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Substrate Specificity of Amine Oxidases

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It has been known for some time that diamines such as putrescine and cadaverine are not oxidized by amine oxidase (Blaschko, Richter & Schlossmann, 1937). This is due to their lack of affinity for the enzyme (Blaschko, 1939). Their breakdown in the mammalian organism, first studied by Udranszky & Baumann (1891), is brought about by the enzyme diamine oxidase (Zeller, 1938a).

The molecular grouping which is present in all substrates of amine oxidase is the amino-group attached to a terminal carbon atom. That the diamines with two such groups have no affinity to the enzyme shows that the presence of the second group in some way interferes with the ability of the first group to react with the enzyme. The nature of this interference is unknown.

We have recently had the opportunity of examining the oxidation of a number of long-chain mono- and diamines by tissue extracts and our results are described in this paper. They represent a contribution to the problem of substrate specificity.

MATERIAL AND METHODS

We wish to thank Dr H. King, F.R.S., for giving us the substances which were examined. They included two monoamines, $\text{CH}_3(\text{CH}_2)_{11}\text{NH}_2$ and $\text{CH}_3(\text{CH}_2)_{17}\text{NH}_2$ and five diamines, $\text{NH}_2(\text{CH}_2)_6\text{NH}_2$, $\text{NH}_2(\text{CH}_2)_8\text{NH}_2$, $\text{NH}_2(\text{CH}_2)_{14}\text{NH}_2$, $\text{NH}_2(\text{CH}_2)_{16}\text{NH}_2$ and $\text{NH}_2(\text{CH}_2)_{18}\text{NH}_2$. As typical substrates of amine oxidase we used tyramine, and of diamine oxidase, cadaverine. The amine oxidase preparation was from rabbit's liver (Blaschko & Duthie, 1945). As source for diamine oxidase we used pig's kidney; two preparations were employed, first, an aqueous extract of the organ dialyzed against distilled water overnight, with one-tenth the volume of $\text{M}/15$ sodium phosphate buffer of pH 7.4 added, and secondly, an acetone-dried preparation. The latter preparation was obtained by passing the organ through a meat mincer and grinding the mince in a cooled mortar. The brei obtained was extracted with cold acetone,

three times the weight of the tissue, and this treatment was repeated three times. The last traces of acetone were pressed out and the preparation was kept in a vacuum desiccator overnight. Coarse particles were removed from the dry powder by passing it through a no. 40 mesh sieve. 1.5 g. of the resulting powder were incubated with 15 ml. of $\text{M}/15$ sodium phosphate buffer, pH 7.4, at room temperature for 50 min. with frequent stirring. The preparation was centrifuged for 5 min. and the supernatant, strained if necessary, in order to remove suspended particles, was used.

The manometric plan need not be discussed in detail. The standard substrate of amine oxidase was tyramine hydrochloride, used in 10^{-2}M concentration, that of diamine oxidase was $0.5 \times 10^{-2}\text{M}$ -cadaverine dihydrochloride. The temperature in all experiments was 38° . The gas phase was oxygen in the amine oxidase experiments and air in those with diamine oxidase. The total reaction volume in each flask was 2.0 ml.

EXPERIMENTAL

(1) Experiments with amine oxidase

(a) *Monoamines*. The compound with 12 carbon atoms had a marked affinity for amine oxidase, and it was a substrate of the enzyme. In an experiment in which 1.6 ml. rabbit liver extract were used there was an additional oxygen uptake of $43\ \mu\text{l.}$ in 15 min. with $0.5 \times 10^{-2}\text{M}$ -dodecylamine. That this was due to the amine oxidase present in the extract is supported by the observation that the amine strongly depressed the oxygen uptake due to tyramine. For instance, 0.5 ml. extract with 10^{-2}M -tyramine alone gave $90\ \mu\text{l.}$ of oxygen consumed in the first 15 min., but with 10^{-2}M -tyramine plus 10^{-3}M -dodecylamine the oxygen uptake was only $4\ \mu\text{l.}$ in the same period.

Octadecylamine, the other monoamine tested, gave entirely different results. There was no oxygen uptake in excess of the blank with $2 \times 10^{-3}\text{M}$ -octadecylamine. This was due to a lack of affinity of the amine for amine oxidase: the oxygen uptake with 10^{-2}M -tyramine was the same in the presence and in the absence of 10^{-3}M -octadecylamine.

* Working with a grant from the Medical Research Council.

(b) *Diamines*. The substances tested fall into two distinct groups, namely, the compounds hexamethylene diamine and octamethylene diamine, and secondly, the long-chain compounds with 14, 16 and 18 CH₂ groups.

The substances of the first group had no affinity for amine oxidase; this is shown by the two experiments of Table 1.

Table 1. *Additional oxygen uptake with tyramine and diamines in rabbit liver extract*

Exp.	Addition	Extra O ₂ (μl., first 15 min.)
I	10 ⁻² M-Tyramine	53
	Ditto + 10 ⁻³ M-NH ₂ (CH ₂) ₆ NH ₂	49
II	10 ⁻² M-Tyramine	49
	Ditto + 10 ⁻³ M-NH ₂ (CH ₂) ₈ NH ₂	49

Table 2. *Oxygen uptake of rabbit liver extract with long-chain diamines*

(1.6 ml. extract; first 15 min. readings. Substrate concentration 0.2 × 10⁻²M.)

Ex- tract alone	Additional oxygen uptake (μl.) with		
	NH ₂ (CH ₂) ₁₄ NH ₂	NH ₂ (CH ₂) ₁₆ NH ₂	NH ₂ (CH ₂) ₁₈ NH ₂
7	62	42	16

The results with the three long-chain diamines were strikingly different. As Table 2 shows, all three were oxidized by the liver extracts. The initial rate of oxidation was greatest for the C₁₄ compound, less for the C₁₆ compound and least for the substance with 18 carbon atoms. That this oxidation was due to amine oxidase is supported by the following observations:

(1) The rabbit liver extracts contained traces only of diamine oxidase; in one experiment the extra oxygen uptakes in the first 15 min. were (μl. O₂):

With 0.5 × 10 ⁻² M-cadaverine	With 10 ⁻² M-tyramine	With 0.5 × 10 ⁻² M-NH ₂ (CH ₂) ₁₄ NH ₂
2.5	65	47

Table 3. *Competition between tyramine and long-chain diamines*

(1.6 ml. of rabbit liver extract. Oxygen uptake for first 15 min.)

Exp.	Addition	Extra O ₂ (μl.)
I	10 ⁻² M-Tyramine	41
	10 ⁻³ M-NH ₂ (CH ₂) ₁₄ NH ₂	19
	Both the above	19
II	10 ⁻² M-Tyramine	49
	10 ⁻³ M-NH ₂ (CH ₂) ₁₆ NH ₂	10
	Both the above	13
III	10 ⁻² M-Tyramine	48
	10 ⁻³ M-NH ₂ (CH ₂) ₁₈ NH ₂	2
	Both the above	9

(2) The oxidation of the C₁₄ diamine was not inhibited in the presence of 10⁻⁴M-semicarbazide.

(3) All three substances showed competition with tyramine (Table 3). The figures given suggest that the affinity decreases in the order: C₁₄ > C₁₆ > C₁₈.

(4) Bernheim & Bernheim (1938) have separated two fractions of amine oxidase from rabbit's liver, one which is insoluble and one which is not spun down in the centrifuge. We found the ratio:

$$\frac{\text{Oxygen uptake with tyramine}}{\text{Oxygen uptake with NH}_2(\text{CH}_2)_{14}\text{NH}_2}$$

approximately the same for the two fractions. This is shown in Table 4.

Table 4. *Relative activities of rabbit liver extract and sediment in oxidizing tyramine and NH₂(CH₂)₁₄NH₂*

	Extra oxygen uptake (μl. O ₂ /20 min.)	
	Sedi- ment	Super- natant
(A) With 10 ⁻² M-tyramine	95	17
(B) With 2 × 10 ⁻² M-NH ₂ (CH ₂) ₁₄ NH ₂	49	9.5
Ratio (A)/(B)	1.9	1.8

(2) *Experiments with diamine oxidase*

A complicating factor in the experiments with preparations from pig's kidney was the presence of both amine oxidase and diamine oxidase. This is why most of the observations were made on acetone-dried preparations, but even here a certain amount of amine oxidase activity was sometimes retained and it was therefore necessary to test the activity of the preparations towards tyramine when amine oxidase had to be ruled out as a factor in these experiments.

(a) *Monoamines*. The two substances with 12 and 18 carbon atoms respectively were not oxidized by the acetone-dried preparations, neither did they affect the rate of oxygen uptake with cadaverine as a substrate. Dodecylamine was tested in 2 × 10⁻³M concentration, octadecylamine in 0.5 × 10⁻³M.

(b) *Diamines*. As in the case of amine oxidase, two groups could be clearly distinguished, the C₆ and C₈ compounds which were found to be substrates of diamine oxidase, and the three compounds with 14, 16, and 18 carbon atoms which were not oxidized.

In one experiment with the diamines in 0.5 × 10⁻²M concentration the oxygen uptakes (μl. O₂) during the first 10 min. were:

Extract alone	Additional oxygen uptake with		
	Cadaverine	Hexa- methylene diamine	Octa- methylene diamine
5	31	20	7

This shows that in the range examined the rate of oxidation decreases with increasing chain length. Zeller (1938*b*) found that with an increasing length of the carbon chain from 2 to 5 carbon atoms there was an increase in the rate of oxidation. The rate of oxidation therefore is at a maximum in cadaverine (pentamethylene diamine). The oxidation of both hexamethylene diamine and octamethylene diamine was due to diamine oxidase because both showed marked competition with cadaverine. This is illustrated by the experiments of Table 5.

Table 5. *Competition between cadaverine, hexamethylene diamine, and octamethylene diamine*

(Acetone preparation of pig kidney. First 15 min. readings.)

Exp.	Addition	Extra O ₂ (μ l.)
I	0.5×10^{-2} M-Cadaverine	19
	0.5×10^{-2} M-Cadaverine + 0.5×10^{-2} M-NH ₂ (CH ₂) ₆ NH ₂	10.5
	0.5×10^{-2} M-NH ₂ (CH ₂) ₆ NH ₂	12
	10^{-2} M-Tyramine	0
II	0.5×10^{-2} M-Cadaverine	27
	0.5×10^{-2} M-Cadaverine + 0.5×10^{-2} M-NH ₂ (CH ₂) ₈ NH ₂	16.5
	0.5×10^{-2} M-NH ₂ (CH ₂) ₈ NH ₂	6.5
	10^{-2} M-Tyramine	0

In the aqueous extract of pig's kidney there was an oxygen uptake with the long-chain C₁₄ diamine, but this was due to the presence of amine oxidase and not to diamine oxidase. With acetone-dried preparations the oxygen uptake with the C₁₄ diamine was very small or completely absent, and the same was true for tyramine. The C₁₆ and C₁₈ diamines were also not oxidized by the acetone-dried preparations. Table 6 shows that the affinity of the

Table 6. *Competition between cadaverine and long-chain diamines*

(Acetone preparation of pig kidney. First 15 min. readings.)

Exp.	Addition	Extra O ₂ (μ l.)
I	0.5×10^{-2} M-Cadaverine	38
	0.5×10^{-2} M-Cadaverine + 0.5×10^{-2} M-NH ₂ (CH ₂) ₁₄ NH ₂	25
	0.5×10^{-2} M-NH ₂ (CH ₂) ₁₄ NH ₂	0
II	0.5×10^{-2} M-Cadaverine	36
	0.5×10^{-2} M-Cadaverine + 0.5×10^{-2} M-NH ₂ (CH ₂) ₁₆ NH ₂	34
	0.5×10^{-2} M-NH ₂ (CH ₂) ₁₆ NH ₂	0
III	0.5×10^{-2} M-Cadaverine	36
	0.5×10^{-2} M-Cadaverine + 0.5×10^{-2} M-NH ₂ (CH ₂) ₁₈ NH ₂	39
	0.5×10^{-2} M-NH ₂ (CH ₂) ₁₈ NH ₂	0

diamines to diamine oxidase decreases with increasing chain length. In the concentration in which they were used the C₁₄ acted as an inhibitor, but there was no inhibition with the C₁₆ and C₁₈ diamines.

DISCUSSION

Some of the results described allow us to define with greater precision the conditions of substrate specificity. The diamines with 14, 16 and 18 carbon atoms were not oxidized by diamine oxidase, but they were substrates of amine oxidase. These observations reveal one of the structural features governing the specificity of diamines for the two enzymes, viz. the length of the polymethylene chain. With short chains (up to C₈) the second amino-group interferes with the affinity for amine oxidase, but with long chains (e.g. C₁₄) the interference disappears and substrate specificity appears. Conversely, short chains (up to C₈) appear to be necessary for substrates of diamine oxidase. That the C₁₄ diamine inhibits the oxidation of cadaverine may indicate that the loss of affinity for diamine oxidase occurs at a greater chain length than the loss of substrate specificity.

A full analysis would require the study of the diamines with an intermediary chain length, which were not at our disposal. But it seems likely that the results so far obtained find their explanation in the different molecular orientation required for substrates of the two enzymes. Zeller (1941, 1942) had already postulated that for oxidation by diamine oxidase it was necessary for both amino-groups of the substrate molecule to react with the enzyme and that this would require an arrangement of the long axis of the molecule parallel with a surface of the enzyme. Such an orientation of short-chain diamines is to be expected on surfaces, e.g. on an oil-water interface; and it would be a necessary condition for the diamine oxidase reaction to occur. With an increasing length of the carbon chain influences which tend to lift the carbon chain off the surface become stronger, viz. the Coulomb forces of attraction between —CH₂— groups of neighbouring carbon chains. These forces, being additive, increase with increasing length of the chain, and in the diamines with 14, 16 and 18 carbon atoms an orientation vertical to the surface might explain why these substances are not substrates of diamine oxidase. In the case of amine oxidase the position would be exactly reversed. The attachment of an amine to this enzyme seems to require an orientation in which the carbon chain stands vertical to a surface. The rate of oxidation of typical substrates of amine oxidase is not affected by the presence of short-chain diamines; this shows that amines which have a tendency to orientate themselves parallel to the surface are not bound by the enzyme.

SUMMARY

1. The oxidation of two straight-chain monoamines, dodecylamine and octadecylamine, and of five diamines with 6, 8, 14, 16 and 18 CH₂ groups by

amine oxidase (rabbit liver) and by diamine oxidase (pig kidney) has been studied.

2. Dodecylamine is oxidized by amine oxidase, whereas octadecylamine is not oxidized.

3. Of the straight-chain diamines, hexamethylene diamine and octamethylene diamine are substrates of diamine oxidase; the three diamines with 14, 16 and 18 carbon atoms are not oxidized by the enzyme.

4. The diamines with 14, 16 and 18 carbon atoms are substrates of amine oxidase.

5. The specificity of substrates of the two enzymes is discussed in connexion with these results.

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The Colouring Matters of Galls. 2

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Whereas it is comparatively easy to collect galls produced by insects, the collecting of galls formed by fungi offers greater difficulties. Furthermore, these galls have not been so extensively studied as insect-galls (Guttenberg, 1905). Thanks to Dr von Wehner, the nephew and assistant of the late Dr von Guttenberg, we were able to obtain reasonable quantities of the six galls investigated by his uncle, which Dr von Wehner collected for us in the vicinity of Graz. Galls of *Ustilago maydis* Guttenberg, on maize collected by one of us at Tifton, Georgia, U.S.A., were found to contain the same pigment as the Austrian galls.

EXPERIMENTAL

The pigments were prepared, identified and investigated as described by Nierenstein & Swanton (1944). They proved known colouring matters described by Nierenstein & Swanton, whose preparations we used for mixed melting-points. The yields in all cases were poor, the maximum obtained being 0.27 %.

Analytical and descriptive data

Melampsorin. Yellow needles, m.p. and mixed m.p. 233–237° produced by *Exoascus amentorum* H. Schneider on the fruits of *Alnus incana* Web. (Found: C, 50.7; H, 5.2; purpurogallin, 40.5; glucose, 63.7 %; mol. wt. 539, 551. Calc. for $C_{23}H_{28}O_{15}$: C, 50.7; H, 5.2; purpurogallin, 40.4; glucose, 63.2 %; mol. wt. 544.)

Raetelin. Red needles, m.p. and mixed m.p.

242–244°, isolated from the galls produced by *Albugo candida* Pers. on the leaves of *Capsella bursa pastoris* L., by *Exobasidium rhododendri* Cram. on the leaves of *Rhododendron hirsutum* L. and *R. ferrugineum* L. (Found: C, 50.6, 50.7, 50.4; H, 5.3, 5.3, 5.4; purpurogallin, 40.5, 40.4, 40.5; glucose, 63.0, 63.3, 63.1 %; mol. wt. 555, 573; 541, 549; 551, 587. Calc. for $C_{23}H_{28}O_{15}$: C, 50.7; H, 5.2; purpurogallin, 40.4; glucose, 63.2 %; mol. wt. 544.)

Xestophanesin. Dark red needles, m.p. and mixed m.p. 265–266°, produced by *Puccinia adoxxae* Haberl. on the leaves, twigs and buds of *Adoxa moschatellina* L. (Found: C, 50.8; H, 5.5; purpurogallin, 40.4; glucose, 63.1 %; mol. wt. 551, 562. Calc. for $C_{23}H_{28}O_{15}$: C, 50.7; H, 5.2; purpurogallin, 40.4; glucose, 63.2 %; mol. wt. 544.)

Pontanin. Yellow needles, m.p. and mixed m.p. 283–287° produced by *Ustilago maydis* Guttenberg on the leaves of *Zea mays* L. (Found: C, 50.6, 50.8; H, 5.3, 5.2; purpurogallin, 40.5, 40.4; glucose, 63.0, 63.2 %; mol. wt. 548, 554; 547, 562. Calc. for $C_{23}H_{28}O_{15}$: C, 50.7; H, 5.2; purpurogallin, 40.4; glucose, 63.2 %; mol. wt. 544.)

In conclusion we draw attention to the use of these colouring matters as low-range indicators (Nierenstein, 1945).

SUMMARY

The isolation and characterization of colouring matters from various galls produced by fungi is described.

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