The Quantitative Isolation of Valine from Amino Acid Mixtures as its Dinitrophenyl Derivative

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The quantitative determination of an amino acid, present in a mixture such as a protein hydrolysate, usually demands its separation; only in a few cases can it be directly determined chemically or enzymically. Amino acids can be separated and determined by paper chromatography but great care is needed to obtain reproducible results of reasonable accuracy (Hanes, 1961; Tigane, Wade, Wong & Hanes, 1961). Fractionation and complete analysis of amino acid mixtures on ion-exchange columns has been described in detail (Moore & Stein, 1954; Moore, Spackman & Stein, 1958) and several automatic analysers are available. Where there are many samples and only one or two amino acids need be determined in each, these methods are not very suitable because they are relatively slow and yet require expensive apparatus that normally handles only one sample at a time.

The ease and speed of separation of the DNP-amino acids reported by Matheson (1963) suggested that one might develop a system of analysis based on them, provided that the various steps could be made quantitative. The present paper describes the application of dinitrophenylation and separation by partition chromatography (Matheson, 1963) to the determination of valine in mixtures of amino acids.

MATERIALS AND METHODS

The preceding paper (Matheson, 1963) gives details of materials, of methods employed to prepare them for use, and of buffers.

Determination of valine. The sample containing 0.1–1.5 μ moles of free valine is evaporated to dryness at the water pump in the bottom of a test tube (15 cm. $\times$ 1.5 cm., with B14 ground-glass joint) which is shaken by hand. Non-volatile acids should be neutralized (with NaHCO_3) if abundant. From now on the analysis is done by the light of a 100 w tungsten-filament bulb. The residue is dissolved or suspended in 0.2 ml. of 10% (w/v) NaHCO_3 and 0.4 ml. of freshly prepared 10% (w/v) fluorodinitrobenzene (British Drug Houses Ltd.) in ethanol is added. The mixture is shaken gently for 2 hr. at room temperature and then the ethanol is removed *in vacuo*. Chloroform (5 ml.) and water (2.5 ml.) are added to the tube, which is stoppered and shaken vigorously. After the formation of separate layers the upper aqueous phase is removed with a teat pipette to a

similar tube. Aqueous extraction is repeated with a further three 2.5 ml. portions. The combined aqueous extracts are acidified with 0.2 ml. of 5N-HCl and extracted similarly with three 3 ml. portions of peroxide-free ether [washed with 5% (w/v) FeSO_4]. The combined ether extracts are evaporated to dryness in a small flask *in vacuo*. A chromatographic column buffered at either pH 8.3 or 8.8 is prepared and the mixture of DNP-amino acids is transferred to it as described by Matheson (1963). DNP-valine (R 0.50 $\pm$ 0.05) is soon visible immediately behind dinitrophenol (R 0.78 $\pm$ 0.05), which is a clear yellow and is normally present in quite large amount. Identification of the DNP-valine band may be confirmed by measurement of R values. Dinitrophenol usually begins to emerge from the column at pH 8.3 after 19 ml. of eluate has passed and continues to about 24 ml.; DNP-valine emerges from about 26 to 31 ml. These volumes are much the same on a column at pH 8.8. Eluate (12 ml.) is collected in a 50 ml. stoppered measuring cylinder, beginning when DNP-valine nears the bottom of the column; 20 ml. of 0.01 M-sodium phosphate buffer (Na_2HPO_4 – Na_3PO_4 in molar ratio 4:1, pH about 11), is pipetted in and the cylinder is put in a water bath at 30° for about 10 min. It is then shaken and returned to the bath until separate layers have formed, when the extinction of a portion of the bottom phase and of a similar blank, made by extracting 12 ml. of buffer-saturated ethyl acetate, are determined at 363 $m\mu$ in a cell of 1 cm. light-path.

If a determination of radioactivity is also to be made, the DNP-valine is recovered quantitatively from the cell with water and combined in the measuring cylinder with the remainder of the original aqueous phase, from which the ethyl acetate phase has meanwhile been removed. The pooled aqueous phases are acidified with 0.2 ml. of 5N-HCl and extracted with four 5 ml. portions of peroxide-free ether. The ether extracts are pooled by evaporating them to dryness *in vacuo* in a small flask. The residue is then plated and counted as described by Syngé & Youngson (1961).

Comments on the analytical method The dinitrophenylation procedure is based on that of Sanger (1949); it gave good recoveries of valine, as is shown below, so no modifications were tested. The mixed dinitrophenyl derivatives are obtained by suspending the reaction product in CHCl_3 and extracting it repeatedly with water. Fluorodinitrobenzene is almost insoluble in water so it remains in the CHCl_3 ; sodium salts of the dinitrophenyl derivatives are partitioned readily into water especially as the final extracts contain very little Na^+ ion. CHCl_3 is preferred to ether at this stage since it emulsifies less readily with DNP-amino acids at alkaline pH and it allows the required aqueous layer to form the top phase, which is more easily removed.

The work reported in the present paper was carried out with columns buffered at pH 8.3. It was later found that DNP- γ -aminobutyric acid does not separate from DNP-valine under such conditions but does so readily at pH 8.8. The absolute R values of the DNP-amino acids other than DNP- γ -aminobutyric acid are very little affected by the change in pH.

The determination of DNP-valine in column eluates is done by extraction into aqueous buffer before measuring the extinction. It should be possible to determine the DNP-valine directly in column eluate after adjustment to a fixed volume. The extraction procedure is only adopted here to remove any salts that might interfere with the subsequent determination of the radioactivity of valine under conditions of 'infinite thinness'.

Extraction of DNP-valine from the eluate is carried out at 30° because the solubility of ethyl acetate in water decreases with rise in temperature so that an extract made at room temperature often becomes cloudy in the spectrophotometer. The amount of DNP-valine extracted falls as the concentration of the extractant rises (Fig. 1); the effect of Na^+ ion is particularly marked. A comparison of tris-HCl buffer, sodium bicarbonate and sodium phosphate buffer showed that the first extracts the least and that the other two are about equally effective. In each case most DNP-valine is extracted by concentrations of 0.005M to 0.05M. Sodium phosphate buffer is preferred since it gives no CO_2 on acidification. A stock 0.1M solution (0.08M with respect to Na_2HPO_4 and 0.02M with respect to Na_3PO_4) is stored at 0° and diluted to 0.01M as required. The relative volumes of organic phase and phosphate buffer used in the trial experiments (Fig. 1) are altered in the analytical method to increase the proportion of DNP-valine extracted. Over 98% is then present in the aqueous layer after a single extraction.

In the spectrophotometric work both the sample and the blank are measured with E equal to 0 when there was no cell in the light-path. This shows whether there is any variation in the blank reading (usually about 0.050). In fact the blanks are fairly constant (± 0.003) with any given batch of solvents etc. The blank value is the same whether or not the 12 ml. portion of buffer-saturated ethyl acetate has been passed through the columns.

RESULTS

Recoveries and standard curves. Known amounts of DNP-valine were dissolved in 12 ml. portions of ethyl acetate saturated with 0.1M-tris-hydrochloric acid-salt buffer, pH 8.3, and extracted as described for column eluates in the Materials and Methods section. The extinction value of each extract and of a blank were read at 363 m μ . A second series of known quantities of DNP-valine was passed through chromatographic columns prepared with the same buffer. The appropriate 12 ml. portions of eluate were collected and extracted. The extinction values of these extracts and of a blank were also measured. Finally, known amounts of valine were dinitrophenylated and carried through the complete procedure. The results (Fig. 2) show that there was no loss of

DNP-valine on passage through the column and that the loss in the dinitrophenylation step was about 3%. The straight line given by those samples of valine that had been carried through the preparation (not the line shown) was used as a

Fig. 1. Effect of concentration of extractant on the partition of DNP-valine between ethyl acetate and aqueous solutions. Into a stoppered 50 ml. measuring cylinder were placed 40 ml. of ethyl acetate, containing 42.6 μg . of DNP-valine, and 10 ml. of the appropriate aqueous solution. The cylinders were put in a water bath at 30° for about 10 min. and were then shaken vigorously and returned to the bath. After settling was complete, a portion of the bottom phase was removed by teat pipette and its extinction at 363 m μ and that of a similar blank containing no DNP-valine were determined. Extractant concentration is given as molarity of tris base, of total phosphate and of sodium bicarbonate respectively. $\circ$, Tris-HCl buffer, pH 8.3; $\blacktriangle$, sodium bicarbonate; $\bullet$, sodium phosphate buffer, pH about 11.

Fig. 2. Effects of dinitrophenylation and of chromatography on recoveries of valine and of DNP-valine; standard curve. Known quantities of valine were dinitrophenylated and carried through the complete procedure. Known amounts of DNP-valine were extracted from ethyl acetate saturated with tris-salt buffer, with or without previous passage through the usual chromatographic column. The DNP-valine content was determined by spectrophotometry of the extracts. Further details are given in the text. $\circ$, Added as DNP-valine, then extracted; $\bullet$, added as DNP-valine, then chromatographed and extracted; $\blacktriangle$, added as valine initially.

Table 1. *Determination of valine in insulin*

Experimental details are given in the text.

Sample no.	1	2	3
Wt. of insulin preparation hydrolysed ($\mu\text{g.}$)	334	555	314
Nitrogen content of sample hydrolysed ($\mu\text{g.}$) (14.5% of N found in batch no. 2189)	48.5	80.5	45.6
Valine found in sample hydrolysed ($\mu\text{g.}$)	31.8	52.3	29.2
Valine content of hydrolysate of batch no. 2189 (g./16 g. of N)	10.5	10.4	10.2
Valine content as residues/insulin molecule (assuming all N in batch no. 2189 is in insulin of mol.wt. 5734)	5.10	5.05	4.97

standard curve in subsequent experiments. From it one can calculate that under the conditions described:

$$152 \times E_{1\text{cm.}} = \mu\text{g. of valine in sample analysed}$$

where $E_{1\text{cm.}}$ is the extinction of the aqueous extract at $363\text{ m}\mu$ in a cell of 1 cm. light-path, less the extinction value of the blank; both sample and blank are read with the spectrophotometer set at E equals 0 with no cell in the light-path.

Recovery of very small amounts of [^{14}C]valine. A solution (0.2 ml.) of [^{14}C]valine (482×10^3 counts/min./mg. of valine; approx. $1.7\text{ }\mu\text{g.}$ of valine/ml.) was plated and counted directly. Twice this volume was carried through the complete dinitrophenylation procedure. No yellow material was visible in the DNP-valine position but the eluate was collected as usual, beginning when all of the strong dinitrophenol band had left the column. The valine determined was less than the experimental error; the DNP-valine fraction gave 318 counts./min compared with 332 counts/min. taken (calculated from the direct count), a recovery of 96% with a sample that contained about $0.7\text{ }\mu\text{g.}$ of valine.

Determination of valine in insulin. The insulin used was from batch no. 2189 manufactured by British Drug Houses Ltd. in November 1955 for the Commission on Proteins of the International Union of Pure and Applied Chemistry. A solution of known concentration (about 1 mg./ml.) was prepared and dispensed by weight. Three portions were evaporated to dryness *in vacuo* in tubes. To each tube was added 1 ml. of constant-boiling hydrochloric acid; the tubes were sealed *in vacuo* and kept at 110° for 45 hr. The hydrolysates were evaporated to dryness and analysed for valine by the dinitrophenylation procedure with a column buffered at pH 8.3. Kjeldahl determinations were carried out on another three portions of the insulin solution. The results in Table 1 show that the determinations on three different hydrolysates agreed closely. Further, the mean values are very close to the values of $10.3\text{ g. of valine/16 g. of N}$

and 5 valine residues/insulin molecule, corresponding to the formula of insulin derived by Sanger and his co-workers (see Ryle, Sanger, Smith & Kitai, 1955).

DISCUSSION

The procedure for dinitrophenylating, separating and extracting amino acids has given recoveries of DNP-valine of over 95%. Krol (1952) and Levy (1954) also found no difficulty in dinitrophenylating amino acids quantitatively, but Schroeder & LeGette (1953) reported losses with several of the amino acids. Part of the losses may have occurred during ether-extraction of fluorodinitrobenzene from the alkaline reaction products, since ether will extract the DNP-amino acids from alkaline solutions to a small extent if the Na^+ ion concentration is high (N. A. Matheson, unpublished work). Krol's (1952) study of the rate of dinitrophenylation of glycine suggests that by extending the dinitrophenylation time from 2 to 4 hr. the percentage recovery of valine might be slightly increased. The small losses that occur at dinitrophenylation and at extraction are fully allowed for by a standard curve for valine put through the complete procedure. The values of specific activities are, however, consistently underestimated by about 2%, because the counts in the discarded ethyl acetate phase are not allowed for. The recoveries of DNP-valine given by the method are still good even when the amount is too small to measure spectrophotometrically, but has to be determined in terms of radioactivity. This experiment also emphasized the value of dinitrophenol as a marker immediately preceding DNP-valine on the column. The range of the method, $0.1\text{--}1.5\text{ }\mu\text{moles}$ of valine, is capable of considerable extension by alteration in the volumes of extractants, the dimensions of the column etc. DNP-valine obeys Beer's law over the entire range of concentrations measured (extinction values of $0\text{--}1.2$).

Valine determinations carried out on a sample of insulin specially purified for the I.U.P.A.C. gave results very close to the theoretical value of 5 valine

residues/molecule of insulin of molecular weight 5734. The results may be slightly less accurate than would appear since Harfenist (1953) has reported that the complete release of valine from insulin is slow. She found the equivalent of 4.73 residues/molecule after hydrolysis in 5.7N-hydrochloric acid *in vacuo* for 24 hr. at 110°, 4.93 residues/molecule after 48 hr. and 5.14 residues/molecule after 96 hr. For isoleucine (to which the single resistant valine residue seems to be bound) the corresponding values were 0.66, 0.80 and 0.97 residue/molecule after 24, 48 and 96 hr. respectively. Between 80 and 93 % of the isoleucylvalyl grouping appears to break down in 48 hr., so the total valine content of the 45 hr. hydrolysate used here should have been equivalent to 4.80–4.93 residues/molecule. For the same batch of insulin, R. Nunnikhoven (personal communication), while working in the Laboratorium voor Physiologische Chemie, Amsterdam, found a valine content of 4.7 residues/molecule after a 22 hr. hydrolysis with 6N-hydrochloric acid *in vacuo* at 110°, which agrees well with Harfenist's (1953) values. The value determined by the dinitrophenylation method is probably 1–5 % too high but it is still an accurate result.

The determination of valine by the method described is relatively simple and rapid. Though one must allow time for dinitrophenylation and extraction, many samples can be treated at a time. Several columns can also be run simultaneously, the number depending on the experience of the operator. DNP-valine emerges from the column in 30–40 min.; there is thus no bottle-neck, as often occurs in separation and analysis of amino acid mixtures by ion-exchange resins and reaction with ninhydrin. The extinction values of DNP-valine

have been read at 410 m μ as well as at 363 m μ with almost as good results. A colorimeter can therefore be used in place of a spectrophotometer.

SUMMARY

1. A method is described for the quantitative determination of valine as its dinitrophenyl derivative. It is simple, accurate and requires no special apparatus. The range is 0.1–1.5 μ moles of valine and several determinations may be done in parallel.

2. Recoveries of valine were good even when less than 1 μ g. of valine was used so that the amounts present had to be determined in terms of radioactivity.

3. The valine content of a purified insulin sample, determined by this method, agreed closely with the calculated value.

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The Metabolism of Propionate in the Isolated Cow's Udder

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Propionate is recognized as one of the principal products of carbohydrate fermentation by the micro-organisms inhabiting the rumen of cows (Elsden & Phillipson, 1948). When [1-¹⁴C]propionate was added to the perfusion blood of the isolated cow's udder, an appreciable specific radio-

activity was observed in the fatty acids containing an even number of C atoms/molecule but highest activity was found in the fatty acids containing an odd number of C atoms/molecule (James, Peeters & Lauryssens, 1956). In the present paper further studies on the metabolism of propionate by the perfused cow's udder are reported. The incorporation of ¹⁴C from [1-¹⁴C]propionate into organic

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