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DETERMINATION OF N-METHYLAMINO ACIDS AND THEIR OPTICAL PURITY WITH AN AMINO ACID ANALYZER*

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SUMMARY

The ninhydrin color constants for N-methylamino acids obtained with a Model 120B Beckman amino acid analyzer were shown to increase 10–20 times to 18–93 % of those for the parent amino acid when the eluting buffer flow rate was decreased from 68 ml/h to 34 ml/h. The optical purity of N-methylamino acids can be established to within one part in 100 by determination with the analyzer of the diastereomeric dipeptides obtained by coupling L-alanine N-carboxyanhydride with the N-methylamino acid as devised for amino acids by MANNING AND MOORE.

INTRODUCTION

N-Methylamino acids react with ninhydrin[1–3], however, a higher temperature[2,4] and a longer reaction time[5,6] are required in order to obtain the maximum color development for these and the α-C-methylamino acids. EBATA *et al.*[5] investigated the determination of N-methylamino acids with a Hitachi KLA Type 2 amino acid analyzer and found that the color constants were 1.4–20 % of those for the parent amino acid, that many gave unsymmetrical peaks, and that the tedious addition of absorbances[7] method had to be used for the calculations. They therefore concluded that the amino acid analyzer was unsuitable for the determination of N-methylamino acids. Our need for a method of determining N-methylamino acids[8] has prompted an investigation of this problem. We have found that a standard Beckman Model 120B amino acid analyzer is a most satisfactory instrument for determining N-methylamino acids if it is operated with a half-normal buffer flow rate***. Under these conditions, the color constants are 18–93 % of those for the parent amino acid, and the usual $(H \times W)/C$ method of calculation[7] can be employed. The constants for two α-C-methylamino acids were also increased substantially.

A second obstacle encountered in developing our new method of synthesis of N-methylamino acids was establishing the optical purity of the products. The optical

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purity of amino acids has generally been determined using stereospecific enzymes such as the amino acid oxidases[9]. This method, unfortunately, is not applicable to N-methylamino acids because suitable enzymes are not available. A more recent approach to the determination of the isomers of amino acids involves the preparation of their diastereomeric dipeptides followed by separation and determination of these by some type of chromatography[10–14]. The method of MANNING AND MOORE[13] in which the dipeptides are prepared by reaction of the amino acid with an optically pure amino acid N-carboxyanhydride and subsequently determined with an amino acid analyzer seemed particularly attractive to us. This method was therefore investigated and it was found that it indeed could be used for determining the optical purity of N-methylamino acids.

MATERIALS AND METHODS

The amino acids were obtained from General Biochemicals Corp., Chagrin Falls, Ohio; sarcosine, N-methyl-DL-leucine, N-methyl-DL-alanine and α-C-methyl-DL-leucine from Cyclo Chemical Corp., Los Angeles, Calif.; α-aminoisobutyric acid from Sigma Chemical Co., St. Louis, Mo.; and L-alanine N-carboxyanhydride from Miles Laboratories Inc., Elkhart, Ind. N-Methyl-L-leucine, N-ethyl-L-leucine and N-methyl-L-phenylalanine were synthesized in our laboratory[8]. The amino acid analyzer was a standard Beckman Model 120B instrument equipped with a 0.9 × 50 cm column of AA-15 resin at 57°. Normal operation conditions refer to a 0.35 *N* sodium citrate buffer, pH 3.28, pumped at a flow rate of 68 ml/h with the ninhydrin solution being pumped at 34 ml/h. The absorption spectra of the products of the ninhydrin reaction with leucine, N-methylleucine and α-C-methylleucine were taken with a Unicam SP-800 recording spectrophotometer. The reaction was carried out in a boiling water bath for 30 min using the method of ROSEN[15].

The L-alanyl dipeptides for the determination of optical purity were prepared essentially as described by MANNING AND MOORE[13]. The sample (100 μmoles) was weighed into a 100 × 10 mm pyrex test tube and 1 ml of ice-cold 0.45 *M* borate buffer, pH 10.4 (prepared by adding 5 *N* KOH to 0.45 *M* boric acid at 0°) and one drop of octanoic acid were added. The tube was taken into a cold room (4°), L-alanine N-carboxyanhydride (12.7 mg; 100 μmoles) was quickly added, and the tube was shaken vigorously on a "Vortex Genie" stirrer for 2 min. The solution was brought to pH 2 with *N* HCl, diluted to 10 ml with water, filtered (Celite) and suitable aliquots were analyzed on the analyzer.

RESULTS AND DISCUSSION

Determination of N-methylamino acids

The results for the chromatography of a few representative N-methylamino acids on the long column of the amino acid analyzer operated under the normal conditions are illustrated in Fig. 1. It is seen that in agreement with a previous report[5], the peaks were all unsymmetrical, and moreover, that the color yields were very low since the sarcosine peak has a height about equal to that given by 0.05 μmole of glycine on our instrument. The variation of the constant $C = (H \times W)/c$ (H = net height of peak = absorbance; W = width of peak at half net height; c =

concentration) with concentration for one of these methylamino acids is shown in Fig. 2. It was obvious that the usual method of calculation using this constant could not be used. We had previously encountered a similar difficulty in the determination of ammonia by a modified procedure[16]. We had found, however, that this difficulty could be circumvented by using for the calculations a plot of $H \times W$ *versus* concentration, which had given a straight line. When this type of plot was made for the N-methylamino acids, as presented in Fig. 3, it was indeed found that straight lines resulted. Therefore, despite all the difficulties alluded to above, N-methylamino acids can be determined with the amino acid analyzer, albeit with only fair reproducibility, if a plot of $H \times W$ *versus* concentration is used for the calculations.

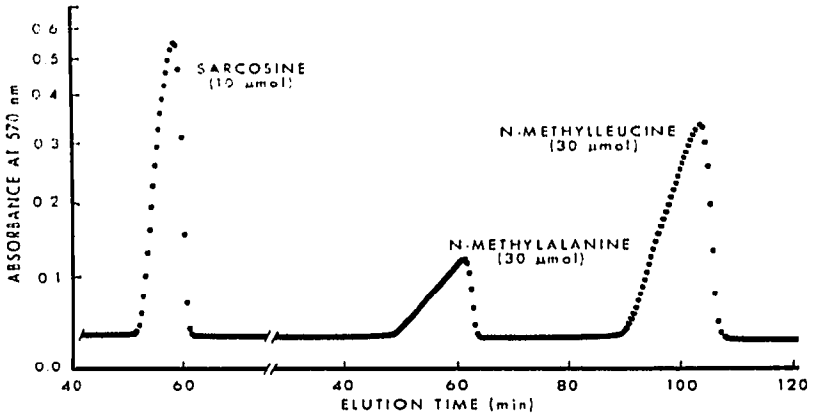

Fig. 1. Chromatography of N-methylamino acids under the normal operating conditions of the Beckman amino acid analyzer.

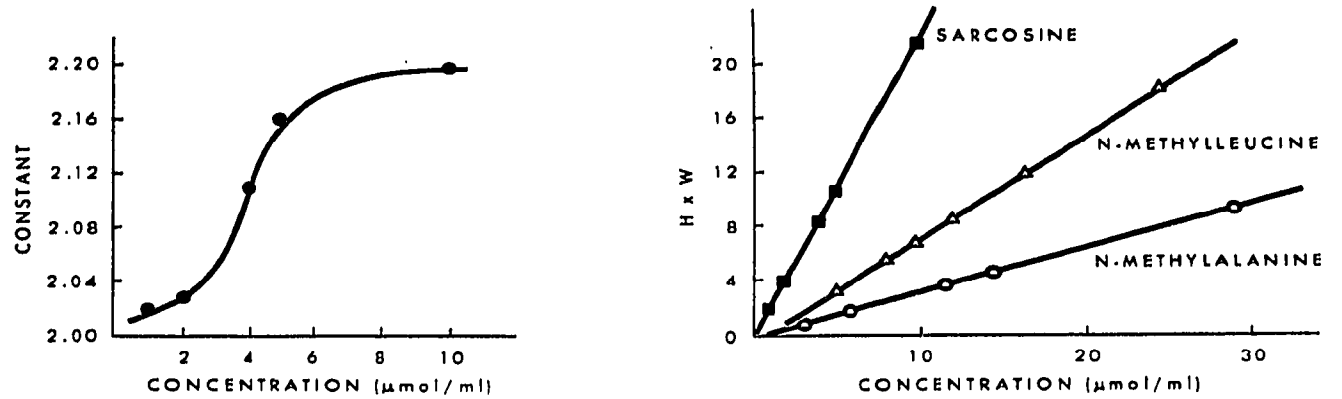

Fig. 2. Variation of constant ($H \times W/c$) with concentration for sarcosine under the normal operating conditions of the analyzer.

Fig. 3. Standard curves for the determination of N-methylamino acids under normal operating conditions of the analyzer.

Not being satisfied with the sensitivity of the method, we then verified that the reason for the low color yields, as suggested[5], was that the reaction of ninhydrin with N-methylamino acids was so much slower than with amino acids. It is seen in Fig. 4 that for sarcosine, the least hindered of all the N-methylamino acids, the reaction was not complete until after about 50 min. Under the same conditions, the reaction with

glycine was complete in 10–12 min. It has also been reported that the reaction of ninhydrin with an α-C-methylamino acid is unusually slow[4]. There was therefore no doubt in our minds that the low color yields obtained for these methylamino acids under the normal operating conditions of the analyzer were in large part due to the fact that the effluent stream remained in the reaction coil for too short a time. A second reason considered to account for the low color yields was the possibility that the colored products formed were not the same as those formed from amino acids and consequently that their absorbance maxima were not at the same wave length. This was therefore examined and it was found that over the range of 350–700 nm, the absorbance spectra of the solutions resulting from the reaction of ninhydrin with leucine, N-methylleucine and α-C-methylleucine were all identical, having maxima at 408 and 570 nm and minima at 458 nm.

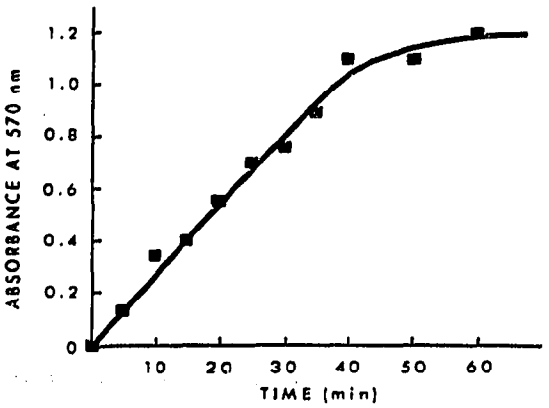

Fig. 4. Variation of the absorbance at 570 nm with time for the reaction of sarcosine with ninhydrin. 0.5-μmole samples of sarcosine were heated in a boiling water bath with cyanide–acetate buffer and ninhydrin reagent according to ROSEN[15]. At the designated times, the cooled mixtures were diluted with isopropanol–water (1:1) and the absorbance at 570 nm was read against a blank.

It was therefore decided to increase the reaction time in the analyzer by decreasing the buffer flow rate by one half, from 68 ml/h to 34 ml/h. This had the effect of increasing the reaction time by 50 % (the ninhydrin flow rate was not changed) and consequently doubling the constants for the amino acids. However, it also had the dramatic effect of increasing the constants for the N-methylamino acids by 10–20 times. The constants for α-C-methylalanine and α-C-methylleucine were also increased 5-fold and that for N-ethylleucine 30-fold. Furthermore, the constants were really constant, and the reproducibility was comparable with that for amino acids. The values of the constants for the N-methylamino acids now varied from 18–93 % of those for the parent amino acids. A comparison of the data for both flow rates appears in Table I.

There is a useful observation to be made from these results. The fact that α-C- and α-N-alkylamino acids react more slowly with ninhydrin can be made use of for identification purposes. A peak on the amino acid analyzer chart paper can be identified as being due to an alkylamino acid if, upon decreasing the buffer flow rate, the increase in the size ($H \times W$) of the peak is greater than the increase observed for an α-amino acid.

TABLE I

CHROMATOGRAPHIC DATA[a] FOR DIFFERENT ELUTING BUFFER FLOW RATES

A 0.9 × 50 cm column of AA-15 resin eluted with 0.35 *N* sodium citrate, pH 4.25, at 57°.

Compound	*Normal; 68 ml/h*		*Half-normal; 34 ml/h*	
	Constant	*Time (min)*	*Constant*	*Time (min)*
Sarcosine	3.0	30	36.3	59.5
Glycine	23.4	33	38.9	67
N-Methylalanine	0.36	28	7.5	54
α-C-Methylalanine[a]	1.8	33	10.2	62
Alanine	24.1	32.5	42.7	61
N-Methylleucine	0.74	34	15.7	63
N-Ethylleucine	0.03	32	1.0	60.5
α-C-Methylleucine	1.95	46.5	11.2	92.5
Leucine	23.9	51	40.5	102
N-Methylphenylalanine	0.66	61	23.5	106
Phenylalanine	22.7	79	40.6	157

[a] α-Aminoisobutyric acid.

Optical purity of N-methylamino acids

Since the details of the method of MANNING AND MOORE[13] were not available at the time, exploratory experiments were first carried out. The procedure using L-alanine N-carboxyanhydride described in MATERIALS AND METHODS is the one which was subsequently adopted in our laboratory for routinely determining the optical purity of amino acids. With amino acids, it gives results similar to those reported[13*], that is, about a 90% yield in the coupling, small amounts of alanine and alanylalanine as by-products, and narrow separable peaks for the diastereomeric dipeptides. The reaction, however, does not proceed so smoothly with N-methylamino acids. It was found that much more alanine and alanylalanine resulted after the reaction of alanine N-carboxyanhydride with N-methylleucine than with leucine. This is due to the generally much lower reactivity of N-methylamino acids towards acylation reagents. This was demonstrated in this case by carrying out the coupling with a mixture containing seven parts N-methylleucine and one part leucine. The dipeptides were formed in a ratio of 1:2 (Ala·MeLeu:Ala·Leu).

A second signifiant difference which was observed when the method of MANNING AND MOORE was applied to N-methylleucine was that the peaks for the dipeptides of the latter were three times as wide (at the half-height) as those for the corresponding unmethylated peptides. This had the effect of decreasing the sensitivity of the method since the peaks of the two diastereomeric methylamino acid dipeptides were not so well separated. Instead of being able to detect one part of an isomer in 1000 parts of the other, as reported[13], we were only able to detect down to one part in 100. However, this was satisfactory for our purpose. The chromatographic data are given in Table II. Note that the N-methylamino acid isomers could actually be

* We are grateful to Dr. S. MOORE for sending us a copy of the manuscript before its publication.

TABLE II

CHROMATOGRAPHIC DATA FOR SOME L-ALANINEDIPEPTIDES

A 0.9 × 50 cm column of AA-15 resin eluted at a flow rate of 68 ml/h with 0.35 *N* sodium citrate, pH 3.28 for 85 min, followed by pH 4.25 buffer.

Compound	*Elution time (min)*	*Color yield ratio* L-D/L-L
L-Ala-L-Ala	144	
L-Ala-D-Leu	196	0.85
L-Ala-L-Leu	207	
L-Ala-D-MeLeu	180	1.06
L-Ala-L-MeLeu	170	
Ala	89	
Leu	141	
MeLeu	87	

determined in the presence of the parent amino acid since there would be no interference by any other peak.

It is worth noting a few of the very attractive features of this method of MANNING AND MOORE for determining the optical purity of amino acids. The amino acid in question need not be crystallized, nor purified, nor even isolated from a solution. The test can be run on a salt solution of the amino acid even in the presence of other amino acids if they or their coupling products do not overlap on the chromatogram with the dipeptide peaks. The information required is the relative color yields of the dipeptides which must be obtained from the racemate since they are different for each pair of diastereomers, and knowledge from some source of the order of elution of the diastereomers since this cannot be predicted.

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