Separation of Water-Soluble N-2,4-Dinitrophenylamino Acids

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1. An account is given of the development of a rapid method for separating water-soluble N-2,4-dinitrophenylamino acids by liquid-liquid partition chromatography. 2. The mechanism of the chromatography is discussed. 3. The *R* values of a number of water-soluble and ether-soluble DNP derivatives are listed.

When DNP-amino acids are partitioned between ether and N-hydrochloric acid, they divide quite sharply into two groups. The ether-soluble compounds include the DNP derivatives of aspartic acid, glutamic acid, hydroxyproline, serine, threonine, glycine, proline, alanine, α -aminobutyric acid, γ -aminobutyric acid, valine, methionine, leucine, isoleucine, phenylalanine and tryptophan, and the bis-DNP derivatives of LL-diaminopimelic acid, meso-diaminopimelic acid, hydroxylysine, *allo*-hydroxylysine, ornithine, lysine and tyrosine. Among the water-soluble compounds are DNP-cysteic acid, DNP-citrulline, DNP-aurine, δ -DNP-ornithine, ϵ -DNP-hydroxylysine, ϵ -DNP-lysine, DNP-arginine, DNP-homoarginine, bis-DNP-histidine, *S*-DNP-cysteine and the almost colourless compounds *O*-DNP-tyrosine and *N*^{im}-DNP-histidine. Methods have already been described that allow the rapid isolation of almost any one of the ether-soluble compounds from all the others (Matheson, 1963, 1965). These methods depend on liquid-liquid partition chromatography of the mixture on a short column. The separations permit accurate quantitative determinations of amino acids (for details and references see Matheson & Sheltawy, 1966). It was therefore thought worth while to try to isolate the individual water-soluble DNP-amino acids with a view to basing quantitative determinations of these amino acids on such separation.

EXPERIMENTAL

Materials. DNP-L-arginine hydrochloride, δ -DNP-L-ornithine and *O*-DNP-L-tyrosine were purchased from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). *S*-DNP-cysteine was purchased from Mann Research Laboratories Inc. (New York, N.Y., U.S.A.). ϵ -DNP-L-lysine was prepared by the method of Sanger & Porter (1948), DNP-aurine by the procedure of Rao & Sober (1954) (see also Munier & Sarrazin, 1963) and *N*^α-DNP-L-histidine by the method of Ramachandran & McConnell (1955). DNP-L-citrulline and DNP-L-homoarginine were

not isolated as such; instead, L-citrulline and L-homoarginine hydrochloride (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) were dinitrophenylated and extracted as described for free lysine (Matheson & Sheltawy, 1966) except that the DNP-citrulline or DNP-L-homoarginine remained in the aqueous phase after ether extraction. They were each extracted from this phase with butan-1-ol and the combined butanol extracts were evaporated to dryness *in vacuo*. The residues were then used for chromatography. DNP-L-cysteic acid was prepared from cysteic acid by the method of Rao & Sober (1954) (see also Bettelheim, 1955). ϵ -DNP-hydroxylysine was prepared either (1) from gelatin or (2) from hydroxylysine. (1) A 5.5 mg. portion of gelatin (Bacto Gelatin; Difco Laboratories Inc., Detroit, Mich., U.S.A.) was dissolved in 1 ml. of 2% (w/v) NaHCO₃, 1.5 ml. of ethanol and 20 μ l. of fluorodinitrobenzene were added and the mixture was shaken for 1 hr. The product was evaporated dry *in vacuo* and hydrolysed for 19 hr. in 6N-HCl. The hydrolysate was diluted to about 10 ml. and extracted three times with 2.5 ml. of ether and then with five 2 ml. lots of butan-1-ol. The butanol extracts were evaporated dry, giving a mixture of ϵ -DNP-lysine, ϵ -DNP-hydroxylysine and one or two other minor coloured components. The ϵ -DNP-hydroxylysine was isolated on a chromatographic column of the type described below. (2) A 212 μ g. portion of hydroxylysine (a mixture of DL and *allo*; Sigma) was dissolved in 1.6 ml. of hot water; excess of CuCO₃ was added and after 10 min. the mixture was cooled and centrifuged. The supernatant was decanted as completely as possible and shaken with 0.5 ml. of 2% (w/v) NaHCO₃, 3 ml. of ethanol and 20 μ l. of fluorodinitrobenzene for 1 hr. The mixture was acidified and H₂S was bubbled through it for 10 min. It was then extracted with three 2.5 ml. lots of ether, which were discarded, and three 3 ml. lots of butan-1-ol, which were pooled and evaporated dry. This dried residue was used as such for chromatography (see also Zahn & Umlauf, 1954).

Phenylurea and ethyl methyl ketone each of laboratory-reagent grade were bought from British Drug Houses Ltd. (Poole, Dorset); phenylurea was recrystallized from water and ethyl methyl ketone was redistilled before use. Hopkin and Williams Ltd. (Chadwell Heath, Essex) supplied the butan-1-ol (analytical-reagent grade). Other materials were obtained or prepared as described previously (Matheson, 1963, 1965; Matheson & Sheltawy, 1966).

RESULTS

The water-soluble DNP-amino acids include DNP-arginine, bis-DNP-histidine, DNP-aurine and DNP-citrulline from dinitrophenylated hydrolysates of proteins or tissues, DNP-cysteic acid from dinitrophenylated hydrolysates of oxidized proteins, and ϵ -DNP-lysine, ϵ -DNP- δ -hydroxy-lysine, δ -DNP-ornithine (arginine can be converted into ornithine while still bound in peptide linkage; Close, Adriaens, Moore & Bigwood, 1953; Hamilton & Anderson, 1954; J. E. Eastoe, unpublished work cited by Eastoe & Kenchington, 1954), *S*-DNP-cysteine (see Zuber, Traumann & Zahn, 1955), *N*tm-DNP-histidine (Siepmann & Zahn, 1964) and *O*-DNP-tyrosine (Zahn & Würz, 1952; Hirs, 1962) from hydrolysates of dinitrophenylated proteins, and possibly *N*^α-DNP-L-histidine produced by the breakdown during acid hydrolysis of *N*-terminal bis-DNP-histidine, bound in DNP-protein (see Zahn & Pfanmüller, 1958).

Most of these compounds partition very strongly in favour of the aqueous phase in the systems used previously for ether-soluble DNP derivatives (Matheson, 1963, 1965) and so remain at the top of chromatographic columns when such systems are employed. However, the incorporation of an alcohol such as butan-1-ol will cause the water-soluble DNP-amino acids to move at finite speeds. In such a system (ethyl acetate-butan-1-ol-0.2M-tris-hydrochloric acid buffer, pH 8.8; 9:1:2, by vol.), the bands formed tail and so they are broad and do not separate well. No other solvent system tested gave much better results.

The use of ion-exchange chromatography was investigated. Ion-exchange resins [Dowex AG1 (X8), Amberlite CG-120 (type II) and Amberlite CG-50 (type II)] did not separate the water-soluble DNP-amino acids satisfactorily, largely because of strong adsorption effects (see also Hirs, Halmann & Kycia, 1965), which produced tailing. The method of Seki (1960), as used by Rao, Carter & Frampton (1963) to isolate ϵ -DNP-lysine, was also tested. Here, elution is carried out first with 3N-hydrochloric acid to remove contaminating substances, then with a mixture of aqueous acid and ethyl methyl ketone to elute the ϵ -DNP-lysine. Marked tailing of the DNP compounds still occurred and ϵ -DNP-lysine and DNP-arginine could not be completely separated.

Ion-exchangers based on dextran were next tested. Adsorption and tailing were much decreased and were frequently absent. Cross-linked *O*-sulphethyldextran (SE-Sephadex) and *O*-diethyl-aminoethyldextran (DEAE-Sephadex) were each tested with a range of buffers, salts, acids and bases in the pH range 1-10 but with limited success. Several of the columns would separate certain of

the water-soluble DNP-amino acids but none would separate DNP-arginine and ϵ -DNP-lysine on a 15-20cm. column, still less ϵ -DNP-lysine and δ -DNP-ornithine.

The successful resolution of ether-soluble DNP-amino acids already reported (Matheson, 1963, 1965) is possible because of the unusually narrow bands formed by these compounds under the conditions used for chromatography. The DNP compounds are thought to exist largely as ions in both phases and such ions of like charge would not associate readily, which might explain the compact bands obtained. Most of the water-soluble DNP-amino acids are zwitterions, however, except at extreme pH values. Zwitterionic molecules containing large flat dinitrophenyl groups seem very likely to associate. Satisfactory chromatography appeared most probable at high or at low pH where one group of the zwitterion was discharged, provided that any ester in the solvent was not hydrolysed rapidly. In tests at low pH, water-soluble DNP-amino acids gave compact bands with a range of *R* values (Table 1); this looked promising. However, the DNP-cysteic acid band was found to be more compact and to have a smaller *R* value when run alone than when run in a mixture with any of the basic water-soluble DNP-amino acids. Further, by paper chromatography in 2-methylpropan-2-ol-aq. ammonia (sp.gr. 0.88)-water (20:1:4, by vol.) (Russell, 1963), the DNP-cysteic acid band isolated from such a mixture always contained the basic DNP-amino acid as well, even though there was also a separate band due to this basic DNP-amino acid alone; DNP-arginine gave particularly heavy contamination. This interaction prevents chromatography at low pH being used in the presence of DNP-cysteic acid, though it appears satisfactory in its absence.

Chromatography at high pH, with 2-methylpropan-2-ol and ethyl acetate forming the organic phase, gave broad diffuse bands that overlapped. However, the effect was found to be due to the electrolyte used to maintain high pH. Originally this was phosphate buffer (0.2M-disodium hydrogen phosphate-0.2M-trisodium phosphate) or ammonia

Table 1. *R* values of some DNP-amino acids at low pH

The column was prepared (Matheson, 1963, 1965) from 4g. of Hyflo Super-Cel and 2.5ml. of bottom phase produced by mixing ethyl acetate and 0.5N-HCl (5:1, v/v). *R* values were constant for DNP-amino acids run alone or in a mixture, except for that of DNP-cysteic acid, which increased in the presence of any of the other compounds.

Compound	<i>R</i>	Compound	<i>R</i>
δ -DNP-L-ornithine	0.22	DNP-L-arginine	0.45
ϵ -DNP-L-lysine	0.32	DNP-L-cysteic acid	0.61

solution. Replacement of these substances by a 0.2M-tris-0.2M-glycine buffer, pH 9.1 (Matheson, 1965), gave much sharper bands, despite the lower pH. There are many possible systems based on this buffer. Those tested contained either ethyl acetate and some other substance such as (A) ethyl methyl ketone, (B) 2-methylpropan-2-ol or (C) phenylurea to increase the *R* value above the value obtained with ethyl acetate alone, or else (D) ethyl methyl ketone forming the organic phase with urea added to the buffer to decrease the high *R* value given by this solvent by itself. In each case, tris-glycine buffer was used. Rather surprisingly, all these systems would separate DNP-arginine, ϵ -DNP-lysine, δ -DNP-ornithine and DNP-cysteic acid. System D was rejected because it was found difficult to pack columns with ethyl methyl ketone alone as the organic solvent. In system C, the organic phase tended to evaporate and leave a deposit of phenylurea on the end of chromatographic columns, so it also was discarded. Systems A and B were both good but system A was preferred since it gave a slightly wider range of *R* values. Each of the four bands produced in system A gave only a single spot on paper chromatography in ethyl acetate-2-methylpropan-2-ol-water-aq. ammonia (sp.gr. 0.88) (14:6:4:1, by vol.).

Recommended procedure. A two-phase mixture is prepared from 40ml. of buffer (0.2M-tris-0.2M-glycine, pH 9.1), 80ml. of ethyl acetate and 120ml. of ethyl methyl ketone. A chromatographic column is packed with a mixture of 2.5ml. of the lower phase and 4g. of Hyflo Super-Cel as described by Matheson (1963, 1965). The sample of mixed DNP-amino acids is made strongly acidic with hydrochloric acid and extracted with ether to remove the ether-soluble DNP derivatives; the aqueous phase is then extracted thoroughly with butan-1-ol, which removes the water-soluble DNP-amino acids but leaves most of the salts in the aqueous layer. The sample is dried *in vacuo* and dissolved in about 0.3ml. of top phase, then loaded on the column and washed in, all as described previously (Matheson, 1963, 1965). The *R* values of some DNP-derivatives on such a column are given in Table 2. The *R* values given for the ether-soluble DNP compounds and for DNP-taurine were measured from the back of their bands, which were sharp; the fronts were often quite diffuse.

DNP-cysteic acid remains on top of the column in the above procedure. To recover it, one may then develop the column with the top phase of ethyl methyl ketone-ethyl acetate-acetic acid-water (30:20:3:7, by vol.). DNP-cysteic acid will then move down slowly (*R* 0.15), and any other DNP-amino acids, if present, will be eluted rapidly (e.g. for DNP-glutamic acid *R* is greater than 1.0).

DNP-taurine forms a rather slow-running band

Table 2. *R* values of some DNP derivatives at high pH

The column was prepared as indicated in Table 1 except that the phase mixture was made from ethyl methyl ketone-ethyl acetate-0.2M-tris-0.2M-glycine buffer (3:2:1, by vol.).

Water-soluble compounds	<i>R</i>
DNP-L-cysteic acid	0
DNP-L-citrulline	0.09
<i>N</i> α -DNP-L-histidine	0.11
DNP-taurine	approx. 0.17
ϵ -DNP-hydroxylysine (DL or <i>allo</i>)	0.20
δ -DNP-L-ornithine	0.27
ϵ -DNP-L-lysine	0.35
DNP-L-arginine	0.58
DNP-L-homoarginine	0.62
<i>O</i> -DNP-L-tyrosine	0.75
Bis-DNP-L-histidine	1.00
Ether-soluble compounds	<i>R</i>
DNP-DL-glutamic acid	0
DNP-DL-serine	0.14
Bis-DNP-L-cystine	0.20
Dinitrophenol	0.42
DNP-L-valine	0.48
DNP-DL-leucine	0.64
DNP-L-isoleucine	0.64
DNP-L-phenylalanine	0.82
DNP-L-tryptophan	0.90

that is sharp at the back but has a very ill-defined front. With heavy loading this compound will contaminate the bands of δ -DNP-ornithine, ϵ -DNP-lysine and even DNP-arginine.

DISCUSSION

Attempts to separate the water-soluble DNP compounds on ion-exchangers with a resin or dextran matrix failed because of adsorption and poor resolution respectively. Electrophoresis at several pH values in a glass tube packed with ballotini beads was also found to have too little resolving power (N. A. Matheson, unpublished work). Chromatography at extreme pH values, where one group of most zwitterions would be discharged, seemed a possible approach. At about pH 0.3, separations were achieved, but if DNP-cysteic acid was present it interacted with basic DNP-amino acids. This was presumably because of electrostatic interaction between the undischarged sulphonic acid group of DNP-cysteic acid and the ionized basic group of the other molecule in which the carboxyl group would be uncharged. DNP-taurine may behave like DNP-cysteic acid, but this was not tested. At pH 9.1, successful separations of the water-soluble DNP-amino acids were again obtained; here, neither DNP-cysteic

acid nor DNP-arginine interfered. The guanidino group of DNP-arginine would still be largely ionized but the presence of a free ionized carboxyl group in the same molecule is probably the reason why it does not interact with other ionized acidic groups. One might expect association of the DNP arginine molecules with each other when both acidic and basic groups are ionized, but, if it occurs, it does not affect the chromatographic behaviour of this compound.

On the recommended type of column, the water-soluble DNP-amino acids in general gave compact well-separated bands comparable with those obtained previously for ether-soluble DNP-amino acids (Matheson, 1963, Plate 1a and 1b). DNP-taurine and to a smaller extent *N*^α-DNP-L-histidine and DNP-citrulline formed bands with sharp backs and very diffuse fronts, so that these compounds could contaminate DNP compounds of higher *R* value. The ether-soluble DNP-amino acids also 'beard' and have *R* values in the same range as the water-soluble ones. They must therefore be removed effectively before chromatography. *S*-DNP-cysteine isomerizes to *N*-DNP-cysteine under alkaline conditions (Zahn & Traumann, 1954). This reaction occurs readily on the recommended chromatographic column, causing spreading of the band. If the mixture to be separated contains appreciable *S*-DNP-cysteine (some hydrolysates of dinitrophenylated proteins, for example) it should be allowed to stand in aq. *N*-ammonia for about an hour with gentle shaking. *S*-DNP-cysteine is converted into *N*-DNP-cysteine, which is then oxidized to bis-DNP-cystine. Bis-DNP-cystine has a low *R* value on the recommended column and, if desired, it can be removed by ether extraction before chromatography. The ϵ -DNP derivatives of hydroxylysine and *allo*-hydroxylysine were not separated on the column described, although under the conditions of Matheson & Sheltawy (1966) the bis-DNP-derivatives of hydroxylysine and *allo*-hydroxylysine and those of *meso*- and *LL*- $\alpha\epsilon$ -diaminopimelic acid were. The behaviour of DNP-homoarginine on the column is reported here, since it might become of importance in the determination of nutritionally available lysine (Mauron & Bujard, 1963). The free ϵ -amino groups of lysine in protein may be guanidinated, giving homoarginine (Roche, Mourgue & Baret, 1954), which is stable to acid hydrolysis. This compound may be estimated by ion-exchange chromatography or else by dinitrophenylation and isolation of DNP-homoarginine on a column similar to that described above (for analogous determinations of serine and total lysine see Matheson & Sheltawy, 1966).

The behaviour of *N*^{im}-DNP-histidine on these columns was not studied because it is virtually colourless in the spectral range where the absorption

of the other DNP-amino acids is measured. There is also disagreement as to its survival during acid hydrolysis (see Zahn & Pfannmüller, 1958; Siepmann & Zahn, 1964; Hirs *et al.* 1965).

Matheson (1965) previously described the use of urea to change the partition of DNP-amino acids in favour of the stationary aqueous phase in partition chromatography and so decrease their *R* values. In the present paper, phenylurea was found to alter the partition in the opposite direction, presumably because it increased the solubility of the non-polar side chains in the organic layer in which it itself was concentrated (for discussions on the effect of urea on the solubility in water of the non-polar groups of amino acids see Nozaki & Tanford, 1963; Wetlaufer, Malik, Stoller & Coffin, 1964).

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