Determination of Amino Acids as 2,4-Dinitrophenyl Derivatives

SERINE AND LYSINE

By N. A. MATHESON AND MARION SHELTAUW

Rowett Research Institute, Bucksburn, Aberdeen

(Received 5 July 1965)

1. Serine and lysine in complicated mixtures, such as protein hydrolysates, are dinitrophenylated quantitatively, isolated chromatographically and determined.
2. The method is accurate, moderately rapid and suitable for a number of estimations in parallel, but uses only normal laboratory apparatus.

When DNP-amino acids are partitioned between ether and aqueous acid, the majority of them are found almost entirely in the ether phase. In previous papers, methods have been described which allow the rapid separation of nearly any one of the common ether-soluble DNP-amino acids from the others on a short chromatographic column (Matheson, 1963*a*, 1965). It has already been shown that the complete series of operations (dinitrophenylation, extraction, chromatographic separation and absorptiometric estimation) can be made quantitative for valine (Matheson, 1963*b*), for aspartic acid and glutamic acid (Portugal, Green & Sutherland, 1963) and for glutamic acid and alanine (M. Bagdasarian, personal communication). It was of interest to see whether such methods could also be applied to the quantitative determination of other amino acids. For this purpose the estimation of serine and lysine was studied. These amino acids were selected because, on chromatography, their *N*-DNP derivatives have *R* values which differ from each other and from those of the ether-soluble DNP-amino acids previously estimated; further, a method for their specific determination would be of value in lipid research and in nutritional studies.

MATERIALS AND METHODS

Chemicals. Fluorodinitrobenzene was purchased from British Drug Houses Ltd., Poole, Dorset, and either used directly or after redistillation *in vacuo* (b.p. 120° at 1mm. Hg). *LL*- α,ϵ -Diaminopimelic acid and *meso*- α,ϵ -diaminopimelic acid were gifts from Dr Elizabeth Work, Twyford Laboratories; δ -L-hydroxylysine (containing 10–20% of δ -D-allohydroxylysine) was prepared by the method of Hamilton & Anderson (1961). DL-Serine and L-lysine hydrochloride for analytical standards were obtained from Merck, Sharp and Dohme, Rahway, N.J., U.S.A. (purity 97–103% by microbial assay), and L-serine of Sigma Grade from Sigma (London) Chemical Co. Ltd. DNP-DL-methionine sulphone and a mixture of DNP-DL-methionine ($\pm$) sulfoxides were prepared by

Dr F. T. Khouw (Levy & Chung, 1955) and DNP-L-glutamine by Dr F. J. Bealing. *NN*^{im}-bis-(DNP)-L-histidine, *NS*-bis-(DNP)-L-cysteine and *NO*-bis-(DNP)-L-tyrosine were purchased from Sigma (London) Chemical Co. Ltd.; *NN'*-bis-(DNP)-L-cystine and *NN'*-bis-DNP-L-ornithine were bought from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Other chemicals were described by Matheson (1963*a*, 1965).

Phosphate buffer. This was prepared by mixing 8g. of KCl, 10.5 ml. of 0.2M-Na₂HPO₄ and 87 ml. of 0.2M-Na₂HPO₄. The pH was between 7.40 and 7.44.

Tris-glycine-urea-salt buffer. The solution contained 0.2M-tris, 0.2M-glycine, 6M-urea and 0.02M-NaCl and had pH about 9.5 (the exact pH is not critical).

Analysed hydrolysates. These solutions were given and analysed by Dr Gabrielle Ellinger (automatic analysis on a Beckman-Spinco analyser) by Dr R. Dawson and by Dr A. Pusztai (manual analysis; Moore, Spackman & Stein, 1958), all of the Rowett Research Institute.

Estimation of serine

Procedure. A sample containing 20–120 μ g. of free serine is evaporated to dryness in a test tube (2 cm. diam.) with a ground-glass joint; 1 ml. of 2% NaHCO₃ and 1.5 ml. of ethanol are added and if necessary the solution is warmed slightly to dissolve any precipitated NaHCO₃. All subsequent operations are carried out in light from a tungsten filament bulb. Fluorodinitrobenzene (20 μ l.) is then added and the mixture is shaken for 0.5–1 hr. at room temperature. (In the work reported in this paper, the samples were actually dinitrophenylated for 3 hr. Tests showed later that, in fact, the reaction was complete for serine in less than 0.5 hr.) The alcohol is removed under reduced pressure and the ether-soluble DNP-amino acids required are obtained free of FDNB,* salt etc. by extraction as described by Matheson (1963*b*), except that the combined aqueous extracts are acidified with 1 ml. of 5N-HCl and that ethyl acetate is used instead of ether in the final extractions. A phosphate buffer (pH 7.4)–ethyl acetate–methyl acetate (1:1:4, by vol.) mixture is prepared, and 2.5 ml. of the bottom phase and 4 g. of Hyflo Super-Cel are used to prepare a chromatographic column in a glass tube (1.1–1.2 cm. internal diam.)

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

as described by Matheson (1963a, 1965). The mixed DNP-amino acids are loaded on the column and the chromatogram is developed with the top phase of the above-mentioned mixture under gravity. The flow-rate is adjusted to 0.5–1.0 ml./min. by altering the head of top phase over the column. *N*-DNP-serine gives a compact band (*R* 0.26) which is eluted after about 44 ml. of mobile phase have emerged. It is collected in a 25 ml. standard flask containing 1 ml. of 2.5 *N*-triethylamine in ethyl acetate and the ultra-violet extinction is read in a 1 cm. cell at 360 m μ against air. A blank prepared from 1 ml. of triethylamine solution and 24 ml. of top phase is read similarly in the same cell, and the reading is deducted.

Comments on the analytical method. The dinitrophenylation method, used for valine, gave low yields with serine (53–65%). Extraction of the DNP-amino acids with ethyl acetate instead of ether at the final stage gave only a slightly higher yield. If DNP-serine itself were carried through the whole process there was appreciable destruction at the dinitrophenylation stage. Redistillation of the FDNB removed certain fast-moving impurities but did not improve the yield. Dinitrophenylation at 40° in an aqueous medium (Fraenkel-Conrat & Singer, 1956) increased the yield to 86–90%, though it could not be further improved. Schroeder & LeGette (1953) found that they could get high yields of DNP-amino acids if they used a much more dilute single-phase system of aqueous alcohol. The conditions they described did not produce a single phase in this Laboratory (see also Lucas, Shaw & Smith, 1963), but when modified slightly they gave an alcoholic solution of amino acid and bicarbonate in which the droplet of FDNB slowly dissolved. Such a system gave very high yields of DNP-serine from serine and reaction was complete in less than 0.5 hr.

If the sample of serine contains much acid, it will be necessary to add NaHCO₃ to neutralise it, as well as adding the NaHCO₃ required for dinitrophenylation. The pH should be about 8 (after removing any CO₂ *in vacuo*) before FDNB is added. It may be tested by adding a drop of 0.04% phenol red, which does not seem to interfere with serine determination.

The chromatographic column described by Matheson (1963a) will separate DNP-serine from other common DNP-amino acids but not from *N*-DNP-hydroxyproline (the *R* value, given for DNP-hydroxyproline at pH 5.4 in that paper, is incorrect). Below pH 6.7, DNP-serine and DNP-hydroxyproline will not separate. Above this pH, they do form distinct bands but their *R* values are inconveniently low. Replacement of part of the ethyl acetate by methyl acetate gave *R* values of the required size, though precipitation of a salt from the buffer sometimes occurred. The use of KCl instead of NaCl in the buffer avoided precipitation and allowed a still greater inorganic salt concentration to be employed; this further improved resolution. Table 1 gives the *R* values of a number of the DNP-amino acids closest to DNP-serine on the chromatogram. Those for diaminopimelic acid were obtained by carrying authentic LL- and meso-diaminopimelic acid through the analytical procedure.

Columns of this type flow very fast; it is advisable to keep the flow-rate below 1 ml./min. or resolution may be impaired. The spectrum of DNP-serine is strongly influenced by pH and, in the eluate, the absorption is of an intermediate type between the acid and alkaline spectra. Collection of the sample in triethylamine ensures that the alkaline spec-

Table 1. *R* values of DNP-amino acids on a column for isolation of DNP-serine

The column was prepared with phosphate buffer (pH 7.40), ethyl acetate and methyl acetate as described in the text.

Compound	<i>R</i>
DNP-DL-aspartic acid	Slow
DNP-DL-glutamic acid	Slow
DNP-DL-methionine ($\pm$) sulfoxides	0.08
<i>N</i> -DNP-L-hydroxyproline	0.10
DNP-L-glutamine	0.15
DNP-meso- α,ϵ -diaminopimelic acid	0.20
<i>N</i> -DNP-L-serine	0.26
DNP-LL- α,ϵ -diaminopimelic acid	0.40
<i>N</i> -DNP-L-threonine	0.40
DNP-glycine	0.49
<i>NN'</i> -bis-(DNP)-L-cysteine	0.57
DNP-DL-methionine sulphone	0.65
DNP-L-proline	0.68
DNP-DL-alanine	0.70

trum is obtained. The absorption maximum is at 359 m μ but its value is fairly constant between 357 and 360 m μ . Determinations were all made at 360 m μ .

Estimation of lysine

Procedure. Dinitrophenylation is carried out as for serine though either peroxide-free diethyl ether or ethyl acetate may be used for the final extraction of the mixed DNP-amino acids. The chromatographic column is prepared from tris-glycine-urea-NaCl buffer, described above (see also Matheson, 1965), shaken with methyl acetate and ethyl acetate (1:2:3, by vol.) to form two phases. It is packed and used as described for the DNP-serine column. *NN'*-Bis-(DNP)-lysine gives a band (*R* 0.30) which has a retention volume of about 42 ml. The material is collected in a 25 ml. standard flask containing 4 ml. of ethanol and the extinction is read in a 1 cm. cell at 355 m μ and at 410 m μ against air. A blank prepared from 4 ml. of ethanol and mobile phase to 25 ml., is read similarly in the same cell and its value is deducted.

Comments on the analytical method. The dinitrophenylation method used gave good results. The reaction was complete in less than 0.5 hr. As with serine, the samples were neutralized, if necessary, before dinitrophenylation.

The chromatographic column used is similar to those described by Matheson (1965) but has an intermediate urea content (6M). The *R* values of the DNP-amino acids closest to bis-(DNP)-lysine are given in Table 2. The *R* values for the hydroxylysines were obtained by carrying hydroxylysine, prepared from gelatin (Hamilton & Anderson, 1961), through the analytical procedure and assuming that the stronger band (81.4% of whole as measured by extinction at 355 m μ) came from the naturally occurring L-hydroxylysine and that the fainter band (18.5% of whole) came from D-allohydroxylysine. As can be seen bis-(DNP)-lysine can easily be separated from the other DNP-amino acids listed (see also Matheson, 1965) except for *NS*-bis-(DNP)-cysteine; the latter is rather close, though visibly separate. However, cysteine itself is unlikely to survive

Table 2. *R* values of DNP-amino acids on a column for the isolation of bis-DNP-lysine

The column was prepared with tris-glycine-urea-NaCl buffer as described in the text. *R* values of DNP derivatives of ornithine, lysine, cysteine and tyrosine were somewhat variable but the relative positions and degree of separation were fairly constant.

Compound	<i>R</i>
<i>NN</i> ^{lm} -bis-(DNP)-L-histidine	0.11
<i>NN'</i> -bis-(DNP)-D-allohydroxylysine	0.13
DNP-DL-phenylalanine	0.15
<i>NN'</i> -bis-(DNP)-L-hydroxylysine	0.18
<i>NN'</i> -bis-(DNP)-L-ornithine	0.18
<i>NN'</i> -bis-(DNP)-L-lysine	0.29
<i>NS</i> -bis-(DNP)-L-cysteine	0.36
<i>NO</i> -bis-(DNP)-L-tyrosine	0.92

hydrolysis in air to form *NS*-bis-(DNP)-cysteine on dinitro-phenylation.

No triethylamine is added to the bis-(DNP)-lysine eluate before reading, since the pH of the column is high enough to ensure that the spectrum is entirely that of the alkaline form of the compound. The eluates were slightly cloudy, however, and blank extinction values tended to vary. The addition of dry ethyl acetate or methyl acetate to the eluate did not prevent this entirely, but ethanol in the receiving flask did.

The spectrum of bis-(DNP)-lysine is similar to those of many DNP-amino acids. There is a maximum at 353m μ (changing little in intensity in the range 352–355m μ) and an almost level portion around 410m μ . The wavelength 355m μ is near the end of the calibrated portion of the wavelength setting on the spectrophotometer used (model SP.600; Unicam Instruments Ltd., Cambridge). Readings were therefore taken at 410m μ also, to check whether extinction values at both wavelengths would give the same result for the lysine present and to find out if a simpler instrument, operating at a visible wavelength, could be used for determination of the yellow colour.

EXPERIMENTS AND RESULTS

Estimation of serine

Recovery experiments and standard curves. Portions of a solution, containing an accurately known amount of *N*-DNP-serine in 0.1N-hydrochloric acid, were weighed out and evaporated to dryness under vacuum. The residues were taken up in the mobile phase used for chromatography of DNP-serine, and made to 25ml. in a standard flask containing 1ml. of 2.5N-triethylamine. These solutions and a solvent blank were read at 360m μ in a 1cm. cell against air and the net extinction values were plotted against the weight of DNP-serine taken. They all fell close to a straight line. In another experiment, known amounts of DNP-serine were chromatographed and then treated similarly. Here too, the

Fig. 1. Calibration curve for serine determination. The amount of serine taken (μ g.) is plotted against the extinction of the final solution at 360m μ in a 1cm. cell. Details are given in the text.

points fell close to a straight line but the slope was about 2% less. This difference may be due to a small loss during manipulation and chromatography or to traces of a coloured impurity in DNP-serine.

When known amounts of pure serine (Merck, Sharp and Dohme) were dinitrophenylated, chromatographed and determined spectrophotometrically, the results were again linear (Fig. 1). When the results were compared with those obtained on DNP-serine after chromatography, the additional loss due to dinitrophenylation and extraction was found to be about 4%. The use of the line in Fig. 1 as the standard curve for serine determination allows for this loss. From the curve, the extinction value multiplied by 147.3 gives the serine content (μ g.) of the sample analysed, under the above conditions. This factor was used in the experiments recorded here, but, later, new calibration curves were set up for serine with Sigma grade L-serine (purity stated to be 100%). These gave a factor of 150.0 in one experiment and 149.6 in another instead of 147.3, but the differences are small.

Tests of the method. Hydrolysates of protein-containing material were obtained in which the amino acids had been determined by the donor by ion-exchange methods. Serine was determined in portions of the hydrolysates by the dinitrophenylation method described and the results are compared in Table 3 to the serine content calculated from the donor's result. This procedure avoided complications due to sampling errors and to variable losses during hydrolysis.

Estimation of lysine

Standard curve. The *NN'*-bis-(DNP)-lysine available (Matheson, 1963a) did not seem to be of

Table 3. Comparison of serine content of hydrolysates determined by ion-exchange methods and by the dinitrophenylation procedure

Donors: Dr Gabrielle Ellinger (E), Dr R. Dawson (D) and Dr A. Pusztai (P).

Donor	Material hydrolysed	Serine in portion analysed ($\mu\text{g.}$)	
		Calc. from donor's value	By dinitro-phenylation procedure
			$\left(\frac{\text{Serine by dinitrophenylation}}{\text{Serine by ion-exchange}} \right) \times 100$
E	Hay	45.0	48.6
			47.3
E	Hay	46.8	49.6
			49.9
E	Maize	77.7	78.9
			79.5
E	Soya-bean protein	43.2	45.7
		64.9	69.5
		86.4	90.4
E	Fish meal	28.9	30.6
		57.7	59.5
		57.7	59.9
		115.4	117.7
D	'Conarachin' fraction of heated peanut meal 5/45	23.6	24.0
		47.1	46.7
D	'Conarachin' fraction of peanut meal	36.8	35.3
		73.5	70.1
P	Bean protein 24	25.9	26.0
		51.8	50.3
		51.8	51.7
P	Bean protein 48	43.5	43.4
			42.8

the highest purity. Its extinction value fell by 8–9% on a single passage through a column of the type described but only by 2% on a second passage. (Since the 2% loss includes losses in the manipulations needed to concentrate the material in the eluate from the first column and to free it from urea, the loss on the second column is probably very small.) A standard curve was set up on lysine itself by dinitrophenylating known amounts of pure lysine hydrochloride and submitting it to the complete procedure. The extinction values at each wavelength (355m μ and 410m μ) fall very close to straight lines (Fig. 2); the lysine content ($\mu\text{g.}$) of an unknown sample is given by $111.1 E_{355}$ or $315.2 E_{410}$.

Tests of the method. Hydrolysates in which the lysine contents were known were obtained as before. The lysine content of portions of these hydrolysates was also determined by dinitrophenylation, either directly or else on the fast yellow material recovered from columns used for serine determination. The readings at 355m μ and at 410m μ , multiplied by the appropriate factor, gave estimates of lysine content which agreed very closely. The means of these estimates are com-

Fig. 2. Calibration curve for lysine determination. The amount of lysine taken ($\mu\text{g.}$) is plotted against the extinction of the final solution in a 1cm. cell at 355m μ (upper curve) and at 410m μ (lower curve). Details are given in the text.

pared with those calculated from the donor's value in Table 4.

DISCUSSION

Replicate determinations of serine or lysine agree very closely, the range of the results on one

Table 4. *Comparison of lysine content of hydrolysates determined by ion-exchange methods and by the dinitrophenylation procedure*

Donors were as identified in Table 3.

Donor	Material hydrolysed	Lysine in portion analysed ($\mu\text{g.}$)		$\left(\frac{\text{Lysine by dinitrophenylation}}{\text{Lysine by ion-exchange}} \right) \times 100$
		Calc. from donor's value	By dinitrophenylation procedure	
E	Hay	55.7	55.5*	99.6
E	Hay	55.9	56.0*	100.2
E	Maize	39.4	39.9*	101.3
E	Soya-bean protein	25.9	26.5	102.3
		51.8	53.7	103.7
D	'Conarachin' fraction of	30.1	30.4	101.0
	peanut meal GN2	60.3	59.6	98.8
D	'Conarachin' fraction of	25.2	26.6	105.6
	heated peanut meal GN13	26.9	30.1	111.9
		26.9	29.2	108.9
		50.1	54.0	107.8
		53.7	58.8	109.5
D	'Conarachin' fraction of	34.2	32.5	95.0
	heated peanut meal 125/5	47.2	47.1	99.8
		67.2	67.8	100.9
D	'Conarachin' fraction of	35.3	35.4	100.3
	peanut meal	70.7	71.1*	100.6
		70.7	69.5*	98.3

* Lysine determined on eluate from a column used to isolate the DNP-serine in the dinitrophenylated sample.

sample usually being about $\pm 2\%$. The agreement between the serine or lysine content found by ion-exchange methods and that found by the dinitrophenylation procedure is also good, though here there are often small systematic differences between analysts. These probably relate to minor errors in calibration and are not important for the present purpose. Small random disagreements also occur occasionally but it should be remembered that the ion-exchange results given here are calculated from a single determination and that the error of this method may sometimes be in the same sense as the error for the dinitrophenylation procedure and sometimes in the opposite direction. One may conclude that the procedure described has about the same accuracy as ion-exchange methods.

Those lysine determinations marked with an asterisk were done on the DNP-amino acids which eluted rapidly from a column used to isolate DNP-serine (i.e. on the samples analysed primarily for serine). They agree with the values for lysine found by ion-exchange as closely as results on samples put directly through a column for the isolation of bis-(DNP)-lysine, so there is no appreciable loss on the DNP-serine column. The use of a single dinitrophenylation step for several amino acids may be possible in other cases also.

The lysine content determined from readings at $355\text{m}\mu$ and $410\text{m}\mu$ agreed well. The spectrophotometer used in this Laboratory could probably be replaced by a simple photoelectric absorptiometer with filters operating in the visible range.

It has been shown that almost any one of the common amino acids, having an ether-soluble DNP derivative, can be isolated rapidly from a mixture of all the others by partition chromatography on a short column (Matheson, 1963*a*, 1965). The columns utilize partition, mainly of ionized molecules, between organic phases formed by esters and buffered aqueous phases held stationary on kieselguhr. Braunitzer & Reuther (1956) employed a similar system in which ionic partition must also have taken place, and used it to separate DNP-amino acids. They isolated a few DNP-amino acids directly, but generally they used group separations to give mixtures which were further resolved by other solvent systems. In this Laboratory, the selective variation in *R* value, needed to isolate a given DNP-amino acid on a column, was achieved by varying the proportion of a particular ester in the organic phase, by adding various amounts of salts or of urea to the aqueous phase or by lowering the pH so that partition of the free acids began to play a significant part. The

dinitrophenylation procedure can probably be made quantitative for those amino acids not so far tested. If so, the method will be generally applicable. It is fairly rapid, requires only common laboratory apparatus and is suited for determining a few amino acids in a large number of samples. However, the amount of work involved depends, to a greater extent than in methods based on ion-exchange resins, on the number of different amino acids to be determined in each sample; moreover a fresh column needs to be used for each sample, since it is quicker to pack a new column (about 20 min. required) than to elute the slower DNP-amino acids.

We thank Dr R. L. M. Synge for his constructive criticism of this paper, Dr Gabrielle M. Ellinger, Dr R. Dawson and Dr A. Pusztai for supplying analysed hydrolysates, Dr Elizabeth Work for her gift of LL-diaminopimelic acid and meso-diaminopimelic acid, Dr F. J. Bealing and Dr F. T. Khouw for certain DNP derivatives, and Miss Isobel Porter for her valuable technical assistance.

REFERENCES

- Braunitzer, G. & Reuther, K.-H. (1956). *Makromol. Chem.* **18-19**, 501.
Fraenkel-Conrat, H. & Singer, B. (1956). *Arch. Biochem. Biophys.* **60**, 64.
Hamilton, P. B. & Anderson, R. A. (1961). *Biochem. Prep.* **8**, 55.
Levy, A. L. & Chung, D. (1955). *J. Amer. chem. Soc.* **77**, 2899.
Lucas, F., Shaw, J. T. B. & Smith, S. G. (1963). *Analyt. Biochem.* **6**, 335.
Matheson, N. A. (1963a). *Biochem. J.* **88**, 146.
Matheson, N. A. (1963b). *Biochem. J.* **88**, 152.
Matheson, N. A. (1965). *Biochem. J.* **94**, 513.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Portugal, A. V., Green, R. & Sutherland, T. M. (1963). *J. Chromat.* **12**, 183.
Schroeder, W. A. & LeGette, J. (1953). *J. Amer. chem. Soc.* **75**, 4612.