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Biodegradation of dl-synephrine: A novel pathway in *Nocardia* sp DM1

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Abstract

Several organisms were tested for their ability to degrade dl-synephrine. One soil pseudomonad and a *Nocardia* sp have been found to efficiently utilise the compound. *Nocardia* sp degraded synephrine by two novel routes; one involving monoamine oxidase and the other involving conversion to *p*-hydroxyphenyl-acetaldehyde by the synephrinase enzyme. The *p*-hydroxyphenyl-acetaldehyde was converted to *p*-hydroxyphenylacetic acid and finally to 2,5-dihydroxyphenylacetic acid which underwent ring fission between C_1 and C_2 atoms. The monoamine oxidase converted synephrine to *p*-hydroxymandelicaldehyde which was finally oxidised to 3,4-dihydroxybenzoic acid through the intermediate formation of *p*-hydroxymandelic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. 3,4-Dihydroxybenzoic acid was cleaved by an oxygenase through an *ortho*fission. The route involving synephrinase was the major degradative pathway. However, the two pathways were found to operate simultaneously.

Key words: *Citrus* plant, degradation, 2,5-dihydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, *Nocardia* sp, phytotoxicity, synephrine.

1. Introduction

Synephrine [*p*-hydroxy-α-(methylaminomethyl) benzyl alcohol], a well-known sympathomimetic amine, is structurally related to epinephrine and is present in high concentrations in *Amaryllidaceae* and *Rutaceae* plants[1–3]. It accumulates to an enormous amount of 2 g/kg (fresh weight) in the leaves of *Citrus reticulata* (Tangerine and cleopatra mandarin varieties)[3]. A pathway was proposed for the biosynthesis of synephrine in *citrus*[4] and the metabolic relationship of synephrine with other phenolic compounds in animals was established[5,6]. Phenolic amines with no demonstrable role in plants apparently are not metabolised and reach the soil with the senescent leaves. It is known that phenolics in general and phenolic amines in particular, if accumulated in soil in considerable amounts, are phytotoxic and result in decreased crop yield. "Soil sickness" of apple and peach orchards are examples of such situation[7]. At present our

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understanding of the role of phenolic amines in animal metabolism and their function in plant systems is fragmentary, and little is known about their mode of disposal and degradation in nature. So far, there has been only one report on the degradation of synephrine by a pathway involving *p*-hydroxyphenylacetaldehyde, *p*-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid as intermediates[8]. The diversity one finds in the metabolic pathways in microorganisms prompted us to look for the degradation of synephrine in other microbial systems. We have screened a number of organisms for their ability to utilise dl-synephrine as the sole carbon and energy source. A soil pseudomonad and a *Nocardia* sp were found to efficiently utilise synephrine. In the present communication, two novel pathways that operate in the degradation of synephrine in the *Nocardia* sp are discussed.

2. Materials and methods

2.1. Chemicals

All the biochemicals were purchased from Sigma Chemical Co., except *p*-hydroxybenzaldehyde which was procured from Koch-Light Laboratories, England. The stock solution of dl-synephrine was prepared by dissolving 25 g of the chemical in a small amount of 1*M* HCl and the volume was made up to 500 ml after neutralising with 1*M* NaOH. The solution was filter-sterilized and stored at 4°C.

2.2. Growth of the organism

A bacterial strain was isolated from the effluents of the organic chemistry laboratories at the Institute which could degrade several aromatic compounds including dl-synephrine. The organism was identified to be a species of *Nocardia* according to Bergey's Manual of Determinative Bacteriology[9]. This organism will be referred to in the text as *Nocardia* sp DM1. The organism was grown on the following nutrient medium. Sucrose 10 g; glucose 5 g; peptone 4 g; yeast extract 1 g; calcium nitrate 0.5 g; $Na_2HPO_4.12H_2O$ 3 g; KH_2PO_4 0.3 g; NH_4Cl 0.2 g; $MgSO_4.7H_2O$ 0.2 g; $FeSO_4.7H_2O$ 0.5 g and 5 ml of the trace element solution containing $MnSO_4.H_2O$ 3 mg; $ZnSO_4.7H_2O$ 10 mg; $CuSO_4.5H_2O$ 1 mg; $Na_2MoO_4.2H_2O$ 1 mg and H_3BO_3 2 mg; distilled water 1000 ml, pH 7.2. The stock cultures were maintained on the same medium containing 2% agar and preserved at 4°C.

A five millilitre preculture after 36 h of growth from the above medium was transferred to 200 ml of the following induction medium. Yeast extract 0.5 g; dl-synephrine 2 g and 5 ml of the trace element solution as mentioned above, distilled water 1000 ml, pH 7.2. The organism was grown at 30°C on a rotary shaker (120 rev./min) and the cells that were harvested at 12 h were used for metabolic and enzymatic studies.

2.3. Utilisation of dl-synephrine

During growth period: The organism was grown for 30 h in the induction medium. During the growth period, the utilisation of synephrine was checked by taking the culture

supernatants at regular intervals[8]. The optical density (OD) was recorded at 290 nm which is the λ_{max} for synephrine in a Shimadzu UV–190 spectrophotometer against water blank.

By washed cell suspensions: Equal amount (1 g) of induced (grown on synephrine) and uninduced (grown only on yeast extract) cells were washed and incubated separately with 100 ml of 50 m*M* sodium phosphate buffer, pH 7.0 containing 100 mg of synephrine. At 30 min intervals, samples were drawn from the flasks and synephrine was estimated as mentioned above.

Oxygen uptake studies: The synephrine-grown cells were suspended in 0.05M sodium phosphate buffer (pH 7.0) to give a concentration of 30 mg (wet weight)/ml.

Oxygen uptake by the whole cells was measured at 30°C in a Gilson respirometer in the presence of 5 μmol of the aromatic substrate as described by Haribabu and Vaidyanathan[10].

2.4. *Isolation and identification of the metabolites*

At different times during growth period, the culture filtrates were analysed for metabolites by acidifying to pH 2.0 with 1*M* HCl and extracting twice with equal volume of peroxide-free ether. The combined ether layers were subjected to the fractionation procedure of Abitha Devi *et al*[8]. The neutral and acidic fractions thus obtained were dried over anhydrous sodium sulphate and evaporated *in vacuo*. The residues were dissolved in a small amount of ether and were subjected to paper and thin-layer chromatography (TLC). The metabolites were identified by comparing their R_f values and colour reactions with those of authentic compounds. The individual products were isolated from paper and TLC plates by eluting with ether. After evaporating the ether, the residues were dissolved in 95% ethanol and UV spectra were recorded in Beckman Model-26 double-beam spectrophotometer. Infrared spectra of the metabolites were recorded in nujol mull in Perkin–Elmer 580 IR spectrophotometer.

2.5. *Enzyme assays*

Cell-free extract of the organism was prepared by sonicating the cell suspension in 0.05*M* phosphate buffer, pH 7.0 (1:3 w/v) for 10 min and the homogenate was centrifuged at 7,000 *g* for 15 min and the supernatant was used as the crude extract. All these operations were carried out at 0–4°C.

Synephrinase exzyme catalyses the conversion of synephrine to *p*-hydroxyphenylacetaldehyde and methylamine[11]. It was assayed following the formation of *p*-hydroxyphenylacetaldehyde according to the method of Abitha Devi *et al*[8]. Protocatechuate dioxygenase was assayed by following the disappearance of protocatechuate by the method of Nair and Vaidyanathan[12] as described previously[10]. Similarly, homoprotocatechuate dioxygenase was assayed following the disappearance of homoprotocatechuate as mentioned above. *p*-Hydroxymandelic acid oxidase (decarboxylating)

was assayed according to the method of Bhat *et al*[13] by monitoring the product, *p*-hydroxybenzaldehyde. *p*-Hydroxymandelic acid dehydrogenase and *p*-hydroxybenzoylformic acid decarboxylase was assayed according to the methods of Kishore *et al*[14].

The following enzymes were assayed polarographically using Gilson oxygraph and all the reactions were performed in 0.05*M* sodium phosphate buffer, pH 7.0 (120 μmol). In every enzyme assay, the total reaction mixture was 1.2 ml which included 0.1 ml of the crude extract and the individual reaction mixture composition is as follows: Monoamine oxidase – dl-synephrine 0.4 μmol; *p*-hydroxyphenylacetate-1-hydroxylase. – *p*-hydroxyphenylacetate 0.4 μmol and NADH 0.4 μmol; 2,5-dihydroxyphenylacetate, – 1,2-dioxygenase, – 2,5-dihydroxyphenylacetate 0.4 μmol; *p*-hydroxybenzoate-3-hydroxylase, *p*-hydroxybenzoate 0.4 μmol, FAD 0.02 μmol and NADPH generating system 0.1 ml (buffer 5μmol, $NADP^+$ 0.2 units, preincubated for 10 min at 30°C). Reactions were started by the addition of respective substrates and the endogenous oxygen consumption was subtracted from the total oxygen uptake at the end of the reaction. All the enzyme assays were carried out at 30°C.

The hydrogen peroxide formed in the cell-free extracts by the monoamine oxidase was monitored according to the method of Loschen *et al*[15]. The decrease in Scopoletin fluorescence was measured in Perkin–Elmer spectrofluorimeter at 350 nm (excitation) and 460 nm (emission).

Protein was estimated in the cell-free extracts by the method of Lowry *et al*[16]. All the specific activities were expressed as n mole (substrate disappeared, or product formed) per min/mg protein.

The mode of protocatechuate ring cleavage was determined by the method of Stanier *et al*[17].

3. Results and discussion

Of various organisms tested, only *Mycobacterium smegmatis*, *Nocardia* sp DMI, *Aspergillus niger* and a bacterial strain isolated from the soil by substrate enrichment culture were found to utilise synephrine as carbon and nitrogen source. Since *Nocardia* sp DMI and the bacterial isolate were found to grow best on synephrine, these two organisms were selected for detailed studies. The results obtained with the *Nocardia* sp DMI are presented in this paper.

The growth curve and the utilisation of dl-synephrine during growth period of *Nocardia* sp DMI is shown in fig. 1. The organism reached stationary phase in 24 h and consumed most of the synephrine added, within 30 h of growth.

3.1. Identification of the products of synephrine metabolism

The R_f values, colour reactions and λ_{max} values of various metabolites isolated from the spent medium of *Nocardia* sp DMI at different times during growth period are given in Table I. UV and IR spectra of the metabolites were compared with those of the authentic compounds and were identified as *p*-hydroxyphenylacetic acid, 2,5-dihydroxyphenylace-

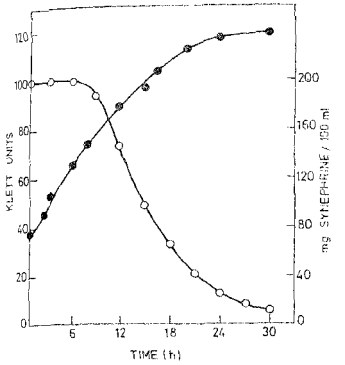

FIG. 1. Growth of *Nocardia* sp DM1 on synephrine (●) and utilisation of synephrine during growth period (○).

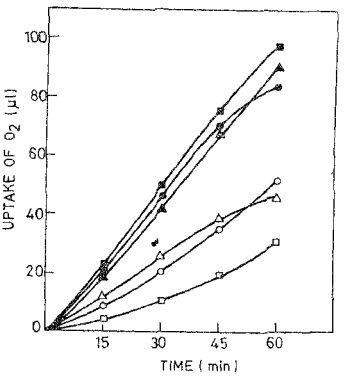

FIG. 2. Respiratory activities of synephrine-grown *Nocardia* sp DM1. Synephrine (■), *p*-hydroxyphenylacetate (▲), 2,5-dihydroxyphenylacetate (●), *p*-hydroxymandelate (△), *p*-hydroxybenzoic acid (○), 3,4-dihydroxybenzoate (□). Endogenous respiration was subtracted.

Table I
Properties of the metabolites formed from synephrine by *Nocardia* sp DM1

Compound	R_f values[+]		Colour reactions[++]		UV maximum (nm) at	Identified as
	A	B	1	2		
I	0.43	0.75	Violet	–	278	*p*-Hydroxyphenyl-acetic acid
II	0.85	0.76	Pale brown	–	295	2,5-Dihydroxyphenyl-acetic acid
III	0.28	0.82	Brick red	–	276	*p*-Hydroxymandelic acid
IV	0.64	0.71	Purple red	Middle chrome	285	*p*-Hydroxybenzaldehyde
V	0.19	0.62	Red	–	254	*p*-Hydroxybenzoic acid
VI	0.10	0.51	Pale steel grey	–	259, 296	3,4-Dihydroxybenzoic acid

+ Solvents A: Isopropanol: Ammonia: Water (8:1:1 v/v); B: 2% Aqueous formic acid.
++ Reagents 1: Diazotized-*p*-nitroaniline followed by 10% aqueous NaOH; 2: 2,4-Dinitrophenylhydrazine followed by 10% aqueous NaOH.

tic acid, *p*-hydroxymandelic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid.

3.2. Oxygen uptake studies

dl-Synephrine-grown cells of *Nocardia* sp DMI readily oxidized dl-synephrine, *p*-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, *p*-hydroxymandelic acid, *p*-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (fig. 2). The oxygen uptake by the cells with the first three of these compounds was considerably higher than the rest of them. However, 3,4-dihydroxyphenylacetic acid was not oxidised to any significant extent. The uninduced cells oxidized only synephrine but could not oxidize any of the other metabolites.

3.3. Utilisation of dl-synephrine by washed cell suspensions

The utilisation of synephrine by the induced and uninduced cells of the *Nocardia* sp DMI is shown in fig. 3. The induced cells utilised synephrine immediately after transferring into buffered synephrine solution (fig. 3). However, the uninduced cells did so only slowly without any lag. These observations suggest that the synephrine metabolising enzymes are constitutively present in relatively low quantities in this organism.

3.4. Enzyme activities in the cell-free extract

Cell-free extracts of synephrine-grown cells showed very high enzyme activities of synephrinase, *p*-hydroxyphenylacetate-1-hydroxylase and 2,5-dihydroxyphenylacetate 1,2-dioxygenase whose specific activities were respectively 34, 105 and 291, while the

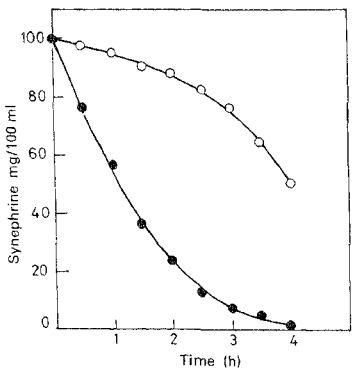

FIG. 3. Utilisation of synephrine by washed cell suspensions of *Nocardia* sp DMI. Synephrine-grown (induced) cells (●), yeast extract-grown (uninduced) cells (○).

enzymes, monoamine oxidase, *p*-hydroxymandelate oxidase (decarboxylating), *p*-hydroxybenzoate-3-hydroxylase and protocatechuate dioxygenase were at very low levels having specific activities 15,11,17 and 13 respectively. We could not demonstrate 3,4-dihydroxyphenylacetate dioxygenase activity in the cell-free extract of the induced cells. Interestingly, only the monoamine oxidase (MAO) activity could be detected in the cell-free extract of the uninduced cells.

Based on the results of the above studies, two pathways have been proposed for the degradation of dl-synephrine by the *Nocardia* sp DM1 (fig. 4). It is known that in animal systems, monoamine oxidase converts catecholamines to the corresponding aldehydes which are subsequently oxidized to the respective acids[18]. It has been suggested that monoamine oxidase may also convert synephrine to *p*-hydroxymandelicaldehyde by a similar reaction[6]. However, an unidentified *Arthrobacter* converted synephrine to *p*-hydroxyphenylacetaldehyde by a novel enzyme, synephrinase, in contrast to the possible conversion to *p*-hydroxymandelicaldehyde[8,11].

Interestingly, in *Nocardia* sp DM1, two different pathways were operating simultaneously (fig. 4) which are entirely different from the previously reported pathway in an unidentified *Arthrobacter*[8]. dl-Synephrine was converted to *p*-hydroxyphenylacetaldehyde by synephrinase which was immediately oxidized to *p*-hydroxyphenylacetic acid. Finally, *p*-hydroxyphenylacetate was hydroxylated to 2,5-dihydroxyphenylacetic acid by *p*-hydroxyphenylacetate-1-hydroxylase, presumably involving an 'NIH' shift as evident from the positions of the two hydroxyl groups in the product. This reaction is in contrast to the previously mentioned reactions in *Arthrobacter synephrinum, Pseudomonas putida, Eschericia coli* and *Acinetobacter* sp where *p*-hydroxyphenylacetic acid is converted to 3,4-dihydroxyphenylacetic acid without any 'NIH' shift[19–23]. The conver-

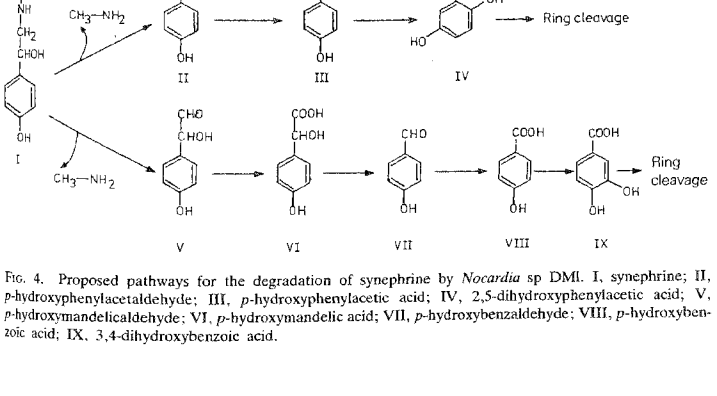

FIG. 4. Proposed pathways for the degradation of synephrine by *Nocardia* sp DM1. I, synephrine; II, *p*-hydroxyphenylacetaldehyde; III, *p*-hydroxyphenylacetic acid; IV, 2,5-dihydroxyphenylacetic acid; V, *p*-hydroxymandelicaldehyde; VI, *p*-hydroxymandelic acid; VII, *p*-hydroxybenzaldehyde; VIII, *p*-hydroxybenzoic acid; IX, 3,4-dihydroxybenzoic acid.

sion of *p*-hydroxyphenylacetic acid to 2,5-dihydroxyphenylacetic acid has also been reported in *Nocardia* spec 43251 by Engelhardt *et al*[24]. The ring cleavage of 2,5-dihydroxyphenylacetate took place between C_1 and C_2 atoms of the benzene nucleus as indicated by the formation of maleyl-acetoacetate. A constitutive nonspecific amine oxidase, possibly an MAO, which was present both in the induced and uninduced cells of *Nocardia* sp DMI converted dl-synephrine to *p*-hydroxymandelic acid. This reaction is similar to the one reported for tyramine degradation by *Aerobacter aerogenes* ATCC 9621[25]. The presence of MAO was also evident from the stoichiometric formation of hydrogen peroxide in the cell-free extracts of induced and uninduced cells. This enzyme accounts for the slow utilisation of synephrine by the washed uninduced cells without any initial lag period (fig. 3). In contrast to the previous reports of the oxidation of *p*-hydroxymandelic acid to *p*-hydroxybenzaldehyde with an intermediate formation of *p*-hydroxybenzoylformic acid in *Pseudomonas*[26,27], in *Nocardia* sp DMI, *p*-hydroxymandelic acid was directly converted to *p*-hydroxybenzaldehyde by *p*-hydroxymandelate oxidase (decarboxylating). A similar enzyme was reported previously from *Pseudomonas convexa* by Bhat *et al*[13]. When we incubated the whole cells with *p*-hydroxymandelic acid, we could not detect any *p*-hydroxybenzoylformic acid in the spent medium. Also, we were unable to demonstrate the activities of *p*-hydroxymandelate dehydrogenase and *p*-hydroxybenzoylformic acid decarboxylase in the cell-free extract of the induced cells. Possibly, *p*-hydroxymandelic acid directly gets converted to *p*-hydroxybenzaldehyde without the intermediate formation of *p*-hydroxybenzoylformic acid. *p*-Hydroxybenzaldehyde was immediately oxidized to *p*-hydroxybenzoic acid. Finally, this acid was ring-cleaved by an oxygenase involving *ortho*fission as indicated by Rothera's test[28].

These results show that dl-synephrine was catabolised by *Nocardia* sp DMI in two different novel pathways operating simultaneously. We checked for the interconversion of *p*-hydroxyphenylacetate to *p*-hydroxymandelic acid as reported by Kishore *et al*[14] in *Aspergillus niger* to see if these two pathways were connected at this point. We could not observe the formation of *p*-hydroxymandelic acid from *p*-hydroxyphenylacetic acid either in the whole cells or in the cell-free extract. Hence, it is unlikely that the two pathways are interconnected *via p*-hydroxyphenylacetic acid and *p*-hydroxymandelic acid. The major degradative pathway of dl-synephrine in this organism is through 2,5-dihydroxyphenylacetic acid, though synephrine was metabolised to a minor extent by the pathway involving MAO as the first enzyme. This conclusion is further strengthened by the fact that the enzymes of the MAO pathway were present at very low levels in the induced cells.

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References

1. STEWART I., NEWHALL, W.F. AND EDWARDS, G.J. — The isolation and identification of l-synephrine in the leaves and fruit of citrus, *J. Biol. Chem.*, 1964, **239**, 930–932.

2. STEWART, I. AND WHEATON, T.A. — l-Octopamine in citrus. Isolation and identification, *Science*, 1964, **145**, 60–61.

3. WHEATON, T.A. AND STEWART, I. — The distribution of tyramine, N-methyltyramine, hordenine, octopamine and synephrine in higher plants, *Lloydia*, 1970, **33**, 244–253.

4. WHEATON, T.A. AND STEWART, I. — Biosynthesis of synephrine in citrus, *Phytochemistry*, 1969, **8**, 85–92.

5. AXELROD, J. — Enzymatic formation of adrenaline and other catechols from monophenols, *Science*, 1963, **140**, 499–500.

6. PISANO, J.J., OATES, Jr. J.A., KARMEN, A., SJOERDSMA, A. AND UDENFRIEND, S. — Identification of *p*-hydroxy-α-(methyl aminomethyl) benzyl alcohol (synephrine) in human urine, *J. Biol. Chem.*, 1961, **236**, 898–901.

7. BORNER, H. — Liberation of organic substances from higher plants and their role in the soil sickness problem, *Bot. Rev.*, 1960, **26**, 393–424.

8. ABITHA DEVI, N., KRISHNAN KUTTY, R., VASANTHARAJAN, V.N. AND SUBBA RAO, P.V. — Microbial metabolism of phenolic amines: Degradation of dl-synephrine by an unidentified *Arthrobacter*, *J. Bacteriol.*, 1975, **122**, 866–873.

9. MCCLUNG, N.M. — In *Bergey's manual of determinative bacteriology*, 8th Edition. Eds. R. E. Buchanon and N.E. Gibbons, William and Wilkins Co., Baltimore, Md., 1974, pp. 726–746.

10. HARIBABU, B. AND VAIDYANATHAN, C.S. — Catabolism of isophthalic acid by a soil bacterium. *FEMS Microbiol. Lett.*, 1982, **14**, 101–106.

11. VEERASWAMY, M., ABITHA DEVI, N., KRISHNAN KUTTY, R. AND SUBBA RAO, P.V. — Conversion of (±)-synephrine into *p*-hydroxyphenylacetaldehyde by *Arthrobacter synephrinum*. A novel enzymic reaction., *Biochem. J.*, 1976 **159**, 807–809.

12. NAIR, P.M. AND VAIDYANATHAN, C.S. — A colorimetric method for determination of pyrocatechol and related substances, *Anal. Biochem.*, 1964, **7**, 315–321.

13. BHAT, S.G. AND VAIDYANATHAN, C.S. — Purification and properties of L-4-hydroxymandelate oxidase from *Pseudomonas convexia*, *Eur. J. Biochem.*, 1976, **68**, 323–331.

14. KISHORE, G., SUGUMARAN, M. AND VAIDYANATHAN, C.S. — Metabolism of DL-(±)-phenylalanine by *Aspergillus niger*, *J. Bacteriol.*, 1976, **128**, 182–191.

15. LOSCHEN, G. FLOHE, L. AND CHANCE, B. — Respiratory chain linked H_2O_2 production in pigeon heart mitochondria, *FEBS Lett.*, 1971, **18**, 261–264.

16. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. AND RANDALL, R.J. Protein measurement with the folin phenol reagent, *J. Biol. Chem.*, 1951, **193**, 265–275.

17. STANIER, R.Y., PALLERONI, N.J. AND DOUDOROFF, M. The aerobic pseudomonads: A taxonomic study, *J. Gen. Microbiol.*, 1966, **43**, 159–271.

18. LEEPER, L.C., WEISSBACH, H. AND UDENFRIEND, S. Studies on the metabolism of norepinephrine, epinephrine and their 0-methyl analogs by partially purified enzyme preparations, *Arch. Biochem. Biophys.*, 1958, **77**, 417–427.

19. ADACHI, K., TAKEDA, Y., SENOH, S. AND KITA, H. Metabolism of *p*-hydroxyphenylacetic acid in *Pseudomonas ovalis*, *Biochim. Biophys. Acta*, 1964, **93**, 483–493.

20. BARBOUR, M.G. AND BAYLY, R.C. Regulation of the meta-cleavage of 4-hydroxyphenylacetic acid by *Pseudomonas putida*, *Biochem. Res. Commun.*, 1977, **76**, 565–571.

21. BLAKELY, E.R., HALVARSON, H. AND KURZ, W. The microbial production and some characteristics of δ-carboxymethyl-α-hydroxymuconic semialdehyde, *Can. J. Microbiol.*, 1967, **13**, 159–165.

22. COOPER, R.A. AND SKINNER, M. A. Catabolism of 3-and 4-hydroxyphenylacetate by the 3,4-dihydroxyphenylacetate pathway in *Escherichia coli*, *J. Bacteriol.*, 1980, **143**, 302–306.

23. SPARNINS, V.L., CHAPMAN, P.J. AND DAGLEY, S. Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid, *J. Bacteriol.*, 1974, **120**, 159–167.

24. ENGELHARDT, G., RAST, H.G. AND WALLNÖFER, P. R. Cometabolism of phenol and substituted phenols by *Nocardia* spec. DSM 43251, *FEMS Microbiol. Lett.*, 1979, **5**, 377–383.

25. HARADA, T. Phenolsulfatase synthesis with tyramine as an inducer by *A. aerogenes*, *Bull. Agric. Chem. Soc. Jap.*, 1957, **21**, 267–273.

26. GUNTER, S.E. The enzymatic oxidation of *p*-hydroxymandelic acid to *p*-hydroxybenzoic acid, *J. Bacteriol.*, 1953, **66**, 341–346.

27. STANIER, R.Y The oxidation of aromatic compounds by fluorescent Pseudomonads, *J. Bacteriol.*, 1948, **55**, 477–494.

28. ROTHERA, A.C.H. Note on the sodium nitroprusside reaction for acetone, *J. Physiol.*, 1908, **37**, 491–494.