the bis-(DNP) derivatives of ornithine, lysine or tyrosine from mixtures as complicated as protein hydrolysates. Certain DNP-amino acids interfere, notably NN^{im} -bis-(DNP)-histidine, DNP-tryptophan and NS -bis-(DNP)-cysteine, but in each case the interference can be removed: bis-(DNP)-histidine by acidification to low pH of the mixture of DNP-amino acids before extraction with ether, tryptophan by hydrolysing the protein in air, and cysteine by oxidation to cysteic acid before or after hydrolysis and before dinitrophenylation. Attempts were made to change the positions of these amino acids relative to the others on chromatographic columns by lowering the pH. Since the pK values of the carboxyl groups of many of the DNP-amino acids differ slightly (Ramachandran & Sastry, 1962), one would expect that a change in pH might allow the formation of different proportions of non-ionized molecules and hence alter the relative R values of these compounds. In practice, however, no significant changes in the relative positions of the DNP-amino acids occurred in the range pH 9.1–8.1 with urea concentrations of 4, 6 or 8 M.

Methods have now been described in this and a previous paper (Matheson, 1963a) that make it possible to isolate, as the DNP derivative, α -aminobutyric acid, γ -aminobutyric acid, ornithine, or almost any one of the usual protein amino acids, from a complicated mixture on a single small chromatographic column. That is, when it has been decided which amino acid is required, one can pack a column that will allow its DNP derivative to be separated completely from the others; each column will usually separate two or three of the DNP-amino acids with the closest R values from each other and from all other common DNP-amino acids. The exceptions are: (i) those amino acids that give a water-soluble DNP derivative (arginine, cysteic acid, histidine and the ω -DNP derivatives of ornithine and lysine) and that are easily removed as a group before chromatography (see Biserte, Holleman, Holleman-Dehove & Sautière, 1960); (ii) cysteine and cystine, which could probably be isolated as cysteic acid; (iii) tryptophan, which has not so far been very clearly separated from phenylalanine; (iv) hydroxylysine, which has not been chromatographed in these experiments and whose behaviour is unknown.

Both dinitrophenylation and chromatographic separation are quantitative in this or modified procedures for those amino acids tested [valine (Matheson, 1963a); aspartic acid and glutamic

acid (Portugal, Green & Sutherland, 1963); glutamic acid and alanine (M. Bagdasarian, personal communication)]. It is not yet known whether other amino acids can be estimated in this way.

The bands of DNP-amino acids are unusually narrow and hence separations are particularly good on these columns. This requires explanation. The factors thought to contribute are ionic partition and high flow rates. The DNP-amino acids are partitioning between the phases as anions, which probably decreases adsorption and interactions such as dimerization. The packing of the column allows high flow rates (about 1 ml./min./cm.² of cross-section area) without spreading of the bands due to failure of the system to come to equilibrium. [This implies that the stationary phase is in a thin film or small droplets, that diffusion within both phases is fast and that distribution of the DNP-amino acids between the phases is also rapid (see also Martin, 1949).] These high flow rates combined with *R* values between 0.2 and 0.7 and small columns mean that the time available for molecular diffusion and spreading on the columns is short, generally less than 1 hr.

The author is indebted to Dr R. L. M. Synge for constructive criticism and valuable comments. He also thanks Mr D. C. Allan, Mr H. G. Mitchell and Miss I. T. Porter for essential technical assistance in this work.

REFERENCES

- Biserte, G., Holleman, J. W., Holleman-Dehove, J. & Sautière, P. (1960). *Chromat. Rev.* **2**, 59.
Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Hotchkiss, R. D. (1941). *J. biol. Chem.* **141**, 171.
James, A. T. & Synge, R. L. M. (1951). *Biochem. J.* **50**, 109.
Lucas, F., Shaw, J. T. B. & Smith, S. G. (1963). *Analyt. Biochem.* **6**, 335.
Martin, A. J. P. (1949). *Symp. biochem. Soc.* **3**, 4.
Matheson, N. A. (1962). *Biochem. J.* **84**, 99P.
Matheson, N. A. (1963a). *Biochem. J.* **88**, 146.
Matheson, N. A. (1963b). *Biochem. J.* **88**, 152.
Matheson, N. A. (1964). *Biochem. J.* **92**, 11P.
Moore, S. (1963). *J. biol. Chem.* **238**, 235.
Moore, S. & Stein, W. H. (1963). In *Methods in Enzymology*, vol. 6, p. 819. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.
Portugal, A. V., Green, R. & Sutherland, T. M. (1963). *J. Chromat.* **12**, 183.
Ramachandran, L. K. & Sastry, L. V. S. (1962). *Biochemistry*, **1**, 75.
Rao, K. R. & Sober, H. A. (1954). *J. Amer. chem. Soc.* **76**, 1328.
Synge, R. L. M. (1950). *Cold. Spr. Harb. Symp. quant. Biol.* **14**, 191.
Wetlaufer, D. B., Malik, S. K., Stoller, L. & Coffin, R. L. (1964). *J. Amer. chem. Soc.* **86**, 508.