Biochimica et Biophysica Acta, 524 (1978) 288—296

BBA 68447

PURIFICATION AND CHARACTERIZATION OF ε-*N*-TRIMETHYLLYSINE L-AMINO OXIDASE FROM *NEUROSPORA CRASSA*

KIM HO DIEP LÊ and VICTOR R. VILLANUEVA

Institut de Chimie des Substances Naturelles, C.N.R.S., 91190 Gif sur Yvette (France)

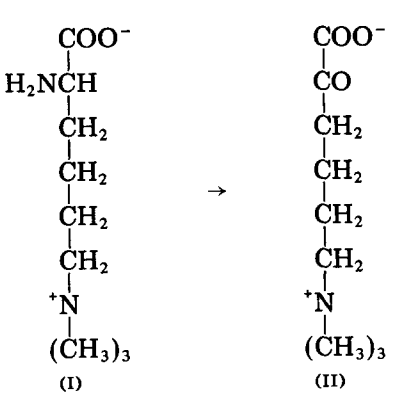
(Received October 28th, 1977)

Summary

ε-*N*-Trimethyllysine L-amino oxidase from *Neurospora crassa* has been purified to electrophoretic homogeneity. A 1500-fold purification was obtained by centrifugation and successive column chromatography on ion-exchange and gel filtration supports. The enzyme has an estimated molecular weight of 160 000. It transforms ε-*N*-trimethyllysine into α-keto, ε-*N*-trimethylhexanoic acid by oxidative deamination. Kinetic studies of this new enzyme are reported and its probable physiological role is discussed.

Introduction

During the course of our studies on the metabolism of ε-*N*-trimethyllysine (I) in *Neurospora crassa*, we isolated a new metabolite produced by its deamination: *q*-keto, ε-*N*-trimethylhexanoic acid (II) [1]. Our efforts were subsequently directed towards the purification and characterization of the deaminase involved in this reaction. A report of our preliminary results has been presented [2].



We now wish to report further results related to this new L-amino acid oxidase which is involved in the metabolism of ϵ-*N*-trimethyl-L-lysine.

Materials and Methods

Reagents were obtained from the following companies: K_2HPO_4, KCl, $(NH_4)_2SO_4$, sodium dodecyl sulfate (SDS) and 2-oxoglutaric acid from Merck, Darmstadt; catalase (beef liver), aldolase (rabbit muscle), L-amino acid oxidase (*Crotalus terrificus*) ovalbumin, bovine serum albumin, glutamate dehydrogenase (beef liver), NAD^+,NADH, FAD and FMN from Boehringer, Mannheim; DEAE-Sephadex A-50 and Sephadex G-50 from Pharmacia, Uppsala; Ultrogel AcA-34 from LKB-France; Resin M-71 from Beckman Instruments France, Gagny.

Cultures. N. Crassa lysine auxotroph (Fungal Genetics Stock center No. 33933) was grown as reported earlier [3] but over a period of 10 days.

Substrate. ϵ-*N*-Tri[^{14}C]methyl-L-lysine was synthesised as described by Cox and Hoppel [4] from α-*N*-acetyl-L-lysine (Calbiochem) and $^{14}CH_3I$ (CEA, Saclay). The radiochemical purity of ϵ-*N*-tri[^{14}C]methyl-L-lysine was controlled by ion-exchange column chromatography and by thin-layer chromatography on SiO_2 and Al_2O_3 using *n*-butanol/methanol/ammonia (1 : 1 : 3, v/v) [1].

Enzyme extraction. All manipulations were carried out at 4°C and 0.1 M K_2HPO_4 (pH 8.9) was used for the extraction and column chromatography development unless otherwise stated. 20 g *N. crassa* (wet weight) were crushed in a mortar with 10 g Fontainebleau sand in 40 ml potassium phosphate and the homogenate was centrifuged at 10 000 × *g* for 15 min. The first supernatant, whose activity was negligible, was discarded. The precipitate was reextracted twice in the same way. The two supernatants were pooled (Extract 1) and centrifuted at 170 000 × *g* for 90 min. The last supernatant (Extract 2) was recovered for further purification.

First DEAE-Sephadex A-50 chromatography. Extract 2 was applied to a DEAE-Sephadex A-50 column (35 × 2 cm) equilibrated with 0.1 M K_2HPO_4 (pH 8.9). The column was then washed out to eliminate contaminating protein with the same buffer. Elution was continued with the same buffer containing 0.4 M KCl (Fig. 1). The fractions containing the active protein were combined and $(NH_4)_2SO_4$ was added to 90% saturation. After centrifugation at 10 000 × *g* for 30 min the supernatant was discarded and the precipitate recovered for further purification.

Ultrogel AcA-34 chromatography. A column (35 × 2 cm) containing gel equilibrated with 0.1 M potassium phosphate (pH 8.9) was used. The $(NH_4)_2SO_4$ precipitate was dissolved in 2 ml potassium phosphate and applied to the column which was eluted with the same buffer. The fractions containing enzyme activity were pooled (Fig. 2).

Second DEAE-Sephadex A-50 chromatography. The active fractions obtained from the preceding step were applied to a DEAE-Sephadex A-50 column (30 × 1.5 cm) equilibrated with phosphate buffer. Fractions were eluted with a linear 400 ml gradient (0—0.4 M KCl) in 0.1 M K_2HPO_4 (pH 8.9). The fractions containing enzyme activity (Fig. 3) were assembled and used for

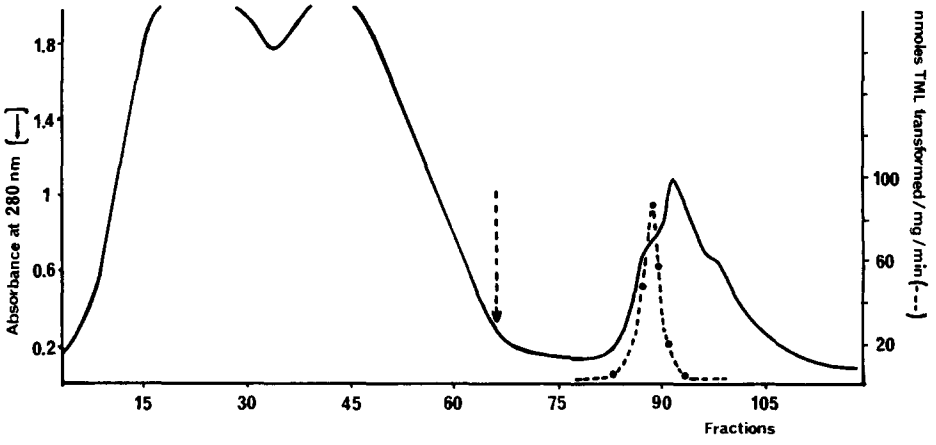


Fig. 1. Chromatography of extract 2 on DEAE Sephadex A-50 (35 X 2 cm). The column was equilibrated and eluted with 0.1 M K_2HPO_4 (pH 8.9). At fraction 66 (arrow) elution of the enzyme was initiated with the same buffer containing 0.4 M KCl. Fractions of 5 ml were collected at a flow rate of 20 ml/h.

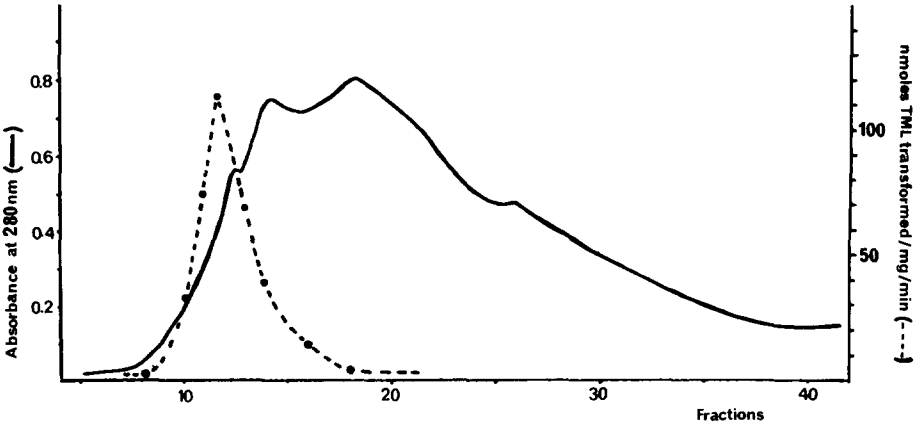


Fig. 2. Ultrogel AcA-34 (35 X 2 cm) chromatography of DEAE-Sephadex fractions. Equilibration and elution with 0.1 M K_2HPO_4 (pH 8.9). Fractions of 5 ml were collected at a flow rate of 12 ml/h.

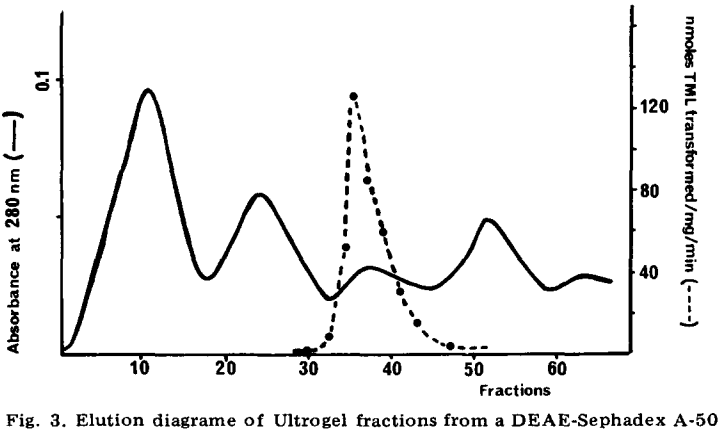


Fig. 3. Elution diagrame of Ultrogel fractions from a DEAE-Sephadex A-50 column (30 X 1.5 cm) with a linear 400 ml gradient (0—0.4 M KCl) in 0.1 M K_2HPO_4 (pH 8.9). Fractions of 5 ml were collected at a flow rate of 25 ml/h.

kinetic studies, terminal NH_2 and amino acid composition determinations.

Enzyme assays. The purification of ϵ-*N*-trimethyllysine L-amino oxidase was followed by measuring the conversion of ϵ-*N*-tri[^{14}C]methyl-L-lysine into α-keto, ϵ-*N*-trimethylhexanoic acid. The reaction mixture contained (in 0.5 ml): 750 nmol ϵ-*N*-tri[^{14}C]methyl-L-lysine (807 000 dpm), 0.1 M potassium phosphate (pH 8.9) and limiting amounts of enzyme to ensure initial rate measurements. The incubations were carried out at 37°C under agitation. The reaction was stopped by the addition of 50 μl 80% trichloroacetic acid. After cooling and centrifuging the supernatants were analysed by automatic ion-exchange column chromatography. A cation-exchange column (9 × 0.9 cm) of Beckman M-71 resin in 0.35 M trisodium citrate buffer (pH 5.15) was used to isolate the keto acid [1]. The radioactivity of the eluate was determined using Bray's solution [5]. The specific activity is expressed as the number of nmol ϵ-*N*-trimethyl-L-lysine transformed per mg protein per min at 37°C.

Protein determination. Protein concentration was determined according to Lowry et al. [6] or Ehresmann et al. [7].

Qualitative and quantitative analysis of amino acids. The amino acid composition of ϵ-*N*-trimethyllysine L-amino oxidase after hydrolysis was determined with the help of an automatic amino acid analyser, Liquimat-Labotron (Kontron, Veilizy-Villacoublay). The analyser was equiped with a column of Durrum ion-exchange resin (DC 6A) and was coupled with an integrator ICAP-10 (LTT, Conflans Sainte Honorine). Tryptophan was assayed according to Liu and Chang [8].

The activity of the enzyme with respect to other amino acids was determined by incubation of 750 nmol substrate (Table III) with 0.9 μg enzyme in 0.5 ml 0.1 M potassium phosphate (pH 8.9) at 37°C for 1 h. The reactions were stopped with 50 μl trichloroacetic acid and, after centrifugation, aliquots of the supernatants were used to quantify the substrate present by automatic amino acid analysis.

NH_2-terminal amino acid determination. The enzyme was dansylated as described by Gray [9]. After dansylation the protein was desalted by passage through a Sephadex G-50 column (7 × 0.5 cm). Elution of the dansylated protein was followed visually with the aid of an ultraviolet lamp. Hydrolysis was carried out in 6 M HCl for 18 h at 110°C. The dansyl amino acids were separated by two-dimensional thin-layer chromatography on 7.5 × 7.5 cm polyamide sheets. Ascending chromatography was performed according to the method of Woods and Wang [10] as modified by Hartley [11].

Polyacrylamide gel electrophoresis. The purification procedure was followed by gel electrophoresis analysis with 7% polyacrylamide gels in the presence of 0.1% SDS [12]. The molecular weight of the protein subunits was determined according to the method of Weber and Osborn [13]. 10% polyacrylamide gels containing 0.1% SDS were used. The experiments were carried out with a GE-4 Pharmacia apparatus.

Analytical gel column chromatography. The molecular weight of the enzyme was determined by gel chromatography according to Andrews [14]. ϵ-*N*-trimethyllysine L-amino oxidase was applied to a Ultrogel AcA-34 column (35 × 2 cm) equilibrated with 0.1 M potassium phosphate (pH 7.5). The column was equilibrated with the following proteins of known molecular weight: catalase, aldolase, L-amino acid oxidase, ovalbumin and bovine serum albumin.

Results

Purification procedure. Preliminary results showed that the enzymic activity of the mycelium increased with the age of the culture and the maximum activity is found in cultures for 10 days [2]. Table I summarizes the results of a typical preparation procedure. The enzymatic preparation had a 1500-fold higher specific activity compared to that of the initial extract. Electrophoresis on polyacrylamide gels showed only one protein band (Fig. 4). During the whole procedure, total enzyme activity increased. This activation could indicate the elimination, through the purification steps, of inhibitor(s) present in the crude extracts. After the second DEAE-Sephadex A-50 chromatography (Fig. 3) the collected active fractions were pooled and used for the present study. At this point the enzyme lost 85% of its activity when kept at 4°C for 1 month in 0.1 M potassium phosphate (pH 8.9) but preincubation for 1 h at 37°C restored full activity.

Effect of pH on the enzyme activity. The enzyme activity was measured in potassium phosphate buffers with pH ranging from 7.0 to 10.5 (at 0.5 pH intervals). The pH optimum was approx. 9.5; however, all our experiments were carried out at pH 8.9.

Effect of enzyme concentration and the time of incubation. Enzyme activity was proportional to the concentration of protein. In the range tested (up to 4.6 mg/l), a linear relationship was obtained between the time of incubation (0—60 min) and enzyme activity. An incubation time of 30 min was chosen for our experiments.

Action of KCl. The introduction of KCl in the incubation medium stimulated the enzyme activity with a maximum of 2.5 times at 0.8 M.

K_m *value.* The K_m value was investigated by the standard assay system using different concentrations of ϵ-*N*-trimethyl-L-lysine. The apparent K_m was obtained from a Lineweaver-Burk plot and was estimated to be 0.11 mM.

Action of -SH group inhibitors. The enzyme activity was partially inhibited when it was preincubated for 30 min with sulphydryl group reagents before incubation under standard conditions. The presence of 1 mM *p*-chloromercuribenzoate resulted in inhibition of 35% while that of 5,5′-dithiobis(2-nitrobenzoic acid) at the same concentration inhibited the reaction by 20%.

TABLE I

PURIFICATION OF ϵ-*N*-TRIMETHYLLYSINE L-AMINO OXIDASE

20 g of *N. crassa* were used.

	Volume (ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/mg per min)	Purification
Extract 1	80	227.2	898	4	1
Extract 2	80	128.8	1309	10	2.5
1st DEAE	50	24.3	1066	44	11
Ultrogel	35	2.5	1143	454	114
2nd DEAE	35	0.16	1005	6246	1561

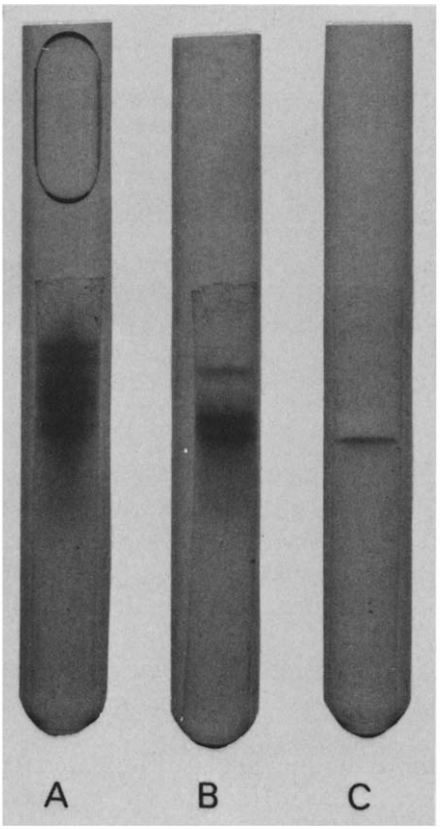


Fig. 4. Electrophoretic analysis of ε-*N*-trimethyllysine L-amino oxidase at various stages during purification: (A) 1st DEAE-Sephadex A-50 fraction. (B) Ultrogel AcA-34 fraction. (C) 2nd DEAE-Sephadex A-50 fraction.

Cofactors. FAD, FMN, NAD and pyridoxal phosphate were tested as cofactors. At 1 mM, only FMN stimulated the enzyme reaction, by almost 50%.

Presence of metal ions. When the enzyme was incubated with 1 mM EDTA in the standard conditions the activity decreased by 30%. This suggests the presence of a metal ion in the molecule of the enzyme.

Reaction mechanism of deamination. The following results show that ε-*N*-trimethyl-L-lysine deaminase is an L-amino oxidase:

ε-*N*-Trimethyl-L-lysine is not deaminated when the reaction is carried out in the presence of nitrogen. In contrast, in the presence of oxygen the amount of keto acid produced is 1.7 times higher than under standard incubation conditions (air). See Table II.

There is no formation of glutamic acid when the enzyme is incubated with ε-*N*-trimethyl-L-lysine (750 nmol) in the presence of 2-ketoglutarate and NAD^+ (each at concentration up to 1 mM).

Using an automatic amino acid analyser the formation of ammonia has been detected in quantity equivalent to the ε-*N*-trimethyl-L-lysine deaminated by enzymic reaction. We have tried to use the so-formed ammonia for the production of glutamic acid by coupling the reaction with that of glutamate dehy-

TABLE II

ACTION OF NITROGEN OR OXYGEN ON THE DEAMINATION REACTION

Duplicate experiments were carried out as follows: 750 nmol of ε-*N*-trimethyl-L-lysine were incubated in three different tubes with 0.9 μg of enzyme in 0.5 ml of 0.1 M potassium phosphate (pH 8.9) during 30 min at 37°C: one bubbled with nitrogen, another with oxygen and the third under air-equilibrated conditions (standard). Results are given as nmol of ε-*N*-trimethyl-L-lysine (TML) transformed per mg of protein per min.

Conditions of incubation	TML transformed (nmol/mg per min)
Standard	5360
In presence of nitrogen	0
In presence of oxygen	9220

drogenase in the presence of 2-ketoglutarate according to Holme and Goldberg [15]. The coupled system was incubated during 30 min, 1 and 2 h. As expected the analysis of the enzymatic incubations showed the formation of glutamic acid in increasing amounts in accordance with the incubation time.

Employing the same incubation conditions as for ε-*N*-trimethyl-L-lysine D-lysine remained unchanged after 3 h.

Thus from the above results we deduce that ε-*N*-trimethyl-L-lysine deaminase is an L-amino-acid oxidase (L-amino acid: O_2 oxidoreductase (deaminating) EC 1.4.3.2).

Enzyme specificity. The percent of various amino acids deaminated by ε-*N*-trimethyllysine L-amino oxidase is shown in Table III. The enzyme activity with ε-*N*-trimethyl-L-lysine is considered as 100%. The enzyme is active towards most of the basic amino acids tested but not towards neutral and acid amino acids.

TABLE III

SPECIFICITY OF ε-*N*-TRIMETHYLLYSINE L-AMINO OXIDASE

Substrates (750 nmol) were incubated with enzyme (0.9 μg) in 0.5 ml of 0.1 M potassium phosphate (pH 8.9) during 1 h at 37°C. The number of nmol of ε-*N*-trimethyl-L-lysine transformed/mg protein per min is considered as 100%.

Substrate	Transformation (%)
ε-*N*-Trimethyl L-lysine	100
L-Lysine	42
D-Lysine	0
L-β-Lysine	44
ε-*N*-Dimethyl-L-lysine	46
ε-*N*-Monomethyl-L-lysine	38
L-Ornithine	53
L-Histidine	30
3-Methyl-L-histidine	15
1-Methyl-L-histidine	50
L-Arginine	30
L-Phenylalanine	28
L-Glycine	0
L-Proline	0
L-Serine	0

TABLE IV

AMINO ACID COMPOSITION OF ε-*N*-TRIMETHYLLYSINE L-AMINO OXIDASE

Average values of 24 and 72 h hydrolysis of three different samples. Methionine was measured as methionine sulfone. Tryptophan was determined after hydrolysis in *p*-toluene sulfonic acid [8]. A value of 40 000 daltons was taken as the subunit molecular weight for purpose of calculation.

Amino acid	mol of amino acid/ mol of subunit	Amino acid	mol of amino acid/ mol of subunit
Asp	25	Met	2
Thr	14	Ile	13
Ser	15	Leu	23
Glu	26	Tyr	7
Pro	15	Phe	12
Gly	20	Try	68
Ala	23	Lys	10
Cys	n.d.	His	8
Val	18	Arg	10

n.d., not determined.

Molecular weight estimation. After treatment with sodium dodecyl sulfate followed by electrophoresis on SDS-polyacrylamide gels [14], ε-*N*-trimethyl-L-lysine oxidase migrates as a single band of 40 000 daltons as determined from the graph of the logarithm of molecular weight against electrophoretic mobility of the marker proteins. However the molecular weight of the native protein determined by gel filtration is approx. 160 000. It is therefore most probable that the enzyme exists as a tetramer consisting of subunits of 40 000.

Amino acid composition. The amino acid composition of the purified enzyme is presented in Table IV based on a molecular weight subunits of 40 000. The analysis revealed a high content of tryptophan.

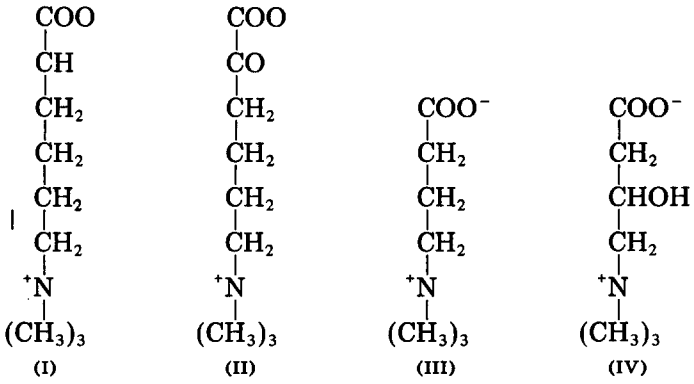
NH_2-terminal amino acid. The NH_2-terminal amino acid analysis was carried out by the dansyl method. The dansyl derivative was separated and identified by thin-layer chromatography as dansyl-tryptophan.

Discussion

In this paper we have shown the occurrence in the mycelium of *N. crassa* of a new L-amino acid oxidase. The method described for its purification is simple and lead to an electrophoretically homogeneous enzymic preparation, the specific activity of which was 1500-fold higher as compared to the initial extract. The enzyme has an optimum pH of 9.5 and is active towards most of the basic amino acids but not towards the neutral and acid amino acids. Whether or not this was due to the enzyme activity itself or to a contaminating enzyme has not yet been established. Significantly, enzymic preparations obtained in others experiments, up to 200-fold purification, were 1.2 times more active with L-lysine as with ε-*N*-trimethyl-L-lysine. As the purification of the enzymatic preparation was increased the enzyme activities were reversed and that toward ε-*N*-trimethyl-L-lysine became higher. Experiments are underway to obtain an enzyme preparation rid of activity on amino acid others than ε-*N*-trimethyl-L-lysine. Molecular weight studies demonstrate that

the enzyme is a tetramer of 160 000 daltons. Amino acid analysis revealed a high content of tryptophan which is also the NH_2-terminal amino acid.

A major reason for carrying out this work was to establish whether this enzyme is or not concerned in the metabolic pathway of carnitine biosynthesis. ϵ-*N*-Trimethyl-L-lysine (I) has been shown to be a precursor in carnitine (IV) biosynthesis via butyrobetaine (III) [4,16,17] but the mechanism of its transformation into butyrobetaine has not yet been established. α-keto, ϵ-N-trimethylhexanoic acid (II), the reaction product of ϵ-*N*-trimethyl-L-lysine with ϵ-*N*-trimethyllysine L-amino oxidase could be a good candidate as an



(I) (II) (III) (IV)

intermediate of carnitine biosynthesis [18]. Further studies are in progress to verify whether this keto acid is or not a precursor of carnitine biosynthesis. In either case, the enzyme reported here like other amino acid oxidases, would play a role in the regulation of the intracellular concentration of ϵ-*N*-trimethyl-L-lysine.

Acknowledgement

This work was supported by grants from Firmenich, S.A. Geneva and CEA, Saclay (for the purchase of labelled compounds).

References

1 Villanueva, V.R. and Lederer, E. (1975) FEBS Lett. 52, 308—311
2 Villanueva, V.R. and Lê, K.H.D. (1976) Phytochemistry 15, 1762—1764
3 Villanueva, V.R. and Lederer, E. (1974) Phytochemistry 13, 2157—2160
4 Cox, R.A. and Hoppel, C.L. (1973) Biochem. J. 136, 1083—1090
5 Bray, G.A. (1960) Anal. Biochem. 1, 279—282
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Ehresmann, B., Imbault, P. and Weil, J.H. (1973) Anal. Biochem. 54, 454—463
8 Liu, T.Y. and Chang, Y.H. (1971) J. Biol. Chem. 246, 2842—2848
9 Gray, W.R. (1967) Methods Enzymol. 11, 139—151
10 Woods, K.R. and Wang, K.T. (1967) Biochim. Biophys. Acta 133, 369—370
11 Hartley, B.S. (1970) Biochem. J. 119, 805—822
12 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
13 Weber, K. and Osborn, M. (1969) J.Biol. Chem. 244, 4406—4412
14 Andrews, P. (1964) Biochem. J. 91, 222—233
15 Holme, D.J. and Goldberg, D.M. (1975) Biochim. Biophys. Acta 377, 61—70
16 Horne, D.W. and Broquist, H.P. (1973) J. Biol. Chem. 248, 2170—2175
17 Tanphaichitr, V. and Broquist, H.P. (1973) J. Biol. Chem. 248, 2176—2181
18 Paik, W.K., Nochumson, N. and Kim, S. (1977) Trends. Biol. Sci. 2, 159