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DETERMINATION OF BRANCHED-CHAIN N-METHYLAMINO ACIDS

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SUMMARY

Ion-exchange chromatography did not resolve N-methyl-leucine from N-methylisoleucine, and paper chromatography only partially resolved N-methyl-isoleucine from N-methyl-*allo*-isoleucine. N-Methylvaline was well resolved by both techniques, a combination of which was used to determine all four amino acids quantitatively.

A spectrophotometric ninhydrin method is described for determining total branched-chain N-methylamino acids. Primary amino acids, if present, are determined in the same solution with 2,4,6-trinitrobenzenesulphonic acid, with which N-methylamino acids do not react, and a correction is applied to the result of the ninhydrin determination.

N-Methyl-D-*allo*-isoleucine was synthesized and its properties were recorded.

INTRODUCTION

Several species of the fungal genus *Fusarium* produce cyclodepsipeptide antibiotics known as enniatins[1]. A given isolate normally produces a mixture of homologous and isomeric enniatins, the hydrolyzates of which contain N-methyl-valine, -leucine and -isoleucine[2]. In order to develop a method for resolving such a mixture obtained from *F. sambucinum*[3], it was first necessary to be able to determine these three amino acids in hydrolyzates obtained from many chromatographic fractions. The paper chromatographic method described earlier[4] appeared to be unnecessarily tedious, and attempts were made to use an automatic amino acid analyzer for this purpose[5]. These efforts, although unsuccessful, led to the discovery of N-methyl-L-*allo*-isoleucine in hydrolyzates of enniatin A from *F. sambucinum*, a result which will be described in detail elsewhere. In this paper, a method is described for determining separately all four branched-chain N-methylamino acids by the use of both paper and ion-exchange chromatography. A simple technique for measuring their total amount in the presence of primary amino acids is also reported.

MATERIALS

L-Isoleucine and N-methyl derivatives of L-valine (MeVal), L-leucine (MeLeu)

and L-isoleucine (Melle) were purchased from Cyclo, Los Angeles, Calif., U.S.A. The D-form of N-methyl-*allo*-isoleucine (Me*a*lle) was synthesized from D-*allo*-isoleucine via the N-benzyl derivative[6]. Other amino acids were obtained from Koch-Light, Colnbrook, Great Britain. Each gave a single peak when examined in the automatic amino acid analyzer, and a single spot on paper chromatograms prepared in the three solvent systems described below.

METHODS

Paper chromatography

Qualitative paper chromatography was performed on Whatman 3MM paper. The following three solvent systems were used, all by the descending technique.

System A. tert.-Butanol–4.25 *N* ammonium hydroxide solution (4:1) (ref. 4).

System B. n-Butanol–acetic acid–water (4:1:5) (ref. 7).

System C. tert.-Amyl alcohol–acetic acid–water (20:1:20) (ref. 8).

N-Benzyl-N-methyl-D-*allo*-isoleucine was detected by spraying the paper with a 2% solution of iodine in chloroform; 0.1 μmole gave a transient yellow spot. All other amino acids were detected with the buffered ninhydrin spray previously described[4]. Papers were heated for at least 30 min at 110°. Spots due to 0.1-μmole amounts of primary amino acids, N-methylamino acids and N-benzyl-*allo*-isoleucine became visible in that order during the heating period, and after 30 min their intensities were similar. N-Benzyl-N-methyl-D-*allo*-isoleucine reacted very weakly, 0.1 μmole being barely detectable.

Determination of N-methylvaline and N-methyl-allo-isoleucine

These amino acids were determined using a Beckman-Spinco (Palo Alto, Calif., U.S.A.) Model 116 automatic amino acid analyzer. The column, 0.9 × 56 cm, was packed with UR-30 resin supplied by the manufacturers, and was operated at 56°. Samples were applied in a volume of 0.25 ml; amino acids were eluted with 0.1 *M* sodium citrate, pH 4.0, flowing at 35 ml/h. Ninhydrin solution was pumped at the rate of 25 ml/h. Peak areas were determined using the "height × width" method of integration described in the manufacturer's instruction manual.

Determination of N-methylvaline and N-methyl-leucine and of N-methylisoleucine plus N-methyl-allo-isoleucine

Sheets (46 × 57 cm) of Whatman 3MM paper were washed chromatographically overnight in diethyl ether. Solutions (5–20 μl containing 0.05–0.25 μmole) of amino acids in water were applied at the origin. The chromatograms were sprayed lightly with water and allowed to dry briefly in air, which resulted in the production of spots that were more compact than when this treatment was omitted. The papers were then suspended in an all-glass chromatographic tank containing the lower phase from system C. After a 2-h equilibration period, they were developed by the descending technique in the upper phase for 50 h, then hung in a fume-hood overnight so as to remove most of the acetic acid. The dried chromatograms were sprayed evenly with a 1% solution of ninhydrin in *n*-butanol (previously equilibrated with 0.05 *M* phosphate buffer, pH 7) and heated at 105° for 40 min. Rectangular pieces of the paper (2.5 × 3.75 cm) containing the ninhydrin-stained spots were excised, coarsely shredded and

eluted by shaking with 3 ml of freshly prepared acetone–0.05 *M* collidine acetate (pH 6.5) (7:3) followed by 2 ml of the same solvent. The combined extracts were centrifuged and their absorbance was determined at 575 nm in a 1-cm cell with distilled water as reference. The absorbance of a blank prepared by eluting an identically sized portion of the chromatogram from a sprayed region containing no amino acid was subtracted from the reading. Blank values were 0.01 absorbance unit or less. Standards (0.05, 0.1, 0.15 and 0.2 μmole) of MeIle were run on every chromatogram. Analyses were performed in duplicate.

Spectrophotometric determination of branched-chain N-methylamino acids

The sample (1 ml), containing 0.1–1 μmole of amino acids, was mixed with 4 *M* acetate buffer (0.5 ml) and Rosen's[9] ninhydrin reagent (0.5 ml). The tube containing the reaction mixture was covered so as to prevent evaporation and heated in a vigorously boiling water-bath for exactly 1 h, then immediately immersed in ice. The cooled solution was diluted with 5 ml of isopropanol–water (1:1) and the absorbance was measured at 575 nm in a 1-cm cuvette with distilled water as reference. The absorbance of a reagent blank was also measured; it did not exceed 0.05 absorbance unit and was usually less than 0.03. All determinations were performed in duplicate.

Primary amino acids were determined separately using 2,4,6-trinitrobenzenesulphonic acid (TNBS)[10], and their amounts were expressed as isoleucine by reference to a standard curve constructed for that amino acid using TNBS. The proportion of the ninhydrin colour due to primary amino acids was then calculated from a standard curve prepared for isoleucine using the ninhydrin technique described above, and this absorbance was subtracted from the ninhydrin absorbance due to total amino acids. Using this "corrected" absorbance, the amount of N-methylamino acids present in the sample was calculated as MeIle using a standard curve constructed for that amino acid by the ninhydrin technique.

RESULTS AND DISCUSSION

N-Methyl-D-*allo*-isoleucine has not previously been described. It was prepared from D-*allo*-isoleucine in an overall yield of 47%. Its properties, and those of the intermediates used in its synthesis, are presented in Table I.

Ion-exchange chromatographic analysis

Ninhydrin reacts with N-methylamino acids more slowly than with primary amino acids, but sarcosine, N-methylalanine, MeLeu and N-methylphenylalanine can be determined with the automatic amino acid analyzer using pH 4.25 citrate buffer flowing at one-half of the usual rate[5]. When the four branched-chain N-methylamino acids were chromatographed in this way, they were incompletely resolved, all four having elution times between 46 and 57 min. The resolution was even poorer in a pH 4.5 buffer, but improved as the pH was lowered. However, MeLeu and MeIle were not resolved at any pH between 3.25 and 4.5. At the lower pH values, elution times were inconveniently long (168 min for MeLeu at pH 3.25) and a buffer of pH 3.85 was selected for determining MeVal and MeaIle. Chromatographic data for the four amino acids are shown in Table II. Elution times were reproducible, and the N-methylamino acids were well separated from the corresponding primary amino acids.

TABLE I

PROPERTIES OF N-METHYL-D-*allo*-ISOLEUCINE AND ITS SYNTHETIC INTERMEDIATES

Synthesis was by reductive alkylation and hydrogenolysis according to the method of Quitt *et al.*[6].

Property	D-*allo-Isoleucine*	*N-Benzyl-*D-*allo-isoleucine*	*N-Benzyl-N-methyl-*D-*allo-isoleucine*	*N-Methyl-*D-*allo-isoleucine*
Yield (%)*	—	80	80	73
M.p. (°C)**	—	251–253 (decomp.)	156–158	288–290 (decomp.)
$[\alpha]_D^{22}$***	−36.2°	−19.7°	−31.0°	−44.1°
N (%): calculated	10.68	6.33	5.95	9.65
found	10.71	6.23	5.91	9.51
R_F§ in system A	0.33	0.67	0.70	0.45
B	0.57	0.83	0.86	0.63
C	0.32	0.74	0.81	0.36

* Yields are of recrystallized material with the properties listed.
** Uncorrected.
*** For 1% solutions in 6 *N* HCl.
§ In paper chromatography using the techniques described in the text.

Thus valine, *allo*-isoleucine, isoleucine and leucine had elution times of 74, 101, 106 and 115 min, respectively, under these conditions. The constants, ($H \times W$) per μmole, were rather variable, but the ratio of the constants for MeVal and Me*a*Ile was extremely reproducible (Table II). In this laboratory, standards of these two amino acids are run routinely before the analysis of an unknown sample.

TABLE II

ION-EXCHANGE CHROMATOGRAPHY OF BRANCHED-CHAIN N-METHYLAMINO ACIDS

A 0.9 × 22 cm column of PA-35 resin was eluted with 0.1 *M* citrate buffer, pH 3.85, at 35 ml/h and 56°. The flow-rate of the ninhydrin reagent was 25 ml/h.

Amino acid	*No. of experiments*	*H × W per μmole*			*Elution time (min)*		
		Highest value	*Lowest value*	*Mean*	*Highest value*	*Lowest value*	*Mean*
MeVal	6	10.2	7.7	8.6	47	44	46
Me*a*Ile	7	9.7	7.3	8.3	56	53	55
MeIle	5	7.4	6.4	6.8	63	60	61
MeLeu	4	34.5	30.5	32.4	64	62	62
MeVal/Me*a*Ile	5	1.05	1.03	1.04	—	—	—

The constants for the three amino acids whose alkyl groups were branched in the β-position were lower by a factor of about four than the value obtained for MeLeu, in which the chain is branched in the γ-position. This effect, attributable to the steric hindrance imposed by β-substitution, might be overcome in part by further decreasing the buffer flow-rate. This was not done because the elution times would then have become inconveniently long.

Paper chromatographic analysis

As MeIle and MeLeu could not be resolved by the amino acid analyzer, they

were determined by quantitative paper chromatography. The solvent system A previously used[4] did not resolve MeLeu sufficiently well from its isomers, although it was more efficient than system B. Excellent resolution was obtained in system C, which permitted the separate determination of MeLeu and of MeIle plus Me*a*Ile as well as providing a check on the amount of MeVal (Table III). For this purpose, chromatograms were stained with the buffered ninhydrin reagent previously employed[4]; the stained areas were eluted with buffered 70% (v/v) acetone, as unbuffered 70% (v/v) acetone gave erratic results.

TABLE III

PAPER CHROMATOGRAPHY OF BRANCHED-CHAIN PRIMARY AND N-METHYLAMINO ACIDS

Whatman 3MM paper was used. Results are expressed as distances moved relative to MeLeu = 100 on descending chromatograms. The composition of solvent systems A, B and C is given in the text.

Amino acids	*Distance moved relative to MeLeu in solvent*		
	System A	*System B*	*System C*
N-Methyl-leucine	100*	100**	100***
Leucine	84	87	92
N-Methylisoleucine	94	98	88
Isoleucine	76	85	82
N-Methyl-*allo*-isoleucine	90	96	81
allo-Isoleucine	66	87	72
N-Methylvaline	74	78	54
Valine	54	65	48

* 29.6 cm in 60 h.
** 28.8 cm in 18 h.
*** 31.7 cm in 60 h.

Results obtained by this method for the purpose of constructing standard curves are presented in Table IV. As reported previously, colour yields were similar to those obtained with primary amino acids[4], and they were identical for all four compounds. In applying this method, therefore, a standard curve constructed for MeIle (Fig. 1) was used in calculating the amounts of MeIle plus Me*a*Ile and of MeLeu (and sometimes of MeVal). As the amounts of MeVal and Me*a*Ile in a sample could be determined by ion-exchange chromatography, the amount of MeIle could be obtained by difference.

In four different crude enniatin hydrolyzates, the MeVal contents were 1.03, 0.764, 0.279 and 0.076 μmole per 0.1 ml when determined in single samples by ion-exchange chromatography. The same samples analyzed by quantitative paper chromatography were found to contain 1.08, 0.842, 0.289 and 0.088 μmole per 0.1 ml, respectively (values are means of duplicate determinations).

Determination of total primary and total N-methyl branched-chain amino acids in one solution

For preliminary monitoring of column chromatographic fractions containing enniatins, a rapid method for determining total N-methylamino acids in hydrolyzates was required. The method of Rosen[9], modified by increasing the heating period to

TABLE IV

PAPER CHROMATOGRAPHIC DETERMINATION OF BRANCHED-CHAIN N-METHYL-AMINO ACIDS

Chromatograms were run on Whatman 3MM paper in *tert.*-amyl alcohol–acetic acid–water (20:1:20), dried and stained with ninhydrin. Spots were eluted in buffered (pH 6.5) 70% (v/v) acetone and the absorbance of the eluates (5 ml) was measured at 575 nm.

Amount of amino acid applied (μmole)	*Absorbance at 575 nm* observed for*			
	*MeVal****	*MeAlle****	*MeIle*****	*MeLeu****
0.05	0.132	0.139	0.142	0.142
0.10	0.264	0.264	0.265	0.260
0.15	0.412	0.414	0.418	0.415
0.20	0.551	0.549	0.550	0.552
0.25	—	—	0.663§	—
Absorbance per 0.1 μmole ± standard deviation (*n*)§§	0.269 ±0.013 (16)	0.273 ±0.015 (16)	0.274 ±0.014 (23)	0.273 ±0.017 (16)

* Values recorded after subtraction of paper blank.
** Each value is the mean of four determinations.
*** Each value is the mean of five determinations.
§ Mean of three determinations.
§§ Calculated from the pooled results for all concentrations. *n* = number of analyses.

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1 h as recommended for sarcosine[5], gave very high blank values. The effects of altering the buffer were therefore investigated. As it had previously been observed[4] that the optimum pH in the ninhydrin reaction is different for primary and N-methylamino

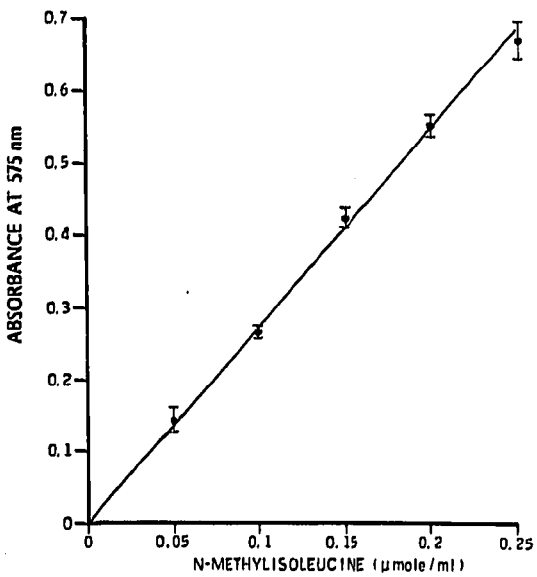

Fig. 1. Standard curve used in determining N-methylamino acids by quantitative paper chromatography. This curve was constructed for N-methylisoleucine using the results in Table IV. Each point is a mean value, the actual range of results being shown by the vertical lines. Details of the method used are given in the text.

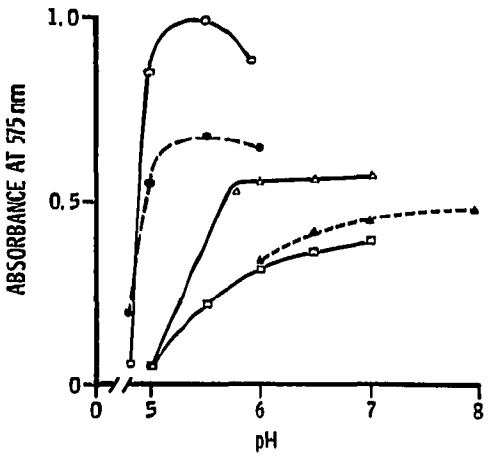

Fig. 2. Dependence upon pH and buffer of the reaction of N-methylisoleucine with ninhydrin. The amino acid (1 μmole in 1 ml) was heated with 0.5 ml of Rosen's ninhydrin reagent and 0.5 ml of the buffer solution in a boiling water-bath for 1 h, then cooled immediately and diluted with 50% isopropanol (5 ml). Absorbance was determined in a 1-cm cuvette against distilled water. Results were plotted after subtracting the reagent blank. Each point is the mean of four results. Molarities of buffer solutions are given in Table V. ○, Acetate; ●, acetate + KCN; △, citrate; ▲, phosphate; □, maleate.

acids, each buffer was investigated over a range of pH values. The results (Fig. 2), obtained using Melle, showed that the optimum pH was a function of the buffer used and, more importantly, that the colour yield using acetate buffer was considerably greater when potassium cyanide was omitted. This observation has been repeated many times. Colour yields obtained with 1 μmole of Melle after heating for 1 h with ninhydrin in various buffers at optimal pH values are shown in Table V. These results show the further advantage of using a simple acetate buffer, *viz.* that reagent blank absorbances after the prolonged heating period were lower by about an order of magnitude than those obtained using the same buffer containing potassium cyanide, or with any other buffer tested.

TABLE V

REACTION OF N-METHYLISOLEUCINE WITH NINHYDRIN IN VARIOUS BUFFERS

The amino acid (1 μmole) was heated in a boiling water-bath with ninhydrin reagent according to Rosen[9], but with a variety of buffers and for 60 min. The rapidly cooled mixtures were diluted with isopropanol–water (1:1) before determining their absorbances. The final volume in each case was 7.0 ml. Each value is the mean of four determinations.

Buffer	*pH**	*Absorbance at 575 nm***			
		Blank	*Sample*	*Sample − blank*	*Sample/blank*
Acetate (4 *M*) + KCN***	5.5	0.415	1.090	0.68	2.6
Acetate (4 *M*)	5.5	0.030	0.997	0.967	33
Citrate (1 *M*)	5.8	0.225	0.780	0.55	3.5
Maleate (0.5 *M*)	6.0	0.330	0.635	0.30	1.9
Phosphate (1 *M*)	6.5	0.490	0.860	0.37	1.8

* At room temperature.
** In a 1-cm cuvette with distilled water as reference.
*** 1 mg per 100 ml.

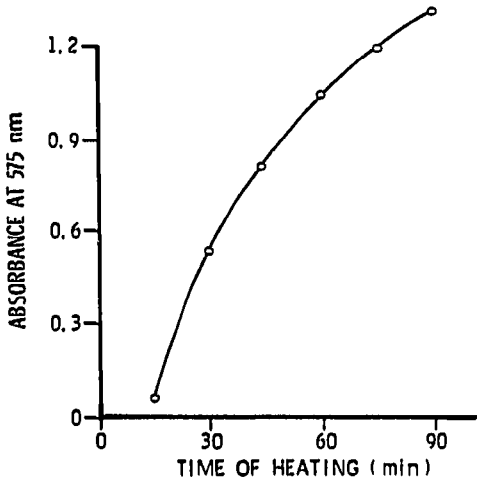

Fig. 3. Reaction of N-methylisoleucine with ninhydrin as a function of heating time. The amino acid (1 μmole in 1 ml) was heated with 0.5 ml of Rosen's ninhydrin reagent and 0.5 ml of 4 *M* acetate buffer, pH 5.5, for the time indicated, and then treated as described in the legend for Fig. 1. Each point is the mean of eight results.

Even after heating in simple acetate buffer for 1 h, absorbance values with MeIle were lower than those expected for the complete reaction of this amino acid with ninhydrin. The time course of colour development was therefore investigated. The results (Fig. 3) showed that a 1-h heating period did not suffice for complete reaction. However, a longer time would have rendered the method unnecessarily tedious, and a standard heating period of 1 h was therefore chosen. The reproducibility of the results for MeIle was satisfactory for a method that measured the product of an uncompleted reaction (Table VI). The colour yields from the four amino acids were compared in a separate experiment in which samples, each containing 1 μmole, were analysed in quadruplicate. The mean results were MeVal 0.941, Me*a*lle 0.916, MeIle 0.935 and MeLeu 0.992 absorbance unit/μmole. Hence the application of the method

TABLE VI

SPECTROPHOTOMETRIC DETERMINATION OF N-METHYLISOLEUCINE

Reactions were carried out in duplicate as described in the text, on separate occasions. The final volume of solutions was 7.0 ml.

Amount (μmole)	*No. of determinations*	*Absorbance at 575 nm*		
		Highest value	*Lowest value*	*Mean ± standard deviation*
0.125	8	0.119	0.100	0.111 ± 0.008
0.250	9	0.254	0.220	0.235 ± 0.011
0.500	9	0.464	0.448	0.453 ± 0.005
0.750	9	0.726	0.702	0.713 ± 0.007
1.000	9	1.106	0.910	0.965 ± 0.059
Absorbance per μmole*				0.932 ± 0.055

* Calculated from the pooled results for all concentrations.

to a mixture of all four amino acids should give results that are accurate to within about 7% when calculated with reference to a standard curve for any one of them. If their approximate proportions are known, a standard curve constructed from a mixture of the amino acids in those proportions would be more appropriate and should give more accurate results.

Hydrolyzates of crude enniatins from *F. sambucinum* contained amino acids that comprised, on a molar basis, about 75% Melle, the remainder being a mixture of the other N-methylamino acids and 2–3% of primary amino acids, mainly isoleucine. In order to determine the total N-methylamino acid content of these hydrolyzates using ninhydrin, the contribution of the more rapidly and strongly reacting primary amino acids to the absorbance had to be determined. No method for the specific measurement of primary amino acids in the presence of their methylated analogues has been described. We observed that the latter do not react with TNBS, at least under the conditions used to determine primary amino acids with this reagent. Therefore, primary amino acids in the hydrolyzates were determined by TNBS[10]. The results were expressed as isoleucine, and a standard curve constructed for that amino acid using the present ninhydrin method was used to calculate its contribution to the ninhydrin absorbance. This figure was then subtracted from the absorbance obtained when the hydrolyzate was analysed by the ninhydrin method. A sample calculation is given below.

Weight of crude enniatin hydrolyzed (reconstituted in 1 ml) = 10.0 mg
Primary amino acids as Ile (TNBS) = 0.075 μmole per 0.1 ml
Absorbance given by 1 μmole of Ile (ninhydrin) = 1.70
$\therefore$ Absorbance due to primary amino acids (ninhydrin) = 0.013 per 0.01 ml
Absorbance due to total amino acids (ninhydrin) = 0.342 per 0.01 ml
$\therefore$ Absorbance due to N-methylamino acids (ninhydrin) = 0.329 per 0.01 ml
Absorbance given by 1 μmole of Melle (ninhydrin) = 0.935

$$\therefore \text{Total N-methylamino acids as Melle} = \frac{0.329 \times 100}{0.935 \times 10} =$$

= 3.52 μmoles/mg crude enniatin.

Standard curves for isoleucine using the TNBS and ninhydrin methods and for Melle by the latter method are shown in Fig. 4.

This method of determining total N-methylamino acids involved approximations and the final result was obtained by difference. The results were therefore compared with those obtained by the more precise paper chromatographic method, in which total N-methylamino acids were calculated as the sum of the four individual compounds present. Six duplicate analyses of one hydrolyzate were performed by each method. The amount found by the difference method was 3.29 $\pm$ 0.22 and by paper chromatography 3.52 $\pm$ 0.16 μmoles of N-methylamino acids per milligram of crude enniatin (these results are means $\pm$ standard deviation).

The methods described should be useful in investigating the composition of peptide mixtures containing residues of branched-chain N-methylamino and primary amino acids. Moreover, the observation that N-methylamino acids do not react with

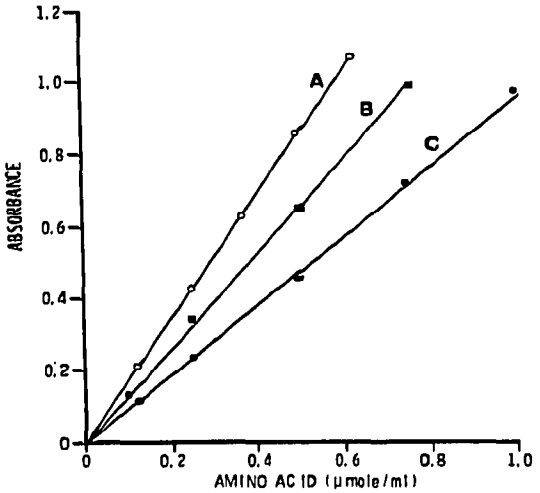

Fig. 4. Standard curves used in determining N-methylamino acids as N-methylisoleu ne and primary amino acids as isoleucine. A, isoleucine determined by ninhydrin, absorbance measured at 575 nm. B, isoleucine determined by TNBS, absorbance measured at 420 nm. C, N-Methylisoleucine determined by ninhydrin, absorbance measured at 575 nm. Details of the conditions used are given in the text.

TNBS is capable of further exploitation, as they do react well with 2,4-dinitrofluorobenzene. The possibility of using this difference to analyze more complex mixtures of primary and N-methylated amino acids is being investigated.

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