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The Oxidation of L-Amino Acids by *Mytilus edulis*

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It is now well established that amino acid oxidases are widely distributed in molluscs. The cephalopod liver contains not only a general D-amino acid oxidase (Blaschko & Hawkins, 1951), but probably also a second oxidase that acts upon D-aspartic and D-glutamic acids (Blaschko & Himms, 1955); D-amino acid oxidase also occurs in the digestive gland of *Helix*.

When other molluscan species were examined, it was found that the digestive gland of *Mytilus edulis* also had amino acid-oxidase activity: DL-methionine, DL-α-aminobutyric acid, DL-norvaline and DL-norleucine were found to be oxidized (Blaschko & Hawkins, 1952). In the same year, Roche, Thoai & Glahn (1952) described an enzyme in the digestive gland of *Mytilus* that oxidized L-arginine and a few other amino acids. Since in the earlier experiments with *Mytilus* (Blaschko & Hawkins, 1952) all the compounds tested were racemic mixtures, it seemed desirable to find out whether the *Mytilus* oxidase had acted upon the D- or the L-forms of the racemic amino acids studied.

In the experiments described below, no evidence for the occurrence of a D-amino acid oxidase in *Mytilus* was obtained. On the other hand, many L-amino acids were oxidized. The substrate specificity of the *Mytilus* enzyme shows characteristic differences from other L-amino acid oxidases that are described in the literature.

MATERIAL AND METHODS

Some of the preliminary experiments were carried out at Plymouth, where freshly caught animals were used. Most of the work was done in Oxford, on specimens sent from Plymouth. The animals were dissected soon after arrival; only animals that appeared to be vigorous were used.

In *Mytilus*, the digestive gland contains parts of the intestine which cannot be separated by dissection. The dissected tissue was weighed, frozen in a mortar, cooled to −10° and then thoroughly ground at room temperature. To each gram of fresh tissue 2 ml. of 0·067 M-sodium phosphate buffer at pH 7·15 was added to give a crude suspension.

In the experiments on substrate specificity, the crude suspension was centrifuged at 3000 *g* for 1 hr. at 0°. The sediment was resuspended in the original volume of phosphate buffer and recentrifuged. The sediment was again suspended in the phosphate buffer and dialysed against the same buffer for 24 hr. at 3°. This procedure gave preparations with a satisfactory enzymic activity and a relatively low oxygen consumption in the 'enzyme blank'.

For the manometric determination of enzymic activity 1 ml. of the *Mytilus* preparation was used in the main compartment of a conical flask, together with 0·6 ml. of the phosphate buffer. The inner tube contained a filter paper and 0·3 ml. of N-KOH, the side bulb 0·4 ml. of either water ('enzyme blank') or of a solution containing 10 μmoles of the L-amino acid to be tested, so as to make the initial substrate concentration 0·005 M. Some of the amino acids were not sufficiently soluble; these were prepared as a suspension of the solid material finely ground in a mortar. This was necessary with cystine (L-, *meso*- and DL-cystine), with DL-homocystine, with DL-α-aminocaprylic acid and with DL-tyrosine. When DL-amino acids were used each flask contained 20 μmoles of the acid.

Unless otherwise stated, the material was obtained from commercial sources. We are indebted to Dr Elizabeth Work for the samples of αα′-diaminopimelic acid and to Professor H. A. Krebs for a number of amino acids prepared by Elks, Hems & Ryman (1948); Dr E. P. Abraham gave us a sample of DL-α-aminoadipic acid prepared by Gaudry (1949).

In the preparation of L-canavanine from Jack beans we followed the advice of Dr E. E. Snell and used in the purification procedure a column of Dowex 50 (×8, 200–400 mesh) in its H form. Crystalline L-canavanine acid sulphate was readily obtained upon seeding with a few crystals given by Dr H. Kihara. Another sample of L-canavanine sulphate was kindly given by Dr E. A. Bell and Professor W. R. Fearon. The total yield of analytically pure L-canavanine acid sulphate from 1 kg. of whole Jack beans was 10·3 g.

In the manometric experiments, the gas phase was O_2 and the temperature 25°. The time course of the oxidation was followed for at least 0·5 hr., but often for much longer, until the enzymic reaction had gone to completion. In most experiments, one flask contained L-arginine as substrate and the initial rate of oxidation of the other amino acids was expressed as a percentage of that of L-arginine. During the initial period, the rate of oxidation was constant.

RESULTS

Properties of the oxidase

In the preliminary studies a crude preparation of the gland was used, in which the tissue had been ground in a mortar with sand. It was soon noted that the enzyme was associated with insoluble material which could easily be spun down on the centrifuge. This was demonstrated in an experiment with a homogenate of the gland tissue in 0·32-molal sucrose, prepared in a glass homogenizer; 5 g. of fresh tissue were used, and the final vol. of the homogenate was 24 ml. This homogenate was centrifuged at 0° and 950 *g* for 20 min. The sediment was resuspended in sucrose so that the final volume was that of the original homogenate. When 1·0 ml. of both supernatant fluid and resuspended sediment were tested with 0·01M L-arginine at pH 7·15, the amounts of oxygen (μl.) consumed in 1 hr. were:

	Without arginine	With arginine	Diff.
Supernatant	32	41	9
Resuspended sediment	16	97	81

In other words, nine-tenths of the enzymic activity was found in the sediment and one-tenth in the supernatant.

An attempt was made to obtain a soluble preparation of enzyme by using a supersonic disintegrator, but even after treatment for 30 min. with vibrations of 25 kc./sec. generated by a 600 w Mullard supersonic generator, the supernatant fluid after high-speed centrifuging was without enzymic activity; most of the activity was recovered in the sediment. It follows that the enzyme is present in a structural element which is readily sedimented and not easily disintegrated.

Substrates of the oxidase

The relative rates of oxidation of the different amino acids tested, in comparison with L-arginine, are shown in Table 1.

Aliphatic monobasic monocarboxylic acids. A number of straight-chain amino acids were found to be oxidized. With L-alanine, the rate of oxygen consumption was very low, but there was an increase with α-amino-*n*-butyric acid, α-aminovaleric acid (norvaline) and α-aminocaproic acid (norleucine, 2-aminohexanoic acid); with norleucine the opti-

Table 1. *Rate of oxidation of amino acids by the* L-*amino acid oxidase of the digestive gland of* Mytilus edulis

The rate of oxidation is expressed in terms of percentage of the rate of oxidation of L-arginine. In these experiments the mean oxygen uptake in 12 min. of the 'enzyme blank' was 3·7 μl. (1·5–6·5) and the total oxygen uptake in the presence of L-arginine was 80·8 μl. (57–98) in the same period.

Substrate	Rate of oxidation
Aliphatic monocarboxylic monoamino acids	
DL-Alanine	4
DL-α-Aminobutyric acid	68
DL-Norvaline	86
DL-Norleucine	114
DL-α-Aminocaprylic acid	35
L-Valine	0
L-Isoleucine	0
L-Leucine	73
DL-Serine	0
DL-Threonine	0
Monocarboxylic diamino acids and other basic amino acids	
DL-αγ-Diaminobutyric acid	0
L-Ornithine	123
L-Lysine	125
DL-Hydroxylysine (synthetic)	28
L-Citrulline	114
L-Arginine	100
L-Canavanine	100
L-Histidine	80
Aromatic and heterocyclic amino acids	
L-Tryptophan	60
DL-5-Hydroxytryptophan	19
L-Proline	0
L-Phenylalanine	108
L-Tyrosine	110
Dicarboxylic diamino acids	
meso-αα′-Diaminopimelic acid	21
LL-αα′-Diaminopimelic acid	21
Sulphur-containing amino acids	
L-Cystine	38
meso-Cystine	40
DL-Homocystine	31
L-Cysteic acid	0
DL-Homocysteic acid	0
L-Cysteinesulphinic acid	0
L-Methionine	151
DL-Ethionine	97
LL-Djenkolic acid	40
Cystathionine (synthetic)	63
Dicarboxylic monoamino acids and amides	
L-Aspartic acid	0
L-Glutamic acid	0
DL-α-Aminoadipic acid	18
DL-α-Aminopimelic acid	19
DL-1-Aminoundecane-1:11-dicarboxylic acid	20
L-Asparagine	49
L-Glutamine	29

mum chain length seems to have been reached, and α-aminocaprylic acid (2-amino-octanoic acid) was more slowly oxidized. This is similar to the findings of Bender & Krebs (1950) on the cobra-venom oxidase; this enzyme also oxidizes norleucine at an optimal rate.

The results with α-aminobutyric acid, norvaline and norleucine confirm earlier observations from these Laboratories (Blaschko & Hawkins, 1952).

Of the amino acids with a branched chain, L-leucine was readily oxidized, but neither L-valine nor L-isoleucine was oxidized. This is reminiscent of the specificity pattern of the cobra-venom enzyme (Bender & Krebs, 1950). The cobra venom, as well as the *Mytilus* enzyme, appears not to act upon amino acids in which the two hydrogen atoms in the β-position are substituted.

Monocarboxylic diamino acids and other basic amino acids. Of the straight-chain aliphatic series, DL-αγ-diaminobutyric acid was not attacked, but the observation of Roche *et al.* (1952) on the oxidation of L-ornithine was confirmed; L-lysine was also readily oxidized.

L-Arginine is the substrate chiefly studied by Roche *et al.* (1952). Of a number of related compounds, L-canavanine was found to be oxidized as rapidly as arginine. The oxidation of L-canavanine has been studied in greater detail.

For the study of the oxidation product derived from L-canavanine, a dialysed washed suspension of *Mytilus* digestive gland was used, which had been prepared in distilled water instead of the phosphate buffer used in all other experiments.

Two experiments were carried out with this preparation. In the first, a manometric experiment with 0·005M L-canavanine as substrate, the reaction came to a standstill in 30 min. The supernatant from this incubation was used for a chromatographic analysis. This showed that all the canavanine had disappeared and that a new compound had been formed which gave an orange-brown colour with the sodium pentacyanoaminoferrate reagent for guanidinoxy compounds described by Fearon & Bell (1955); canavanine gives a magenta colour with this reagent. For the descending chromatography, phenol saturated with water was used in an atmosphere saturated with NH_3. At $24 \pm 1°$ the R_F of the reaction product, presumably α-oxo-δ-guanidinoxybutyric acid, was 0·83, and that of canavanine 0·68.

A parallel experiment was set up with the same enzyme preparation for the isolation of a reaction product as its 2:4-dinitrophenylhydrazone. An extract from 9 g. of fresh tissue was incubated at 25° for 45 min. with 68·5 mg. of L-canavanine acid sulphate, neutralized with NaOH. The total volume of the reaction mixture was 50 ml.; a 250 ml. Büchner flask was used which was agitated in the manometer bath while oxygen was passed through, just above the surface of the liquid. The reaction mixture was centrifuged at 3000 *g* for 30 min. at 0° and the supernatant was filtered through a filter paper. A portion (10 ml.) of 1 % 2:4-dinitrophenylhydrazine in 2N-HCl was added. The mixture was left in the refrigerator at 2° overnight. On the next day an orange-coloured precipitate had separated. This was washed with water and dried. The crude *α-oxo-δ-guanidinoxybutyric acid* 2:4-*dinitrophenylhydrazone* had m.p. 212°. (Found: C, 37·6; H, 4·1. $C_{11}H_{13}O_7N_7$ requires C, 37·2; H, 3·7 %.) Attempts to recrystallize the product brought about some decomposition, as shown by a lowered melting point.

Heterocyclic compounds. The oxidation of L-tryptophan by the *Mytilus* enzyme is in agreement with findings by Roche *et al.* (1952); we have already reported that DL-5-hydroxytryptophan is also readily oxidized (Blaschko & Hope, 1955). The oxidation of both tryptophan and 5-hydroxytryptophan leads to the formation of a brown pigment; this is reminiscent of the pigment formation which occurs when tryptamine or 5-hydroxytryptamine is oxidized by amine oxidase (Pugh & Quastel, 1937; Blaschko & Hellmann, 1953).

Dicarboxylic diamino acid. Apart from the sulphur-containing amino acids, which are discussed below, αα′-diaminopimelic acid was examined. Three forms of the acid were tested: the unresolved synthetic compound, the LL-form and the *meso*-form. All three were readily oxidized; however, the initial rate of oxidation was not maintained, so that an accurate measurement of the total amounts of oxygen consumed could not be made.

Sulphur-containing amino acids. Of the substrates examined by Blaschko & Hawkins (1952), methionine was the only one of which the D- and the L-forms were available. L-Methionine was very rapidly oxidized. With the D-form, there was a small oxygen uptake, which soon came to a standstill. That this oxygen uptake was due to a contamination of the sample with a small amount of the L-form is supported by the observation that a similar, small uptake of oxygen occurred when the sample was incubated with cobra venom.

L-Cysteic acid was not oxidized by the *Mytilus* preparation.

All the dicarboxylic amino acids of this group that were tested were found to be substrates; these were cystine (LL- and *meso*-form), homocystine, djenkolic acid and cystathionine.

Dicarboxylic monoamino acids and their amides. Neither L-aspartic acid nor L-glutamic acid was a substrate, but their amides were found to be oxidized.

The three long-chain dicarboxylic amino acids, DL-α-aminoadipic (C_6), DL-α-aminopimelic (C_7) and DL-1-aminoundecane-1:11-dicarboxylic (C_{13}) acids, were readily oxidized, all at approximately the same rate.

Total oxygen uptake

The total amount of oxygen taken up was not determined in every experiment, but with many of the substrates tested the reaction was followed to completion. With L-arginine and with many other substrates, the total amount of oxygen taken up was close to the theoretical, i.e. one-half molecule of O_2 for one molecule of L-amino acid added. Tryptophan consistently gave oxygen uptakes higher than the theoretical amount.

Other species

No significant increase over the blank oxygen consumption was observed when preparations of the digestive glands of *Pecten maximus* and of *Mya arenaria* were incubated with L-arginine, L-methionine, L-phenylalanine, L-leucine and L-valine. With a preparation from the digestive gland of *Cyprina islandica* the amounts of oxygen taken up during the first 30 min. of incubation were: without substrate, 5·5 μl.; with 0·01 M L-arginine, 14·5 μl.; with 0·01 M L-leucine, 16 μl.; with 0·01 M L-methionine, 28 μl. It seems therefore that *Cyprina islandica* contains an enzyme similar to that of *Mytilus*, but the preparation is less active.

The presence of a D-amino acid oxidase in the digestive gland of *Helix aspersa* (Blaschko & Hawkins, 1952) was confirmed. An experiment was performed with a preparation of this gland, prepared as described for *Mytilus*. The incubation was carried out at 37·5°, and each manometric flask contained the equivalent of 533 mg. of fresh tissue. The oxygen consumed in the first 10 min. was: without substrate, 15 μl.; with 0·01 M L-leucine, 17·5 μl.; with 0·01 M D-leucine, 104 μl.; with 0·01 M L-methionine, 17 μl.; with 0·01 M D-methionine, 112 μl.

DISCUSSION

The experiments reported in this paper show that the specificity of the *Mytilus* enzyme differs from that of the cephalopod enzyme and that of *Helix aspersa*: the *Mytilus* enzyme is a L-amino acid oxidase. Methionine was the only one of the four substrates tested by Blaschko & Hawkins (1952), of which both the D- and the L-forms were available. Only the L-form was oxidized at a significant rate. It seems likely that the enzyme responsible for the oxidation is identical with the L-arginine oxidase described by Roche *et al.* (1952), but this has not been fully investigated.

The study of the substrate specificity of the *Mytilus* enzyme has shown a number of analogies to that of other known oxidases of this group, but there are also differences. Many basic amino acids were oxidized, among them L-lysine and L-canavanine. Of the basic amino acids tested, only αγ-diaminobutyric acid was not oxidized.

The relative rates of oxidation of the straight-chain monoamino acids are reminiscent of the specificity pattern of the cobra-venom enzyme; this enzyme also has an optimum for α-aminocaproic acid (Bender & Krebs, 1950). Of the three branched amino acids tested L-leucine was the only one oxidized; this, too, is a similarity between the *Mytilus* and the cobra-venom enzyme. However, the cobra-venom enzyme oxidizes neither ornithine nor lysine.

The L-amino acid oxidase of *Neurospora crassa* studied by Bender & Krebs (1950) and by Thayer & Horowitz (1951) is in many ways similar to the *Mytilus* enzyme; it also acts on basic amino acids, including canavanine. However, there are differences: L-isoleucine is oxidized by the *Neurospora* enzyme.

Only one of the dicarboxylic diamino acids which did not contain sulphur was oxidized; this is αα′-diaminopimelic acid. This amino acid is known to be also a substrate of the L-amino acid oxidases of both *Neurospora crassa* and snake venoms (Work, 1955). Of the sulphur-containing amino acids of this group, all (cystine, homocystine, djenkolic acid and cystathionine) were found to be oxidized. L-Cystathionine is also oxidized by the *Neurospora* enzyme (Thayer & Horowitz, 1951).

The experiments reported raise the question as to the functional significance of the L-amino acid oxidase in *Mytilus*. It seems possible that in this species oxidative deamination may be an important pathway of degrading L-amino acids. However, as the digestive gland of *Mytilus* is in such intimate connexion with the lumen of the alimentary tract, the possibility that the oxidase is a bacterial enzyme cannot be fully excluded; Florkin (1952) has suggested that the cellulase found in molluscs is a bacterial enzyme.

In connexion with the localization of the *Mytilus* oxidase, it is of interest that the enzymic activity is so readily spun down by centrifuging. This is a property in which the *Mytilus* enzyme differs from all other amino acid oxidases hitherto described.

SUMMARY

1. The amino acid oxidase of the digestive gland of *Mytilus edulis* has been studied; it is shown that this enzyme acts upon L-amino acids.

2. The study of the substrate specificity of this enzyme shows that many amino acids are oxidized.

3. Earlier observations on the occurrence of a D-amino acid oxidase in *Helix aspersa* are confirmed.

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Studies in Detoxication

68. THE METABOLISM OF [^{14}C]NITROBENZENE IN THE RABBIT AND GUINEA PIG*

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In the previous paper on the metabolism of nitrobenzene in this series (Robinson, Smith & Williams, 1951), about 50% of an oral dose of nitrobenzene was accounted for as urinary metabolites. However, only the *p*-aminophenol and total nitro compounds excreted could be accurately determined, the amounts of the other metabolites formed being approximately assessed by paper chromatography and isolation procedures. Using nitrobenzene randomly labelled with ^{14}C in one carbon atom, it has now become possible to estimate accurately all the previously known metabolites, to demonstrate the existence of and identify the mercapturic acid suspected by Robinson *et al.* (1951) as a metabolite of nitrobenzene, and to show the formation of two new minor metabolites, namely carbon dioxide and nitroquinol. It has also been possible to measure the elimination of metabolites such as carbon dioxide, aniline and unchanged nitrobenzene in the expired air of the animal and to determine the amount eliminated in the faeces or retained in the tissues. It will be shown that some 85–90% of a single dose of nitrobenzene can now be accounted for.

MATERIALS AND METHODS

Melting points are corrected.

Preparation of [^{14}C]*nitrobenzene.* [^{14}C]Benzene (5 g.; Radiochemical Centre, Amersham) was added in small amounts to a mixture of HNO_3 (10 g.) and H_2SO_4 (15 g.), keeping the temperature below 45°. After keeping the mixture for 0·5 hr. at 0° the nitrobenzene layer was separated, and the remaining acid layer was diluted to 100 ml. with water and extracted with ether (2 × 10 ml.). The extract was added to the nitrobenzene and the mixture was washed with 10 ml. of water and then twice with 10 ml. of 10% (w/v) aqueous NH_4Cl. After drying over anhydrous $CaCl_2$, the ether was evaporated and the nitrobenzene distilled, b.p. 208–211° (yield 6·4 g., 69%). By isotopic dilution experiments it was shown that the non-volatile residue contained 24·5% of the radioactive benzene as *m*-dinitrobenzene, 2·4% as *o*-dinitrobenzene and 0·25% as *p*-dinitrobenzene.

Nitroquinol, m.p. 132° (Elbs, 1893); 3-nitrocatechol, m.p. 85°, and 4-nitrocatechol, m.p. 174° (Dakin, 1909); *o*-nitrothiophenol, m.p. 57° (Lecher & Simon, 1922); *m*-nitrothiophenol, m.p. 26°, and *p*-nitrothiophenol, m.p. 77° (Bennett & Berry, 1927); L-phenylmercapturic acid, m.p. 142° (Parke & Williams, 1951); and *trans-trans*-muconic acid, m.p. 300° (decomp.) (Ingold, 1921), were also prepared. Other known compounds required for isotope dilution experiments were purchased or prepared by standard methods and purified to constant m.p.

Animals. The [^{14}C]nitrobenzene contained about 100 μc/ml. of radioactivity, and was administered, alone or suitably diluted with inactive nitrobenzene (A.R.), b.p. 209–211°, to rabbits by stomach tube, and in one case to a guinea pig by intraperitoneal injection. After dosing, each rabbit was placed in a metabolism chamber through which a current of air was drawn into ethanol at −50° to trap any unchanged nitrobenzene or aniline, and thence into CO_2 absorbers (Parke & Williams, 1953). The expired CO_2 was collected only in Expts. 4 and 5 (see Table 1). In all the experiments the animals were removed from the chamber not later than 30 hr. after dosing.

* Part 67: Mead, Smith & Williams (1955).