Journal of Chromatography, 383 (1986) 271–280
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3351

DETERMINATION OF $^{15}NH_3$ BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

APPLICATION TO THE MEASUREMENT OF PUTRESCINE OXIDATION BY HUMAN PLASMA

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(First received April 3rd, 1986; revised manuscript received July 24th, 1986)

SUMMARY

A convenient and sensitive method for the determination of $^{15}NH_3$ has been developed. Ammonia was purified from sample solutions by a modified microdiffusion method, derivatized with pentafluorobenzoyl chloride to pentafluorobenzamide (PFBA) and determined by gas chromatography–mass spectrometry using a multiple ion detector. PFBA was eluted from the gas chromatographic column within 2 min and resulted in a simple mass fragmentation pattern. The $^{15}N/^{14}N$ ratio was accurately determined with picomole amounts of PFBA by measuring the molecular ions of PFBA and [^{15}N]PFBA. The method was applied to the assay of putrescine oxidation by human plasma. $^{15}NH_3$ was produced by incubating ^{15}N-labelled putrescine with plasma. The $^{15}NH_3$ production was time dependent and strongly inhibited by the addition of aminoguanidine, an inhibitor of diamine oxidase. Exceedingly high $^{15}NH_3$ production from [^{15}N]putrescine was observed in the plasma from pregnant women. In contrast, only trace amounts of $^{15}NH_3$ were formed in the plasma from normal men and non-pregnant women. The method seems to be applicable to various biological systems that produce ammonia as a metabolic product.

INTRODUCTION

Ammonia is formed through the process of many biological reactions, including the oxidation or deamination of various nitrogenous compounds. The use of ^{15}N appears to have special advantages for the investigation of these reactions, particularly for studies tracing the metabolic fate of nitrogenous compounds in living systems. In the determination of $^{15}NH_3$, two methods, i.e., isotope mass spectrometric and optical emission spectrometric methods, are available at present [1,2]. These methods have been extensively employed in biomedical and agricultural

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fields. However, owing to a lack of sensitivity, their application has been limited to the analysis of samples that contain relatively large amounts of ammonia. In these methods, conversion of ammonia to nitrogen gas is a prerequisite prior to the ^{15}N determination [1,2]. As contamination of nitrogen gas from air results in serious errors in the measurement of ^{15}N abundance ratios, a highly sophisticated technique is needed for accurate ^{15}N determination [3].

In order to develop a more convenient and sensitive method, we adopted gas chromatography–mass spectrometry (GC–MS). The method consists in two steps: (1) derivatization of ammonia with pentafluorobenzoyl chloride (PFBC) to pentafluorobenzamide (PFBA), and (2) determination of PFBA by GC–MS. With the use of a multiple ion detector system, the amount of ammonia derivative and the $^{15}N/^{14}N$ ratio could be simultaneously determined, and excellent sensitivity, which permitted the measurement of the $^{15}N/^{14}N$ ratio at the picomole level, could be achieved.

We applied this technique to the measurement of putrescine oxidation by diamine oxidase (histaminase) (E.C. 1.4.3.6), one of the significant biological oxidations that produce ammonia [4]. Diamineputrescine is a precursor of the polyamines spermidine and spermine, which are intimately related to cell growth and development [5]. Therefore, the oxidation of putrescine, together with other metabolizing systems such as acetylation [6,7] or conjugation to peptides [8,9], could be of key importance in the regulation of polyamine biosynthesis. The specific and sensitive method using ^{15}N-labelled putrescine described here should provide a useful tool for in vitro or in vivo studies of putrescine metabolism in living systems.

EXPERIMENTAL

Chemicals

$(^{15}NH_4)_2SO_4$ with various percentages of ^{15}N and potassium [^{15}N]phthalimide (99% ^{15}N) were obtained from Shoko (Tokyo, Japan). PFBC was purchased from Aldrich (Milwaukee, WI, U.S.A.) and stored dry at 0–5 °C in a sealed container to protect it from ammonia. Ammonia-free water, which had been deionized and distilled twice, was used throughout. Other organic and inorganic chemicals were the purest grade available from commercial sources.

[^{15}N]Putrescine, $^{15}NH_2(CH_2)_4{}^{15}NH_2$, was chemically synthesized in the following way. Potassium [^{15}N]phthalimide (1 g, 5.4 mmol) was mixed with 1,4-dibromobutane (0.54 ml, 2.5 mmol) dissolved in 10 ml of dimethylformamide and heated at 80 °C for 26 h. After the solvent and unreacted 1,4-dibromobutane had been removed using a vacuum evaporator, 10 ml of chloroform were added to the reaction mixture, which was then washed with distilled water three times. After evaporation of chloroform, 70 ml of concentrated hydrochloric acid were added to the residue and heated under reflux for 48 h, followed by cooling to 0 °C. After filtering off precipitated phthalic acid, the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of distilled water and applied to a Dowex 50W-X8 (H^+) column (20×1.5 cm I.D., 100–200 mesh). The product was eluted with 150 ml of 4 *M* hydrochloric acid

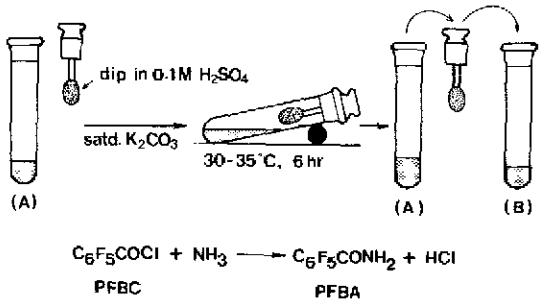


Fig. 1. Schematic diagram of the separation and derivatization of ammonia. Tube A contains 2 ml of sample solution and tube B contains 2 ml of 5% sodium hydrogen carbonate solution and 15 μl of PFBC. The paddle-shaped portion of the glass stopper is finely ground and dipped in 0.1 *M* sulphuric acid so that diffused ammonia is recovered over its surface. The ammonia trapped in acid is derivatized to PFBA in tube B.

after the column had been washed with 100 ml of distilled water and 150 ml of 0.3 *M* hydrochloric acid. The pooled fraction was evaporated under reduced pressure and the residue was dissolved in a small volume of distilled water. The [^{15}N]putrescine dihydrochloride was crystallized from 90% ethanol. The final product gave a yield of approximately 40%.

Separation and derivatization of ammonia

Prior to derivatization, ammonia was purified and trapped in acid by a modification of a microdiffusion method as shown schematically in Fig. 1. A 2-ml volume of sample solution containing ammonia was placed in a glass centrifuge tube (A) equipped with a stopper. The glass stopper was specially devised for recovering ammonia; the paddle-shaped portion of the stopper is finely ground so that when dipped into acid it retains an acid film over its surface. The ground portion of the stopper was dipped into 0.1 *M* sulphuric acid and the tube was tightly capped with this stopper immediately after addition of 1 ml of saturated potassium carbonate solution. The tube was slanted and left for 6 h at 30–35°C. Recovery tests showed that 95–100% of ammonia was trapped in sulphuric acid during this incubation period. The ammonia adsorbed on the acid-coated portion of the stopper was derivatized with PFBC according to the method of Makita et al. [10], with a slight modification as follows. The glass stopper was immediately transferred into another glass centrifuge tube (B) containing 2 ml of 5% sodium hydrogen carbonate solution and 15 μl of PFBC. The mixture was shaken for 5 min at room temperature, followed by shaking with 5 ml of ethyl acetate for 10 min to extract the resulting PFBA into the organic phase. After removal of the aqueous phase, the ethyl acetate was washed once with 2 ml of 6% phosphoric acid, dried over anhydrous copper sulphate and 1–5 μl of the organic phase were taken for GC–MS analysis.

Equipment

GC–MS was performed using an Hitachi M-80 double-focusing mass spectrometer equipped with a multiple ion detector. The GC column, made of Pyrex glass

(2 m×3 mm I.D.), was silanized before use with a 10% solution of hexamethylenedisilazane in toluene, and washed with methanol and acetone. The column was then packed with 3% OV-1 silicone on 80–100-mesh Chromosorb W AW DMCS. Helium was used as the carrier gas at a flow-rate of 60 ml/min. The injector, oven and separator temperatures were 280, 200 and 280 °C, respectively.

Electron-impact mass spectra and mass fragmentograms were obtained using a 20 eV ionizing energy, 220 °C ion-source temperature, 340 μA filament current and 3 kV accelerating voltage. The intensity of each mass fragment ion was recorded by the multiple ion detector, and the peak area of the fragment ion was measured by the integrator. The percentage of ^{15}N was calculated using the equation of VandenHeuvel and Smith [11] and the $^{15}NH_3$ content was calculated by multiplying the amount of ammonia in the sample solution by the percentage of ^{15}N obtained.

Assay of diamine oxidase

Diamine oxidase activity was assayed by measuring $^{15}NH_3$ liberated from ^{15}N-labelled putrescine. Human blood obtained from healthy volunteers was collected in a glass tube containing EDTA disodium salt (4 mg per 5 ml of blood) and immediately centrifuged at 1500 *g* for 5 min. The plasma was used for the present diamine oxidase assay. The standard incubation mixture contained 0.4 ml of 0.1 *M* phosphate buffer (pH 7.2), 0.1 ml of 20 m*M* ^{15}N-labelled putrescine and 0.5 ml of plasma (total volume 1.0 ml). In the blank, [^{15}N]putrescine was omitted from the reaction mixture, but added at the end of the incubation period. The reaction was carried out for 1 or 2 h at 37 °C with continuous shaking and terminated by adding 1 ml of 15% trichloroacetic acid. Proteins were removed by centrifugation and the clear supernatant fluid was employed for the determination of $^{15}NH_3$ by GC–MS.

RESULTS

In order to perform GC–MS analysis, ammonia was converted into another volatile and stable compound. Among the derivatization reagents tested, PFBC was found to be suitable for the quantitative derivatization of ammonia. The reaction was completed at room temperature by mechanical shaking for 5 min. The resulting PFBA was stable in ethyl acetate for several months and showed good resolution in GC–MS analysis. To avoid adsorption of PFBA on the surface of the stationary phase or the glass column, a Silyl-8 column conditioner (Pierce, Rockford, IL, U.S.A.) was usually employed prior to the application of the samples to the GC column. As shown in Fig. 2, PFBA was eluted from the OV-1 column within 2 min at 200 °C, and gave a simple mass spectrum consisting of two intense ion peaks at *m/e* 211 (M^+, $C_6F_5CONH_2$) and *m/e* 195 (C_6F_5CO), and relatively weak ion peaks at *m/e* 167 (C_6F_5) and *m/e* 168. PFBA prepared from the ammonia which contained ^{15}N (Fig. 2B) resulted in an additional peak at *m/e* 212 due to the molecular ion of [^{15}N]PFBA ($C_6F_5CO^{15}NH_2$). Therefore, the molecular ions at *m/e* 211 and 212 were used for determination of ^{15}N abundance ratio, and the intense fragment ion at *m/e* 195 was selected for quantifying

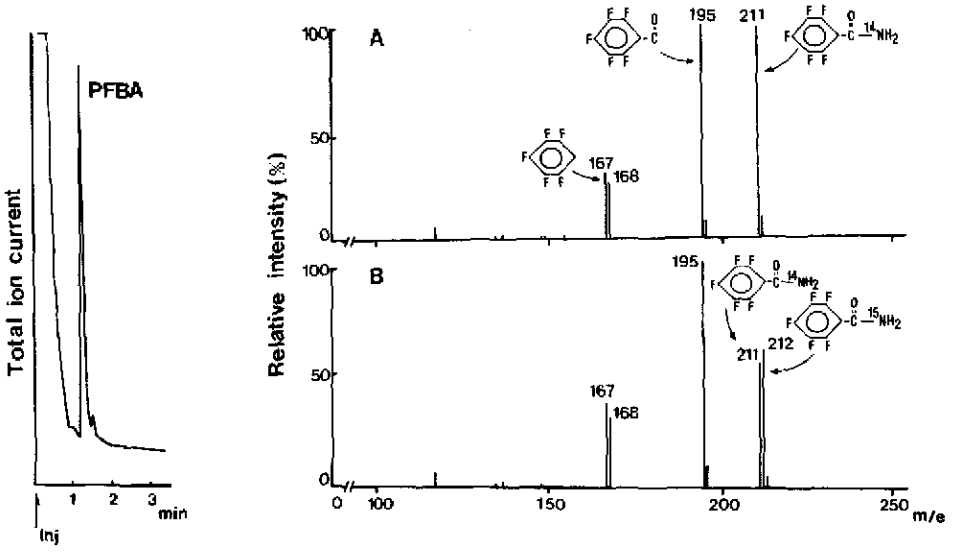


Fig. 2. Total ion current chromatogram and mass spectrum of PFBA. PFBA was prepared from a standard solution of ammonium sulphate with a natural abundance of ^{15}N (A) or with 50.8% of ^{15}N (B).

PFBA. These ions were simultaneously monitored by the multiple ion detector.

Fig. 3 shows mass fragmentograms of the *m/e* 195, 211 and 212 ions of PFBA prepared from a standard ammonium sulphate solution (A) and from a sample

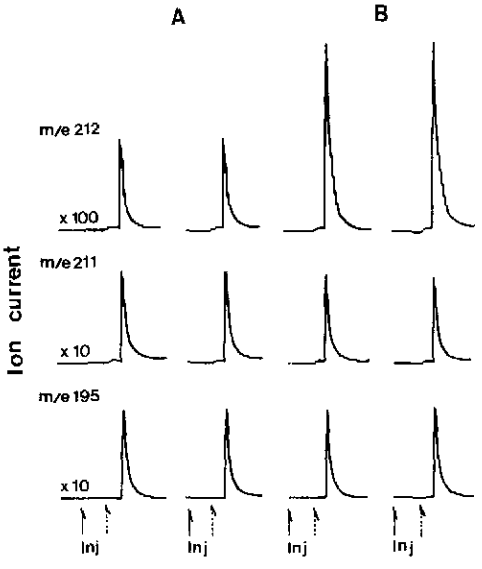


Fig. 3. Mass fragmentogram of *m/e* 195, 211 and 212 ions of PFBA. PFBA injected into GC column was analysed by mass fragmentography under the conditions described under Experimental. Each ion peak was synchronously recorded by the multiple ion detector. The dotted half-arrows show the point of opening of a pre-cut valve attached just before the ion separator. (A) PFBA from a standard solution of ammonium sulphate; (B) PFBA from a sample solution containing ^{15}N.

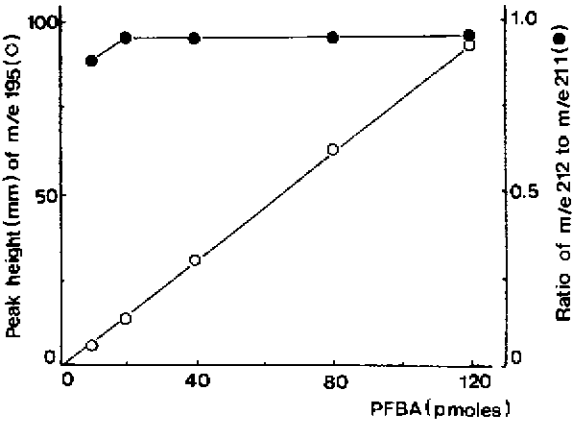


Fig. 4. Calibration graph for *m/e* 195 ion and ratios of *m/e* 212 to *m/e* 211 ion. Various concentrations of PFBA prepared from authentic ammonium sulphate with 50.8% of ^{15}N were injected into the GC column and analysed by mass fragmentography. Peak height (mm) of *m/e* 195 ion on the recorder (○) and peak-area ratios of *m/e* 212 to *m/e* 211 ion (●) are plotted against concentration of PFBA injected into the GC column.

solution containing ^{15}N (B). The ion peak of *m/e* 212 observed in the standard PFBA is attributable to natural ^{15}N in the PFBA molecule, as no background ions were eluted from the OV-1 column. In the calculation of the percentage of ^{15}N in the samples, the ratio of the *m/e* 212 to *m/e* 211 peaks ($^{15}N/^{14}N$ ratio) from this standard PFBA was employed as a reference with a natural abundance of ^{15}N.

In order to determine the optimal amount of PFBA necessary for a precise ^{15}N determination, various concentrations of PFBA with the same percentage of ^{15}N were subjected to GC–MS analysis. Fig. 4 shows the $^{15}N/^{14}N$ ratios and calibration graph for the fragment ion *m/e* 195 plotted against concentration of PFBA. A linear relationship was obtained between the amount of PFBA injected and the detector response (peak height) of this fragment ion. The $^{15}N/^{14}N$ ratios, which were calculated from the areas of the molecular ion peaks *m/e* 211 and *m/e* 212, were constant above 20 pmol of PFBA.

The precision of the method was then tested by repeated analyses of PFBA. The results from ten consecutive analyses of 40 pmol of PFBA revealed that fragmentation of PFBA molecules under a 20 eV ionizing energy was highly reproducible and the measurement of ionic intensity by the multiple ion detector was sufficiently reliable; the coefficients of variation for the measurement of the peak height of *m/e* 195 and for that of the peak-area ratio of *m/e* 212 to *m/e* 211 were 1.7% and 0.4%, respectively. To obtain a calibration graph for ^{15}N concentration, PFBA was prepared from authentic ammonia solutions with different percentages of ^{15}N, and analysed by mass fragmentography. Below 30% of ^{15}N, a good correlation was found between the percentage of ^{15}N employed and the observed values. Above 30% of ^{15}N, however, the observed values deviated slightly from the theoretical values. Therefore, in the analysis of the samples with high ^{15}N concentration, a correction to the ^{15}N calibration graph was necessary.

In the present method of determining ammonia by measuring fragment ions of

an ammonia derivative, there are uncontrollably variable factors such as the efficiency of the derivatization of ammonia with PFBC and the efficiency of the extraction of the derivative into organic phase. In order to correct for these factors, standard solutions in which the ammonia concentration was accurately defined were routinely analysed according to the same procedure as summarized in Fig. 1. The total amounts of ammonia in the samples were always calculated from a comparison with these ammonia standards run simultaneously.

To validate the method, the ammonia content in plasma prepared from the same subject was determined both by the GC–MS method and by the microdiffusion–spectrophotometric method using Nessler reagent [12].

No significant difference was found between the values obtained by these different methods: means ± S.E.M. of four determinations were 86.7 ± 2.2 nmol/ml of NH_3 in plasma for the GC–MS method and 84.7 ± 3.8 nmol/ml for the microdiffusion–spectrophotometric method. The results indicate the applicability of the present GC–MS method to the quantitative determination of ammonia in biological materials.

The method was further applied to the assay of plasma diamine oxidase, which produces ammonia from putrescine. ^{15}N-Labelled putrescine was incubated with the plasma collected from normal men and pregnant and non-pregnant women. The ammonia generated during the reaction was trapped in sulphuric acid, derivatized to PFBA and determined by GC–MS. Typical mass fragmentograms of PFBA prepared from these samples are shown in Fig. 5. In the reaction with the plasma from pregnant women, a high $^{15}N/^{14}N$ ratio, i.e., high putrescine-oxidizing activity, was observed (Fig. 5d). In contrast, only trace of the activity was detected with the plasma from normal men and non-pregnant women (Fig. 5b and c). The addition of aminoguanidine, an inhibitor of diamine oxidase, to the reaction mixture of pregnancy plasma strongly repressed the $^{15}NH_3$ production from [^{15}N]putrescine (Fig. 5e). As shown in the mass fragmentogram for a pregnant woman (Fig. 5d), the ion peak at *m/e* 195 reappeared after the elution of PFBA. This peak disappeared when aminoguanidine was added to the assay mixture. From electron-impact and chemical ionization mass spectra, the structure of the compound eluting after PFBA was determined to be 1-pentafluorobenzoyl-[1-^{15}N]-3-pyrroline, which is formed through [^{15}N]putrescine oxidation via γ-aminobutyraldehyde [4].

The pregnancy plasma was used to establish the characteristics of the standard assay of diamine oxidase. No significant difference in the enzyme activity was observed in the blood samples collected either in the morning or in the evening from the same woman. As shown in Fig. 6, the production of $^{15}NH_3$ from [^{15}N]putrescine was proportional to the amount of pregnancy plasma. The oxidation of [^{15}N]putrescine proceeded in a linear fashion for at least 2 h with the use of 2 m*M* [^{15}N]putrescine and it was ascertained that $^{15}NH_3$ production was greatly inhibited by an addition of 20 μM of aminoguanidine (Fig. 7).

The activities of diamine oxidase with EDTA–plasma were very similar to those with serum. Therefore, either EDTA–plasma or serum was available for the assay of putrescine oxidation in human blood. The use of excess of EDTA, however, should be avoided, as diamine oxidase is generally known as a copper-containing

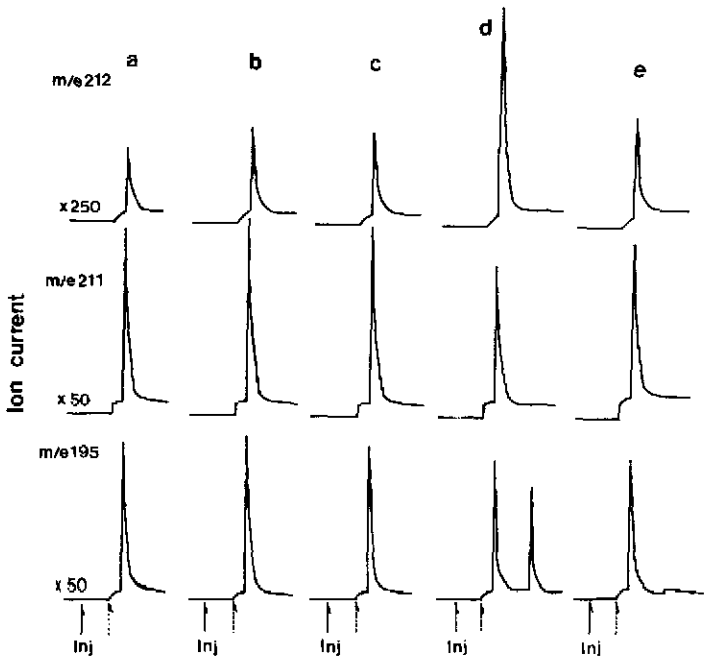
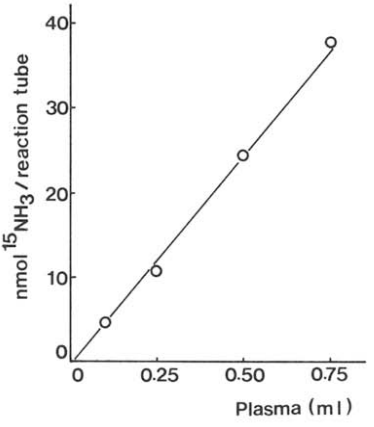


Fig. 5. GC–MS analysis of $^{15}NH_3$ formed through oxidation of [^{15}N]putrescine by human plasma. ^{15}N-Labelled putrescine was incubated with human plasma. The assay mixture contained 0.4 ml of 0.1 *M* phosphate buffer (pH 7.2), 0.1 ml of 20 m*M* [^{15}N]putrescine and 0.5 ml of plasma (total volume 1.0 ml). The reaction was carried out at 37°C for 2 h with continuous shaking and terminated by an addition of 1 ml of 15% trichloroacetic acid. In the blank, [^{15}N]putrescine was added to the assay mixture at the end of the incubation period. The ammonia generated during incubation was trapped in acid and derivatized with PFBC according to the procedure summarized in Fig. 1, and the resulting PFBA was analysed by mass fragmentography. The dotted half-arrows show the point of opening of a pre-cut valve attached just before the ion separator. (a) Blank; (b) normal man (age 36 years); (c) non-pregnant woman (age 23 years); (d) pregnant woman (age 25 years, 24 weeks pregnant); (e) d + 20 μ*M* of aminoguanidine.

enzyme and there is a possibility that excess of EDTA may inactivate the enzyme by chelate formation with copper(II). In the preparation of plasma, we recommend using EDTA at a concentration below 5 m*M*.

DISCUSSION

Although the accuracy of the present GC–MS method is inferior to that of isotope mass spectrometry, which permits the determination of the natural abundance ratio of ^{15}N, the sensitivity and analytical speed were greatly improved. The $^{15}N/^{14}N$ ratio and the amount of ammonia derivative could be determined within 2 min with as little as 20 pmol of ammonia derivative by synchronous monitoring of ion peaks (Figs. 3 and 4). The GC–MS method was shown to be highly reproducible and the $^{15}N/^{14}N$ ratio could be measured with a very low coefficient of variation (below 0.5%). The procedure used for the derivatization of ammonia does not need a special technique such as the preparation of a discharge tube as in optical emission spectrometry [3], and the reaction product



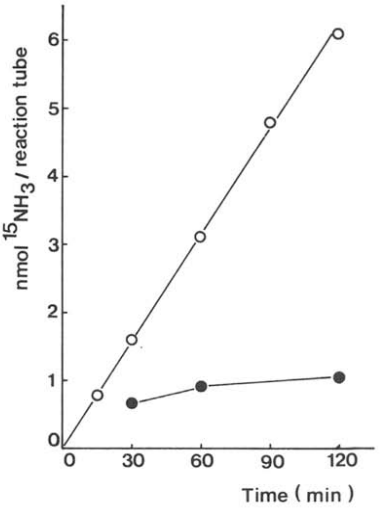


Fig. 6. Effect of plasma concentration on [^{15}N]putrescine oxidation. The assay mixture contained 0.1 *M* phosphate buffer (pH 7.2), 0.1 ml of 20 m*M* [^{15}N]putrescine and various concentrations of plasma (woman, age 25 years, 24 weeks pregnant) in a final volume of 1.0 ml. The reaction was carried out at 37°C for 2 h. $^{15}NH_3$ formed from [^{15}N]putrescine was analysed by mass fragmentography of PFBA as in Fig. 5.

Fig. 7. Time course of [^{15}N]putrescine oxidation by human plasma and effect of aminoguanidine. The assay mixture contained 0.1 *M* phosphate buffer (pH 7.2), 0.1 ml of 20 m*M* [^{15}N]putrescine and 0.5 ml of plasma (woman, age 31 years, 32 weeks pregnant) in a final volume of 1.0 ml. The reaction was carried out at 37°C in the absence (○) or presence (●) of 20 μM of aminoguanidine. $^{15}NH_3$ formed from [^{5}N]putrescine was analysed by mass fragmentography of PFBA as in Fig. 5.

(PFBA) can be successively injected into the GC column at a constant oven temperature; in practice, the samples were applied to the instrument every 4 min. Hence the method appears to be suitable for the rapid analysis of the samples containing small amounts of ammonia. It should be noted, however, that the $^{15}N/^{14}N$ ratio is variable below 20 pmol of PFBA, as shown in Fig. 4, particularly for samples with very low ^{15}N concentrations, owing to the background noise which becomes prominent under the conditions of high sensitivity employed for the detection of *m/e* 212 ion peak. For the accurate analysis of these samples, the injection of 50–100 pmol of PFBA is desirable.

With practical and clinical uses in mind, the technique was applied to the assay of plasma diamine oxidase (histaminase). Putrescine, a typical substrate for this enzyme, is decomposed by oxidative deamination to form ammonia and γ-aminobutyraldehyde [4]. Our method is based on the measurement of $^{15}NH_3$ formed from [^{15}N]putrescine, and therefore is considered to be highly specific for this enzyme. The results indicated that the method was sensitive enough to detect plasma diamine oxidase and, in agreement with previous observations [13,14], pregnancy plasma showed striking putrescine-oxidizing activity (Fig. 5). A radiochemical technique [15], which measures [^{14}C]-1-pyrroline formed from [^{14}C]putrescine via spontaneous cycling of γ-aminobutyraldehyde, is possibly the most sensitive method so far reported. However, as already pointed out by

several workers [16,17], the method does not always measure the full activity of this enzyme when it is applied to tissue that shows high aldehyde-metabolizing activity, as γ-aminobutyraldehyde is further metabolized, for instance, to γ-aminobutyric acid without 1-pyrroline formation. Our method, although less sensitive than the radiochemical method, may be useful for such biological materials.

It has been generally accepted that various growth stimuli bring about a marked elevation of the putrescine level in tissues [5]. Perin et al. [17] reported that in rapidly growing tissues there is also an increase in putrescine catabolism through an enhancement of diamine oxidase activity. In fact, high diamine oxidase activity has been detected in certain human tumours [18,19] and in the blood or effusion fluids of patients with various cancers [20,21]. Measurement of the turnover rate or tracing the metabolic fate of putrescine in the human body seems to have considerable clinical utility as a test for the early detection of abnormalities in a variety of diseases, including cancer. In this regard, the use of [^{15}N]putrescine is especially valuable, as radio-labelled compounds generally cannot be administered to humans for safety reasons. The present technique might be also useful for in vivo studies designed to examine the decomposition of [^{15}N]putrescine injected into circulating human blood.

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