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# Preparation of 2-aminomuconate from 2-aminophenol by coupled enzymatic dioxygenation and dehydrogenation reactions

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**2-Aminomuconate is an intermediate in the oxidative metabolism of tryptophan in mammals. The compound is not commercially available, and studies of its metabolism have been prevented by the lack of a chemical synthesis and the instability of the molecule. We report here the formation of 2-aminomuconate from 2-aminophenol by the coupled action of 2-aminophenol 1,6-dioxygenase and 2-aminomuconic semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45, and isolation of the product by anion exchange chromatography. The overall procedure was completed within 3 h with a yield of 62%. The availability of the dicarboxyl $\alpha$-amino acid provides the basis for investigation of the physiological function of 2-aminomuconate in the neuropathologically significant oxidative metabolism of tryptophan.**

**Keywords:** biocatalysis; 2-aminomuconate; 2-aminophenol; *Pseudomonas*; dioxygenase; dehydrogenase

## Introduction

Interest in the catabolic pathway of degradation of l-tryptophan has grown steadily over the last decade due to the role of quinolinate and other metabolites in several neuropathological conditions [1,2,7,8,10,14,16]. The neurotoxin, quinolinate, is formed nonenzymatically from 2-amino-3-carboxymuconic semialdehyde in mammalian tissues. 2-Amino-3-carboxymuconic semialdehyde is enzymatically converted to 2-aminomuconate (2-aminohexa-2,4-diene-1,6-dioate), via 2-aminomuconic semialdehyde (Figure 1). The effect of the enzymatic pathway on non-enzymatic accumulation of quinolinate in the central nervous system has not been investigated [7]. Furthermore, 2-aminomuconate itself may also have a significant neurophysiological function in the central nervous system by virtue of the fact it is a dicarboxylic $\alpha$-amino acid, and is similar to other neural excitatory amino acid agonists or antagonists [3]. Another concern is the neurotoxicity of ammonia, released from 2-aminomuconate during the metabolism of tryptophan in the central nervous system. The lack of the availability of 2-aminomuconate has severely limited experimental approaches to investigation of these aspects of mammalian neurophysiology. For instance, Yokoi *et al* [18] examined the effect of kynurenine metabolites administered into the right cerebroventricle on the electrocorticogram of rats to establish the role of the metabolites in brain function. The metabolites included 2-aminophenol, picolinic acid (spontaneously produced from 2-aminomuconic semialdehyde), and 2-ketoadipic acid (produced enzymatically from 2-aminomuconate). But the effect of 2-aminomuconate was not reported.

Recently, we have found that 2-aminomuconate is one of the intermediates in the pathway for the biodegradation of nitrobenzene by the bacterium *Pseudomonas pseudoalcaligenes* JS45 [6]. 2-Aminomuconate is produced from 2-aminophenol by the action of 2-aminophenol 1,6-dioxygenase and 2-aminomuconic semialdehyde dehydrogenase [4,6]. We have prepared 2-aminomuconate with the two enzymes either in crude extracts or in the fractions from a DEAE-Sepharose column, and separated it by anion exchange chromatography for use in investigating the properties of 2-aminomuconate deaminase from *P. pseudoalcaligenes* JS45 [5,6]. However, attempts to prepare the material in large quantities for general use failed because the partially purified dioxygenase was unstable even during storage at −70°C. In crude extracts the dioxygenase was relatively stable, but the presence of 2-aminomuconate deaminase precluded accumulation of 2-aminomuconate. We report here an improved method for the preparation of 2-aminomuconate by combination of the 2-aminophenol 1,6-dioxygenase from an *E. coli* clone and the dehydrogenase from *P. pseudoalcaligenes* JS45.

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## Materials and methods

### Bacterial strains and growth conditions

*Pseudomonas pseudoalcaligenes* JS45 was grown in BLKN medium with nitrobenzene at 30°C [12]. Plasmid pNBZ14 containing the 2-aminophenol 1,6-dioxygenase gene of *P. pseudoalcaligenes* JS45 inserted into pUC18 was provided by John Davis, Tyndall AFB, FL, USA (unpublished). *E. coli* DH5$\alpha$/pNBZ14 was grown in Luria broth (Difco, Detroit, MI, USA) containing 50 $\mu$g ml$^{-1}$ ampicillin at 37°C. Cells were harvested by centrifugation, washed with 25 mM potassium phosphate (pH 7.0), and stored at −70°C until used.

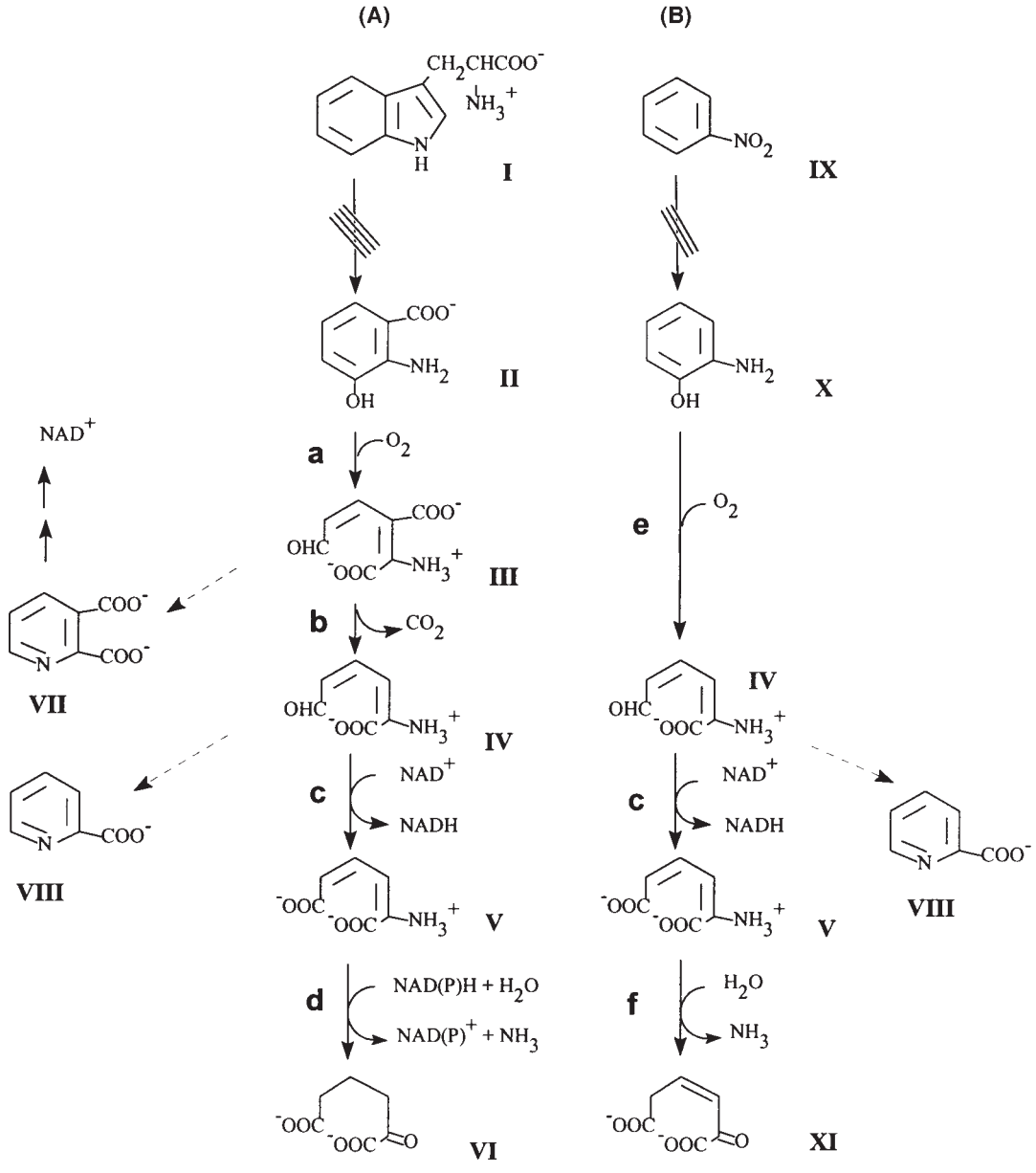


**Figure 1** Pathways involved in formation of 2-aminomuconate in l-tryptophan degradation in mammals (A) and in degradation of nitrobenzene in the bacterium *Pseudomonas pseudoalcaligenes* JS45 (B). I, tryptophan; II, 3-hydroxyanthranilate; III, 2-amino-3-carboxymuconic semialdehyde; IV, 2-aminomuconic semialdehyde; V, 2-aminomuconate; VI, 2-ketoadipate; VII, quinolinate; VIII, picolinate; IX, nitrobenzene; X, 2-aminophenol; XI, 2-oxohex-3-ene-1,6-dioate. a, dioxygenase; b, decarboxylase; c, dehydrogenase; d, reductase; e, dioxygenase; f, deaminase. Dotted arrow, spontaneous reaction.

### *Preparation of enzymes*

Partially purified 2-aminomuconic semialdehyde dehydrogenase was obtained from *P. pseudoalcaligenes* JS45 as reported previously [5]. Cells of *E. coli* DH5$\alpha$/pNBZ14 expressing 2-aminophenol 1,6-dioxygenase were suspended in phosphate buffer (25 mM, pH 7.0) and broken by two passages through a French pressure cell at 20 000 p.s.i. The suspension was centrifuged at $20\,000 \times g$ for 30 min and the pellets were discarded. The crude extract was stored at –70°C. The crude extracts or a fraction that precipitated between 50% and 70% ethanol [11] was used as the source of 2-aminophenol 1,6-dioxygenase.

### *Preparation and isolation of 2-aminomuconate*

In a total of 8 ml, the initial reaction mixture contained 10 mM potassium phosphate buffer (pH 7.5), crude extracts of pNBZ14 (0.3 mg protein), partially purified 2-aminomuconic semialdehyde dehydrogenase (0.3 mg protein), and $NAD^+$ (1.6 $\mu$mol). The reaction was initiated by the addition of 2-aminophenol. A total of 3.2 $\mu$mol of 2-aminophenol was added slowly over 20 min. In the same time 1.5 mg more crude extract of pNBZ14 was also added in three aliquots. The NADH oxidase activity in the crude extract of pNBZ14 replenished the $NAD^+$ reduced during the course of the reaction. The reaction was completed in

20 min. The final volume of the reaction mixture was 8.2 ml.

The reaction mixture was diluted to 24 ml with Tris-HCl buffer (final concentration: 10 mM, pH 9.5). The mixture was passed through a Centriprep-3 tube (Amicon, Beverly, MA, USA) to remove proteins. The filtrate was loaded on a Hitrap Q column (two 5-ml columns in series, Pharmacia, Uppsala, Sweden); the column was washed with 30 ml of 10 mM Tris-HCl buffer (pH 9.5) and 2-aminomuconate was eluted with a 100-ml linear gradient of 0–0.3 M NaCl in the same buffer. 2-Aminomuconate was eluted in fractions 14 and 15 (5.0 ml each).

### *Chemicals*

All chemicals were from Sigma (St Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) unless stated otherwise.

## Results and discussion

### *Instability of 2-aminomuconic semialdehyde*

2-Aminomuconic semialdehyde is reported to be unstable and spontaneously converted to picolinic acid [9,11,12,15,17]. The extent of the instability has not been investigated. A molar absorbance coefficient of 2-aminomuconic semialdehyde was previously estimated to be 15.1 $mM^{-1}$ $cm^{-1}$ at 380 nm [11,12] and the production of 2-aminomuconic semialdehyde, as measured by the increase in $A_{380}$, was used to determine the $K_m$ of 2-aminophenol 1,6-dioxygenase for 2-aminophenol. In the present study, the time course of formation of 2-aminomuconic semialdehyde from 2-aminophenol catalyzed by 2-aminophenol 1,6-dioxygenase revealed that the maximum absorbance at 380 nm increased with increasing enzyme concentration (Figure 2). At high enough concentrations, the maximum absorbance was reached almost immediately after the addition of 2-aminophenol and there was no plateau of absorbance. The observation indicated that 2-aminomuconic semialdehyde was extremely unstable, and it is not appropriate to determine the dioxygenase activity by the formation of the product. The maximum absorbance at 380 nm did not vary substantially from pH 7.0 to 8.0 in 25 mM potassium phosphate or from pH 7.0 to 8.8 in 25 mM Tris-HCl. The molar absorbance coefficient of 2-aminomuconic semialdehyde which we calculated from the maximum absorbance with excess dioxygenase was $25.9 \pm 0.8$ $mM^{-1}$ $cm^{-1}$ (mean ± standard deviation, $n = 7$) at 380 nm.

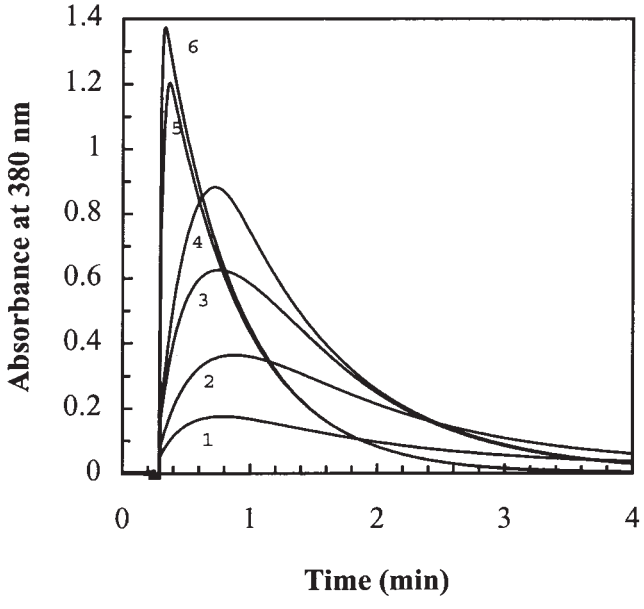


**Figure 2** The time course of formation of 2-aminomuconic semialdehyde. The reaction was initiated by addition of 0.05 $\mu$mol of 2-aminophenol in 1 ml of reaction mixture (50 mM potassium phosphate, pH 7.5) at about 15 s. The partially purified 2-aminophenol 1,6-dioxygenase was added in the amounts of 2.8, 5.6, 11.2, 22.4, 56, and 112 $\mu$g of protein from curves 1–6 in order.

### *Properties of 2-aminomuconate.*

Because of the instability of 2-aminomuconic semialdehyde, we rechecked the molar extinction coefficient of 2-aminomuconate with excess 2-aminomuconic semialdehyde dehydrogenase to prevent the extremely fast spontaneous conversion of 2-aminomuconic semialdehyde to picolinic acid. With a dioxygenase activity of about 0.024 $\mu$mol $min^{-1}$ and a dehydrogenase activity of 5.3 $\mu$mol $min^{-1}$ in a 1-ml assay, only the peak at 326 nm was observed but no transient absorbance at 380 nm appeared. The results indicated that 2-aminomuconic semialdehyde did not accumulate, but was immediately transformed to 2-aminomuconate (data not shown). The molar extinction coefficient of the 2-aminomuconate thus formed was $17.8 \pm 0.4$ $mM^{-1}$ $cm^{-1}$ (mean ± standard deviation, $n = 4$) at 326 nm in 50 mM potassium phosphate buffer (pH 7.5), slightly higher than the previously reported value of 16.5 $mM^{-1}$ $cm^{-1}$ [9,13]. The wavelength of maximum absorbance of 2-aminomuconate as well as the molar extinction coefficient did not change over a pH range of 6.5–13 (6.5–8.0 in 50 mM potassium phosphate, 7.0–9.5 in 50 mM Tris-HCl, and 13 in 0.1 M KOH). 2-Aminomuconate is more stable at higher pH (Figure 3). The half-life changed from 30 min at pH 6.5 to 290 min at pH 9.5. At

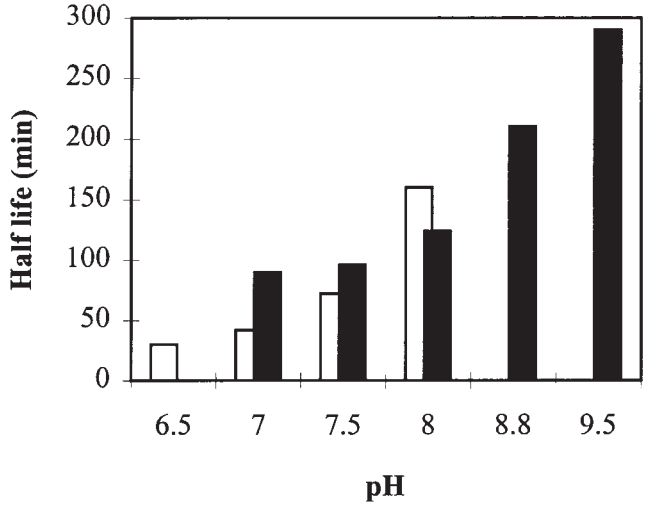


**Figure 3** Influence of pH on spontaneous hydrolysis of 2-aminomuconate. The concentration of 2-aminomuconate was 0.034 mM. Light bars represent 50 mM potassium phosphate buffer, and dark bars 50 mM Tris-HCl buffer.

lower pH, a rapid spontaneous deamination of 2-aminomuconate was accompanied by a shift of the maximum absorbance to a shorter wavelength. The amino group was also lost when the 2-aminomuconate solution was boiled at neutral pH. The previously reported absorbance shifts of 2-aminomuconate from 325 nm at pH 8.0 to 333 nm at pH 13 [6,9] seems to have been a result of trace contamination with the product of deamination, 4-oxalocrotonate, which has a maximum absorbance at 350 nm under alkaline conditions.

### *Preparation and isolation of 2-aminomuconate*

The crude extracts of pNBZ14 and partially purified dehydrogenase were used to prepare 2-aminomuconate. 2-Aminophenol 1,6-dioxygenase has a broad pH optimum from 7–9 [11], whereas 2-aminomuconic semialdehyde dehydrogenase has optimum activity around pH 7.3 in phosphate buffer, and the activity was dramatically decreased when the pH of the buffer was higher than 8. Therefore, we carried out the reactions leading to the formation of 2-aminomuconate at pH 7.5 and isolation of the product was conducted at pH 9.5. To reduce the spontaneous conversion of the intermediate semialdehyde to picolinate, 2-aminophenol was added into the reaction mixture slowly, and the starting 2-aminophenol 1,6-dioxygenase concentration was low. With the protocol described in the Materials and Methods, 2.7 $\mu$mol of 2-aminomuconate was produced from 3.2 $\mu$mol of 2-aminophenol. The total amount of 2-aminomuconate after purification by Hitrap-Q column was 2.0 $\mu$mol, indicating an overall yield of 62%. The elution profile and the spectrum of fraction 15 (diluted 1/10) indicated that 2-aminomuconate was pure (Figure 4). The concentration of 2-aminomuconate in fraction 15 was 0.34 mM. 2-Aminomuconate was relatively stable at such concentrations, the half-life was 20 h at room temperature, and only 30% was lost when the solution was stored on ice for 50 h. Freezing and thawing of the solution at pH 9.5 (Tris-HCl) resulted in decomposition of about 2% of the material, whereas 50% was lost after similar treatment at pH 8.0 in potassium phosphate buffer [5].

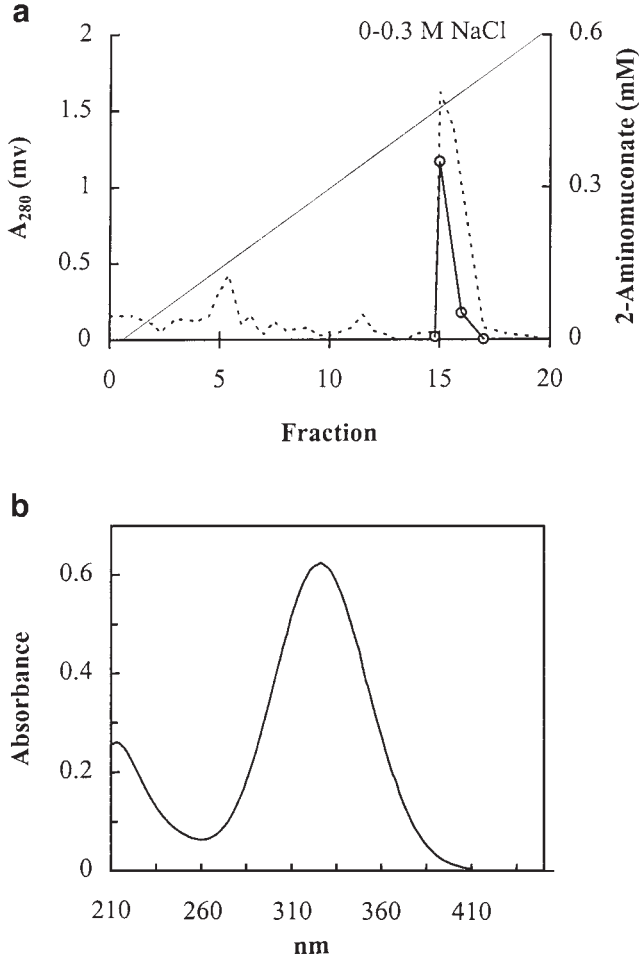


**Figure 4** Elution profile of 2-aminomuconate from Hitrap Q column (a) and the spectrum of 2-aminomuconate (b). The dotted curve in panel (a) indicates the absorbance of fractions at 280 nm. Open circles indicate the concentration of 2-aminomuconate. The spectrum in panel (b) was obtained by addition of 0.1 ml of fraction 15 to 0.9 ml 10 mM Tris-HCl (pH 9.5).

The procedure presented here seems suitable to provide 2-aminomuconate for general research. The advantage of using crude extracts rather than partially purified 2-aminophenol 1,6-dioxygenase is that the dioxygenase in crude extracts is stable during storage. The high dioxygenase activity in crude extracts also eliminated the requirement for ferrous salt and ascorbate [11], which may introduce impurities in the 2-aminomuconate solution. In addition, the crude extract contained NADH oxidase activity, which not only replenished $NAD^+$, but also reduced the interference of NADH, which eluted just ahead of 2-aminomuconate during anion exchange chromatography.

The solution of 2-aminomuconate is sufficiently stable to allow shipment and storage frozen. Alternatively, a two-enzyme preparation and a small anion exchange column as well as related chemicals could be supplied as a kit to produce the chemical immediately prior to use. The availability of 2-aminomuconate should facilitate research on the metabolism and physiological functions of 2-aminomuconate in mammalian tissues, especially in the central nervous systems.

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## References

1 Blight A, EC Leroy, Jr and MP Heyes. 1997. Quinolinic acid accumulation in injured spinal cord: time course, distribution, and species differences between rat and guinea pig. J Neurotrauma 14: 89–98.
2 Ceresoli G, P Guidetti and R Schwarcz. 1997. Metabolism of [5-$^3$H]kynurenine in the developing rat brain *in vivo*: effect of intrastriatal ibotenate injections. Dev Brain Res 100: 73–81.
3 Collingridge GL and RAJ Lester. 1989. Excitory amino acid receptors in the vertebrate central nervous system. Pharmacol Rev 40: 143–210.
4 He Z, JK Davis and JC Spain. 1998. Purification, characterization, and sequence analysis of 2-aminomuconic 6-semialdehyde dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. J Bacteriol 180: 4591–4595.

5 He Z and JC Spain. 1998. A novel 2-aminomuconate deaminase in the nitrobenzene degradation pathway of *Pseudomonas pseudoalcaligenes* JS45. J Bacteriol 180: 2502–2506.
6 He Z and JC Spain. 1997. Studies of the catabolic pathway of degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45: removal of the amino group from 2-aminomuconic semialdehyde. Appl Environ Microbiol 63: 4839–4843.
7 Heyes MP, CY Chen, EO Major and K Saito. 1997. Different kynurenine pathway enzymes limit quinolinic acid formation by various human cell types. Biochem J 326: 351–356.
8 Heyes MP, K Saito, CY Chen, MG Proescholdt, TS Nowak, Jr, J Li, KE Beagles, MA Proescholdt, MA Zito, K Kawai and SP Markey. 1997. Species heterogeneity between gerbils and rats: quinolinate production by microglia and astrocytes and accumulations in response to ischemic brain injury and systemic immune activation. J Neurochem 69: 1519–1529.
9 Ichiyama A, S Nakamura, H Kawai, T Honjo, Y Nishizuka, O Hayashi and S Senoh. 1965. Studies on the metabolism of the benzene ring of tryptophan in mammalian tissues. II. Enzymic formation of $\alpha$-aminomuconic acid from 3-hydroxyanthranilic acid. J Biol Chem 240: 740–749.
10 Kerr SJ, PJ Armati, LA Pemberton, G Smythe, B Tattam and BJ Brew. 1997. Kynurenine pathway inhibition reduces neurotoxicity of HIV-1-infected macrophages. Neurology 49: 1671–1681.
11 Lendenmann U and JC Spain. 1996. 2-Aminophenol 1,6-dioxygenase: a novel aromatic ring-cleavage enzyme purified from *Pseudomonas pseudoalcaligenes* JS45. J Bacteriol 178: 6227–6232.
12 Nishino SF and JC Spain. 1993. Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. Appl Environ Microbiol 59: 2520–2525.
13 Nishizuka Y, A Ichiyama and O Hayaishi. 1970. Metabolism of the benzene ring of tryptophan (mammals). Meth Enzymol 17A: 463–491.
14 Okuda S, N Nishiyama, H Saito and H Katsuki. 1996. Hydrogen peroxide-mediated mediated neuronal cell death induced by an endogenous neurotoxin, 3-hydroxykynurenine. Proc Natl Acad Sci USA 93: 12553–12558.
15 Que L Jr. 1978. Extradiol cleavage of *o*-aminophenol by pyrocatechase. Biochem Biophys Res Commun 84: 123–129.
16 Sanni LA, SR Thomas, BN Tattam, DE Moore, G Chaudhri, R Stocker and NH Hunt. 1998. Dramatic changes in oxidative tryptophan metabolism along the kynurenine pathway in experimental cerebral and noncerebral malaria. Am J Pathol 152: 611–619.
17 Takenaka S, S Murakami, R Shinke, K Hatakeyama, H Yukawa and K Aoki. 1997. Novel genes encoding 2-aminophenol 1,6-dioxygenase from *Pseudomonas* species AP-3 growing on 2-aminophenol and catalytic properties of the purified enzyme. J Biol Chem 272: 14727–14732.
18 Yokoi I, Y Nishijima, A Uchida, H Kabuto, N Yamamoto and N Ogawa. 1998. Effects of kynurenine metabolites on the electrocorticographic activity in the rat. J Neural Transm 105: 147–160.