Degradation of (−)-Ephedrine by *Pseudomonas putida* Detection of (−)-Ephedrine: NAD$^+$-oxidoreductase from *Arthrobacter globiformis*

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(−)-Ephedrine, (−)-Ephedrine: NAD$^+$-oxidoreductase, *Pseudomonas putida, Arthrobacter globiformis*

A bacterium utilizing the alkaloid (−)-ephedrine as its sole source of carbon was isolated by an enrichment-culture technique from soil supplemented with 4-benzoyl-1,3-oxazolidinon-(2). The bacterium was identified as *Pseudomonas putida* by morphological and physiological studies. The following metabolites were isolated from the culture fluid: methylamine, formaldehyde, methylbenzoylcarbinol (2-hydroxy-1-oxo-1-phenylpropane), benzoid acid, pyrocatechol and *cis, cis*-muconic acid. A pathway for the degradation of (−)-ephedrine by *Pseudomonas putida* is proposed and compared with the degradative pathway in *Arthrobacter globiformis.*

The enzyme, which is responsible for the first step in the catabolism of (−)-ephedrine could be demonstrated in extracts from *Arthrobacter globiformis.* This enzyme catalyses the dehydrogenation of (−)-ephedrine yielding phenylacetylcarbinol/methylbenzoylcarbinol and methylamine. It requires NAD$^+$ as cofactor and exhibits optimal activity at pH 11 in 0.1 M glycine/NaOH buffer. The K_m value for (−)-ephedrine is 0.02 mM and for NAD$^+$ 0.11 mM, respectively. No remarkable loss of activity is observed following treatment with EDTA. The enzyme has been shown to react with a wide range of ethanolamines. A slight enrichment was obtained by ammonium sulphate precipitation. The name (−)-ephedrine: NAD$^+$-oxidoreductase (deaminating) is proposed.

Introduction

In a previous paper we reported about a strain of *Arthrobacter globiformis,* isolated by an enrichment-culture technique from soil and which has the capacity of utilizing the alkaloid (−)-ephedrine as its sole source of carbon [1].

In the following paper we present another strain with the ability of utilizing (−)-ephedrine. This strain, identified as *Pseudomonas putida,* was isolated from soil supplemented with 4-benzoyl-1,3-oxazolidinon-(2), a structural analogue of (−)-ephedrine. From the culture fluid of *Pseudomonas putida* a number of metabolites was isolated, allowing us to propose a pathway for the degradation of (−)-ephedrine.

Furthermore we have identified and characterized (−)-ephedrine: NAD$^+$-oxidoreductase from *Arthrobacter globiformis,* the enzyme, which initiates the catabolism of (−)-ephedrine.

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Materials and Methods

Chemicals

(−)-Adrenaline, d,l-aminopropanol, 2-amino-2-methylpropanol, 2-methylaminoethanol, N,N-dimethylethanolamine, 1-aminopropanol-(2) and 2-ethylaminoethanol were obtained from E. Merck AG, Darmstadt, Germany. 4-Benzoyl-1,3-oxazolidinone-(2) was obtained form Nordmark-Werke GmbH, Hamburg, Germany. (−)-Norephedrine, (−)-methylephedrine, (±)-suprifene, 1-phenylpropandion-(1,2) and 2-methylamino-1-phenyl-propanon-(1) were supplied by Knoll AG, Ludwigshafen, Germany. 1-Phenyl-propandiol-(1,2) was synthesized according to Foltz and Witkop [2] and methylbenzoylcarbinol according to Auwers *et al.* [3].

Media

Mineral salts medium (*Pseudomonas putida*) 0.68 g KH_2PO_4; 1.77 g K_2HPO_4; 0.14 g NaCl; 0.1 g $CaCl_2 \times 6\,H_2O$; 0.2 g $MgSO_4 \times 7\,H_2O$; 2.5 mg $FeSO_4 \times 7\,H_2O$; 2.9 mg H_3BO_3; 1 mg $CoCl_2 \times 6\,H_2O$; 1.0 mg $CuSO_4 \times 5\,H_2O$; 2.5 mg $Na_2MoO_4 \times 2\,H_2O$; 1,2 mg $ZnSO_4 \times 7\,H_2O$; 0.2 mg $Na_2WO_4 \times 2\,H_2O$; 0.5 mg

$MnSO_4 \times 4\,H_2O$. 1 g/l (−)-Ephedrine × HCl (as a source of carbon) was added to this nitrogen free medium and up to 0.25 g/l $(NH_4)_2SO_4$, if required.

The mineral salts medium for *Arthrobacter globiformis*, growth conditions and other facts are as described previously [1].

Microorganisms and growth conditions

Arthrobacter globiformis, *Pseudomonas* spec. NCIB 9133 for the enzymatic determination of methylamine and *Pseudomonas putida* B_1.

The isolation of *Pseudomonas putida* B_1 is described under "results". Its characterization and identification was carried out according to the "Manual of Microbiological Methods" [4] and Bergey's Manual of Determinative Bacteriology" [5]. Cultures were grown in 10-l glas fermenters with stirring and an air-supply of 160 l per min. All metabolic studies were performed with the wild type strain.

Separation and identification of metabolites was carried out as described previously. (−)-Ephedrine [6], formaldehyde [7], methylamine [8], phenylacetylcarbinol [9], methylbenzoylcarbinol [10], pyrocatechol [11] and β-ketoadipate [12] were determined quantitatively with known procedures.

Preparation of crude extract from Arthrobacter globiformis. Cells were harvested by centrifugation, washed and suspended in the two fold quantity of 50 mM phosphate buffer pH 7.5 and then disrupted by ultrasonication at 4 °C for 6 minutes. The sonicated extract was centrifuged at 4 °C and 25000 × g for 20 minutes and the supernatant was stored at −20 °C before use.

Enzyme purification procedure

Gelfiltration was carried out with Sephadex G 100, G 150 and G 200. 4 ml of the crude extract were layered on top of the bed (column 2.5 cm × 75 cm), before elution with 50 mM phosphate buffer pH 7.5.

DEAE-A-25 and QAE-A-25 cellulose were applied for anion exchange chromatography. 5 ml of the crude extract were applicated to a column (2.5 cm × 8 cm), which then was eluted with a linear gradient of NaCl (0−1.0 M) in 50 mM phosphate buffer pH 7.5 at 4 °C.

Ultrafiltration was carried out with filters DM 5, PM 10 and PM 30 from Amicon. The extract was pressed through the filter by nitrogen at 4 °C.

For ammonium sulphate precipitation the crude extract was diluted by the addition of 50 mM phosphate buffer pH 7.5 to 15 mg protein/ml and ammonium sulphate was added in portions.

For saccharose gradient centrifugation 1.3 ml crude extract (40 mg protein) were layered on 12.6 ml of a gradient (5−20% saccharose) in 50 mM phosphate buffer pH 7.5 and centrifuged at $2.5 \times 10^5 \times g$ and 4 °C for 60 h in a Beckman SW 40 rotor.

Assay method

The assay method for (−)-ephedrine: NAD^+-oxidoreductase (deaminating) is based on the measurement of NADH formed during the enzymatic reaction.

One unit of (−)-ephedrine: NAD^+-oxidoreductase is defined as the amount of enzyme that catalyses the formation of 1 µmol NADH per minute at 25 °C under the conditions specified.

Usually the incubation mixture contained:

2.0 ml glycine/NaOH buffer (100 mM, pH 11.0),
0.05 ml NAD (100 mM) and
0.20 ml crude extract from *Arthrobacter globiformis*.

The reaction was started by addition of 0.05 ml of the substrate (an aqueous solution of 3.68 mM (−)-ephedrine).

Another method for the determination of enzymatic activity is based on the liberation of methylamine during the enzymatic reaction.

Conditions and reactants are the same as just mentioned except that (−)-ephedrine is used at a 10 fold higher concentration. The mixture is incubated for 1 minute at 25 °C and then heated in a boiling water bath for 5 minutes, before determination of methylamine [8].

Enzymatic production of phenylacetylcarbinol and methylbenzoylcarbinol

The assay solution for detecting the production of phenylacetylcarbinol (PAC) and methylbenzoylcarbinol (MBC) contained 2.0 ml phosphate buffer (50 mM, pH 7.5),

0.05 ml NAD (100 mM),
0.20 ml crude extract from *Arthrobacter globiformis* and
8.0 mg (−)-ephedrine × HCl.

Instead of (−)-ephedrine were also used:

(−)-norephedrine × HCl,
(+)-norephedrine × HCl,
(−)-norpseudoephedrine × HCl,
(+)-norpseudoephedrine × HCl and
(−)-methylephedrine × HCl.

The reaction mixture was incubated for 5 min at 30 °C, heated in a boiling water bath for 5 minutes, cooled immediately, extracted with methylene chloride and finally purified by thin-layer chromatography on silica-gel with methylene chloride as solvent. The purified mixture of phenylacetylcarbinol and methylbenzoylcarbinol is estimated quantitatively as described by Gröger and Erge [9]. The separation of phenylacetylcarbinol and methylbenzoylcarbinol by paper-chromatography as described by Suchy and Fedoronko [10] is a time-consuming method. A quick approximate calculation of rates can be obtained by comparison of the IR-CO-peaks at 1690 cm^{-1} (PAC) and 1720 cm^{-1} (MBC).

Results

Isolation of Pseudomonas putida B_1

The bacterium was isolated from a soil sample of Hohenheim supplemented with 4-benzoyl-1,3-oxazolidinon-(2). The sample had been incubated for two months at 30 °C. The nutritional test revealed that the bacterium was also able to utilize (−)-ephedrine as sole source of carbon.

Classification of the bacterium

Cultivation on nutrient broth medium and on yeast extract, peptone medium leads to circular, elevated, smooth, moist, light pale, stinking colonies. Incubation on (−)-ephedrine mineral salts medium for three days at 30 °C yields colonies with a diameter of 4−5 mm. Under the microscope slightly oval shaped rods of 2−3 µm length and 1 µm width can be seen. The bacteria are motile, showing monopolar, multitrichous flagellation as demonstrated by electron-microscopy. In media with less than 10 mg/l iron a diffusible blue-green fluorescent pigment is produced. Optimal temperature for growth lies at 25 to 30 °C under aerobic conditions and no growth at 42 °C and little growth at 4 °C is observed. No organic growth factors are required, the bacteria grow on minimal medium. Without any source of nitrogen no growth takes place. The physiological tests showed results as follows: Gelatine: no liquefaction, litmus milk: no production of acid, starch is not hydrolized, no production of indole from tryptophan, methyl-red test: negative, Voges-Proskauer-test: negative, no production of H_2S, urease: positive, catalase: positive, cytochromoxidase: positive. Growth characteristics are as follows: no growth is observed on lactate, mannit, adonit, dulcit, meso-inosit, raffinose, rhamnose, arabinose, maltose, galactose, ethanol, starch, trehalose, geraniol, (+)-ephedrine, (+)-norephedrine, (+)-pseudoephedrine, (+)-norpseudoephedrine, (−)-pseudoephedrine, (−)-norpseudoephedrine, methylamine, dimethylamine and ethylamine. Good growth takes place on fructose, glucose, xylose, mannose, saccharose, glycerol, pyruvate, 2-ketogluconate, benzylamine, d,l-valine, β-alanine, d,l-arginine, (−)-ephedrine, (−)-norephedrine, (−)-methylephedrine and (±)-suprifene.

The identification of the bacterium according to the "Manual of Microbiological Methods" [4] and "Bergey's Manual of Determinative Bacteriology" [5] led to the classification into the fluorescent species of the group of *Pseudomonads*, the isolated bacterium being identical with *Pseudomonas putida*. For laboratory use we called the bacterial strain *Pseudomonas putida* B_1.

Degradation of (−)-ephedrine by Pseudomonas putida B_1. Metabolic studies

Fig. 1 shows the growth-curve of a *Pseudomonas putida* B_1 culture and the appearence of metabolites. Wet cells (8 g) of *Pseudomonas* pregrown on (−)-ephedrine for 36 h were suspended, immediately after centrifugation in 10 l of mineral salts medium, supplemented with (−)-ephedrine. This culture was aerated (160 l air pm) and stirred (160 rpm) vigorously.

Methylamine, formaldehyde, methylbenzoylcarbinol, benzoic acid and pyrocatechol were found in the culture medium supplemented with (−)-ephedrine. For the detection of *cis, cis*-muconic acid and β-ketoadipate the culture medium was supplemented with pyrocatechol.

Methylamine was only detectable during the degradation of (−)-ephedrine and not in old cultures. The maximum concentration found was 0.03 mM. The bacterium was able to use both methylamine and (−)-ephedrine as sole source of nitrogen.

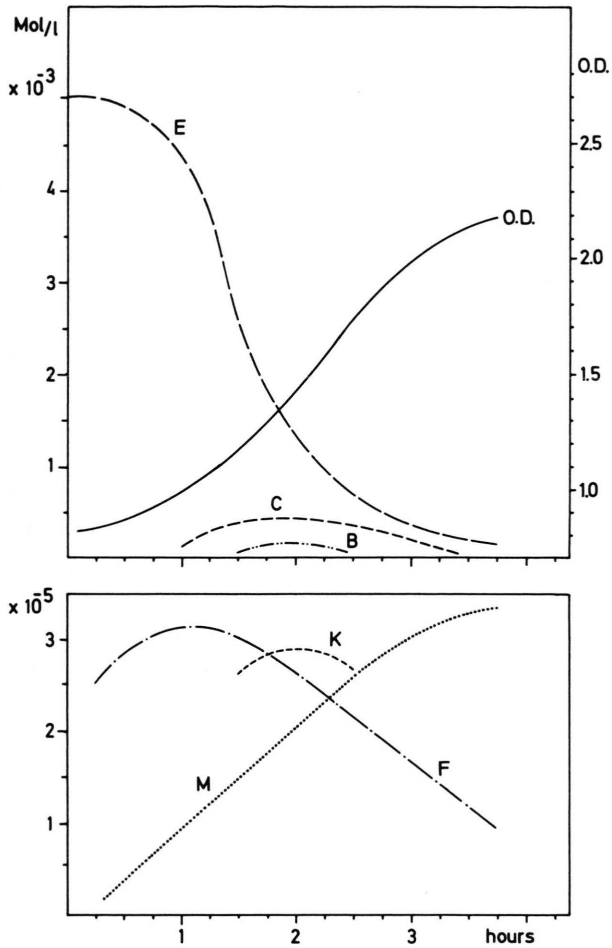

Fig. 1. Time-course of (−)-ephedrine dissapearance, accumulation of metabolites and growth of a culture of *Pseudomonas putida* B_1. E: (−)-ephedrine; M: methylamine; F: formaldehyde; K: pyrocatechol; C: methylbenzoylcarbinol; B: benzoic acid; OD: optical density.

Formaldehyde could be found only if the (−)-ephedrine medium was aerated vigorously. It was not detectable in cultures having already metabolized the greatest part of (−)-ephedrine. Its maximum concentration was found to be 0.03 mM.

Methylbenzoylcarbinol like formaldehyde, was only detectable if the cultures were supplied with great amounts of air. Its maximum concentration was found to be 0.04 mM. Phenylacetylcarbinol, the tautomeric form of methylbenzoylcarbinol was not detected.

Benzoic acid was detected regularly. Its maximum concentration was found to be 0.16 mM.

Pyrocatechol in relatively high concentrations was found in cultures growing in Erlenmeyer flasks on a rotary shaker during the degradation of (−)-ephedrine. Its maximum concentration was found to come up to 0.87 mM.

Cis, cis-muconic acid and *β-ketoadipic acid* were found when the bacterium was grown on pyrocatechol as its sole source of carbon. Therefore a 10-l fermenter was filled with 5 l of mineral salts medium and 10 g of cells, freshly harvested from a culture which had been grown on benzoic acid for 36 h. After 5 minutes pyrocatechol (5 g) was added to the culture medium and the medium was aerated and stirred vigorously (200 l air pm; 200 rpm). After one hour a sample was taken and examined, showing to contain 0.35 mM *cis, cis*-muconic acid and 5.8 mM *β*-ketoadipate. *Cis, cis*-muconic acid was isolated, *β*-ketoadipate was estimated according to Ottow and Zolg [12]. After 2.5 h pyrocatechol was no longer detectable in the culture medium.

Enzymatic studies

In addition to metabolic investigations we have tried to find enzyme activities with a specific function in the catabolism of (−)-ephedrine. In numerous cases this method has proven effective for the elucidation of a metabolic pathway. We have focussed our attention on the key enzyme of (−)-ephedrine-degradation and which catalyses the first step of the catabolic pathway.

Detection and some properties of (−)-ephedrine: NAD⁺-oxidoreductase (deaminating)

1. Preparation of enzyme extracts

In cells of *Pseudomonas putida* B_1 no or only neglectible enzyme activity could be detected, whereas cell extracts of *Arthrobacter globiformis* showed reasonably high activities. Best activity values (7×10^{-2} u/mg) were obtained by ultrasonic treatment of the cells for 5 to 6 minutes at 4 °C.

2. Induction of enzyme activity

No enzyme activity was detectable in cells grown on media with glucose or acetate as carbon source, whereas growth on (−)-ephedrine as the carbon source yielded in cells, which contained enzyme activity.

3. Products and stoichiometry of the enzyme reaction

With (−)-ephedrine and NAD^+ as substrates three products of the enzyme reaction could be detected: NADH, methylamine and phenylacetylcarbinol or its tautomeric form methylbenzoylcarbinol. The amount of NADH and methylamine was determined quantitatively and the ratio was found to be 1.0:1.1 (NADH: methylamine).

4. Some factors influencing enzyme activity

a) Cofactors

NAD^+ was found to be an absolute cofactor for enzyme activity: if a crude extract was dialysed for 20 h against phosphate buffer no formation of methylamine was measured, unless the extract was supplemented with NAD^+. Attempts to replace NAD^+ by $NADP^+$, FAD or FMN were without success.

b) Metal ions and EDTA

A 5 minute preincubation of the dialysed extract with Fe^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, Al^{3+} or NH_4^+, each at a concentration of 1 mM, did not exhibit any influence on enzyme activity. Dialysis of the crude extract against 1 mM EDTA for 20 h resulted in 35% loss of activity and only 5% of enzyme activity were lost by an incubation of the crude extract for 5 min with 1 mM EDTA.

c) pH-value

Maximum enzyme activity is measured in a 100 mM glycine/NaOH buffer between pH 10 and pH 11.5. Only 60% of maximum activity is obtained at pH 9.5 and pH 12.2, respectively.

d) Buffer

At a given pH-value the nature of the buffer seems to be without any significant influence on enzyme activity as shown for phosphate, tris and glycine/NaOH buffer. Variation in buffer concentration from 40 to 150 mM is also without influence on enzymatic activity.

e) Substrate concentrations (K_m-values)

From the double-reciprocal plot of enzyme activity against (−)-ephedrine concentration (Fig. 2) K_m for (−)-ephedrine was determined as 0.02 mM and V_{max} as 0.1 µ/mg. Maximum activity was measured at an (−)-ephedrine concentration of 0.08 mM, at higher concentrations substrate inhibition was observed.

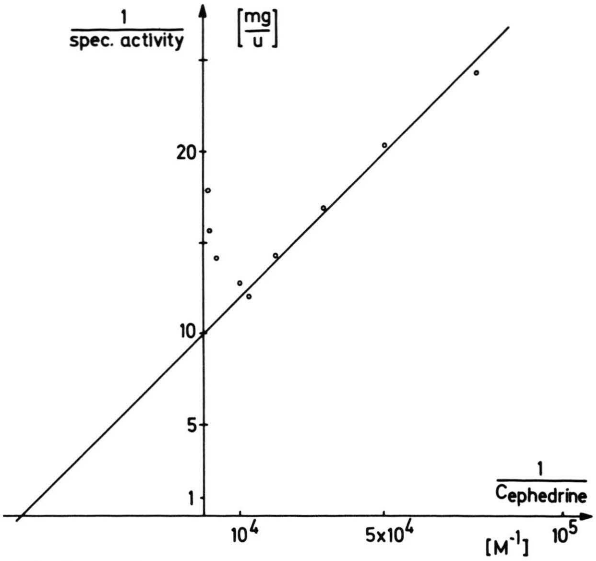

Fig. 2. Double reciprocal plot of enzyme velocity versus (−)-ephedrine concentration at a constant NAD^+-concentration of 2.2 mM.

The K_m-value for NAD^+ (at a constant (−)-ephedrine concentration of 0.08 mM) was found to be 0.11 mM. Increasing concentrations of NAD^+ did not inhibit enzyme activity.

5. Enzyme stability

a) Effect of temperature

The crude extract can be stored at −20 °C for at least 6 months without any appreciable loss of activity. Heating the enzyme extract for 2 minutes at 54 °C destroys enzymatic activity completely.

b) Effect of pH-value

At a temperature of 4 °C the enzyme exhibited maximum stability at pH 7.5 and 8.5, respectively. Within 24 h 20% of activity was lost when the enzyme was stored at these pH-values. 68% were lost at pH 6.8, 53% at pH 7.2 and 67% at pH 10.

c) Effect of dilution

When stored at 4 °C diluted extracts are significantly less stable than concentrated extracts. Within 24 h an extract with a protein concentration of 16.5 mg/ml lost 20% of the original activity, an extract with 11.0 mg/ml protein lost 47% and an extract with 8.2 mg/ml protein lost 80% of the activity.

Enzyme specificity

22 different compounds bearing structural similarities with (–)-ephedrine were tested as substrate in the enzyme reaction. Table I summarizes the results of this study. Aromatic compounds were assayed at a concentration of 0.08 mM, which had been found to give highest activity with (–)-ephedrine as the substrate. Non-aromatic compounds, which at this concentration were only converted at a very slow rate, were applied at the ten-fold higher concentration of 0.8 mM.

When chiral norephedrines were used as substrates for (–)-ephedrine: NAD$^+$-oxidoreductase, the carbinol compounds could be detected in the enzyme incubation mixture. The amount of the carbinols formed was determined quantitatively and found to be as high as the amount of NADH formed during the reaction.

The preparation of about 4 mg of the carbinol compounds was achieved enzymatically. By means of IR spectroscopic measurement this product was shown to consist of a mixture of about 90% phenylacetylcarbinol and 10% of methylbenzoylcarbinol.

Enzyme purification

The highest increase in specific activity was achieved by ammonium sulphate precipitation. The fraction corresponding to 57% to 62% saturation in ammonium sulphate showed a specific activity of 0.61 u/mg, corresponding to a 9.1 fold enrichment.

A two fold enrichment was obtained by centrifugating the crude extract for 4 h at $200 \times 10^3 \times g$.

No effect on the specific activity was achieved with ultracentrifugation in a saccharose gradient at $200 \times 10^3 \times g$ for 60 h. Without success remained attempts to purify the enzyme by gel filtration on Sephadex G-100, G-150 and G-200. The greatest part of enzyme activity was lost during this procedure.

By chromatography on DEAE and QAE-cellulose using a linear NaCl gradient the enzyme could be eluted between 0.17 to 0.27 M NaCl. Specific activity, however, was only slightly higher than in the crude extract.

The attempt to concentrate diluted extracts by membrane filtration using Amicon ultrafiltration cells failed. In the concentrated extracts no activity was detectable.

Compound	Specific activity [10^{-2} u/mg]	Compound	Specific activity [10^{-2} u/mg]
(–)-ephedrine	7.50	2-methylamino-ethanol	0.80
(+)-ephedrine	4.80	2-ethylamino-ethanol	0.00
(+)-pseudoephedrine	31.10	N,N-dimethyl-ethanolamine	0.00
(–)-pseudoephedrine	3.37	DL-threonine	0.00
(–)-methylephedrine	3.07	ethanolamine	3.22
(–)-sympatol	40.50	DL-2-aminopropanol	3.60
(–)-adrenaline	3.30	1-aminopropanol-(2)	3.50
(–)-norephedrine	2.55	2-amino-2-methyl-propanol-(1)	0.40
(+)-norephedrine	11.50		
(+)-norpseudo-ephedrine	4.80		
(–)-norpseudo-ephedrine	0.00		
(1S : 2R)-1-phenyl-1,2-propandiol	0.00		
(±)-suprifene	34.50		
(–)-2-methylamino-1-phenyl-propanon-(1)	0.00		

Table I. Substrate specificity of (–)-ephedrine: NAD$^+$-oxidoreductase.

Discussion

The metabolites, isolated from the culture medium of *Pseudomonas putida* B_1, allow us to propose a pathway for the degradation of (−)-ephedrine (Fig. 3).

Methylbenzoylcarbinol (**III b**) and methylamine (**II**) are the products of the first step in the degradation of (−)-ephedrine (**I**). As shown by growth tests methylamine is utilized by *Pseudomonas putida* B_1 as a nitrogen but not as a carbon source. Methylamine is probably converted into ammonia and formaldehyde (**II a**), which is also detectable in the culture fluid. Although formaldehyde is not used as a growth substrate, a rapid disappearance of this compound is observed. In the crude extracts of *Pseudomonas putida* B_1 a high activity of formaldehyde dehydrogenase (42 u/mg) could be demonstrated, suggesting that formaldehyde disappears from the culture medium by oxidation. Methylbenzoylcarbinol (**III b**) probably undergoes a cleavage into acetaldehyde (**V**) and benzaldehyde (**IV**), the latter compound subsequently being oxidized to benzoic acid (**VI**). Benzoic acid is finally metabolized via the wellknown ortho-cleavage pathway, as demonstrated by the detection of the major intermediates pyrocatechol (**VIII**), *cis, cis*-muconic acid (**IX**) and β-ketoadipate (**XII**). The pathway for the degradation of (−)-ephedrine as postulated in Fig. 3 for *Pseudomonas putida* B_1 resembles very much that of *Arthrobacter globiformis* [1].

Attention may be focussed on some differences: in *Arthrobacter globiformis* predominantly the tautomeric form of methylbenzoylcarbinol, namely phenylacetylcarbinol could be isolated from the culture medium. Formaldehyde was not detectable in the medium of *Arthrobacter*, because methylamine was accumulated quantitatively in the culture medium and not further metabolized. Finally, acetaldehyde which is one of the cleavage products of carbinol was only detectable in the cultures of *Arthrobacter globiformis*.

In the crude extracts of *Arthrobacter globiformis* an enzymatic activity, capable to attack the parent growth substrate (−)-ephedrine, could be detected. Studies on the products and the stoichiometry of this enzyme reaction agreed well with the results of the metabolic studies. As products of the reaction methylamine (**II**) and both tautomeric forms of the carbinols (**III a** and **III b**) were determined. NAD^+ was found to be absolutely necessary for enzymatic

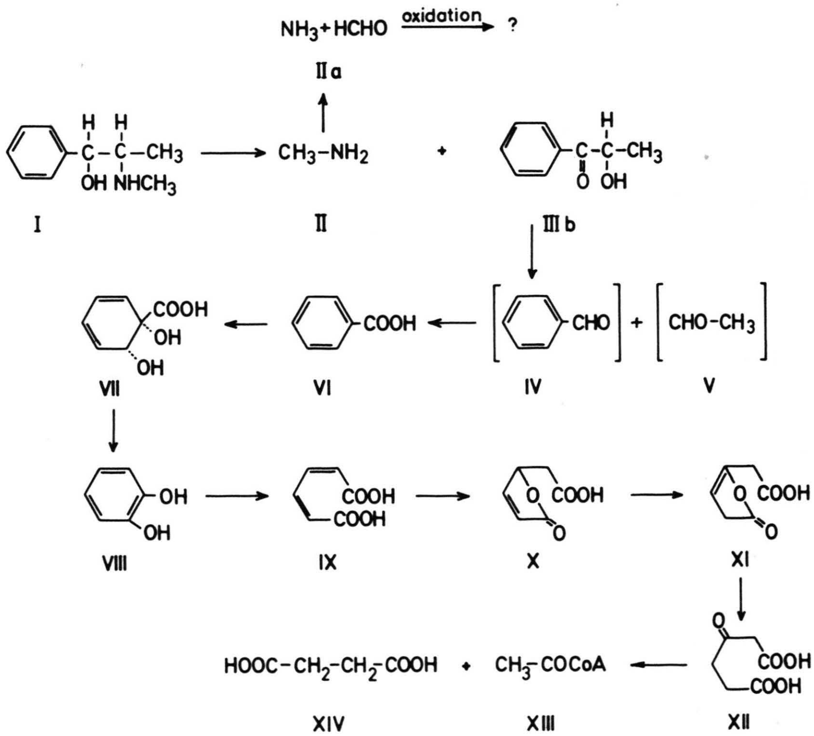

Fig. 3. Proposed pathway for the degradation of (−)-ephedrine in *Pseudomonas putida* B_1.

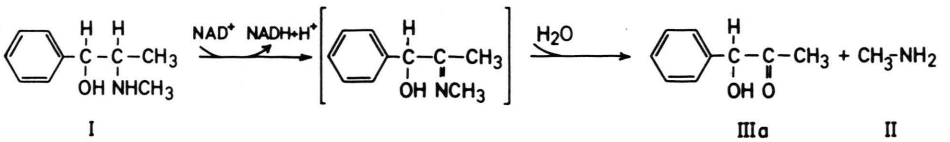

Fig. 4. Proposed reaction sequence for (−)-ephedrine: NAD^+-oxidoreductase (deaminating).

activity. The molecular structure of (−)-ephedrine allows an attack of an NAD^+-oxidoreductase at either C-1 (with the CH-OH group) or at C-2 with the $CH\text{-}NHCH_3$ group. In the first case as an intermediate product 2-methylamino-1-phenylpropanone-(1) should be postulated. This compound was shown by Skita *et al.* [13] to be relatively stable in aqueous solution. Since no intermediate product could be detected neither in the enzymatic reaction, nor in metabolic studies, we assume that the enzyme in the initial step attacks (−)-ephedrine at $CH\text{-}NHCH_3$ yielding 2-methylimino-1-phenyl-propanol-(1), which is immediately hydrolyzed forming methylamine (**II**) and phenylacetylcarbinol (**III a**) (Fig. 4). According to this mechanism we suppose that the enzyme catalysing the first step in (−)-ephedrine degradation belongs to the oxidoreductase of group 1.5.1. (acting on −CH−NH) rather than to those of group 1.1.1. (acting on CH−OH). Summarizing stoichiometric studies and mechanistic considerations we propose the name (−)-ephedrine: NAD^+-oxidoreductase (deaminating) (E. C. 1.5.1.×) for this enzyme.

A thorough investigation of the enzyme's properties was hampered by the relatively high instability of the oxidoreductase, thus preventing the purification of the enzyme to homogeneity. We could show, that (−)-ephedrine-oxidoreductase is sensitive to inactivation by dilution and by exposure to elevated temperature.

Fractionated ammonium sulphate precipitation resulted in a 9 fold enrichment in specific activity, other purification methods like gel chromatography, anion exchange chromatography, ultracentrifugation, saccharose gradient centrifugation and ultrafiltration were without success.

Some enzyme parameters were determined in the partially purified extract. Enzyme activity does not depend on the presence of metal ions. The pH-optimum lies at alkaline pH-values between 10 and 11.5.

The K_m for (−)-ephedrine was determined to be 0.02 mM and for NAD^+ 0.11 mM, respectively. (−)-Ephedrine at higher concentrations exerts significant substrate inhibition.

Studies on substrate specificity revealed that the enzyme is not very specific, being able to catalyse the conversion of a considerable number of structurally related compounds. Some substrate analogues like (+)-norephedrine, (+)-pseudoephedrine, (−)-suprifene and (−)-sympatol are dehydrogenated at a considerably higher rate than (−)-ephedrine itself. These results lead us to conclude, that the enzyme due to its broad substrate specificity plays a role in the metabolism of quite a number of different aromatic and aliphatic amino alcohols.

Acknowledgements

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