The L-Amino-acid Oxidase of *Neurospora*

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Bender, Krebs & Horowitz (1949) and Bender & Krebs (1950) reported that *Neurospora crassa* can produce an L- α -amino-acid oxidase in addition to the D- α -amino-acid oxidase previously described by Horowitz (1944). The L-amino-acid oxidase was present not only in the mycelium but also in the clear culture fluid, whilst the D-enzyme only occurs in the mycelium. The quantity of the L-enzyme formed was found to be dependent on the composition of the medium and to vary from strain to strain. The present paper is an extension of preliminary work by Dr A. E. Bender and is concerned with the factors influencing the production of the L-amino-acid oxidase, and with the properties of the enzyme, including the nature of the prosthetic group. A somewhat similar study has been simultaneously carried out by Thayer & Horowitz (1951) in the Kerekhoff Laboratories of Biology, Pasadena, U.S.A.

EXPERIMENTAL

Strains. The twenty strains of *Neurospora* used are shown in Table 1.

Conditions of culture. The basal medium was essentially that of Horowitz & Beadle (1943). It contained (final concentrations) 2% sucrose, 0.5% ammonium tartrate, 0.1% NH_4NO_3 , 0.1% KH_2PO_4 , 0.01% NaCl , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% CaCl_2 and the following quantities of trace elements per litre: $\text{Na}_2\text{B}_4\text{O}_7$, 0.05 mg.; Na_2MoO_4 , 0.04 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 mg.; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg. and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 9 mg. Biotin, casein hydrolysate (low vitamin casein supplied by Genatosan Ltd., and hydrolysed according to McIlwain & Hughes, 1944) or other substances were added as indicated in the text. The culture flasks, either conical flasks, Roux bottles or the mould culture flasks of Clayton, Hems, Robinson, Andrews & Hunwicke (1944), were filled with liquid medium to a depth of about 1 cm. and autoclaved for 15 min. at 15 lb. pressure. Conidia from subcultures grown on agar slopes were used for inoculation except with strain CMI 3411 which did not regularly sporulate; it was propagated by transferring about a milligram of wet mycelium from subcultures grown on 10 ml. liquid medium in 25 ml. conical flasks. The growth temperature was 25°.

Determination of oxidase activity. O_2 consumption was determined in Warburg manometers of about 25 ml. volume at 30° and pH 8.3 in 0.033 M-pyrophosphate with a total fluid volume of 4.5 ml. The gas phase was O_2 and the centre well contained 0.2 ml. 2 N-NaOH and a roll of filter paper. The enzyme and substrate were placed in separate compartments and mixed after thermal equilibrium was established. In testing for the L-amino-acid oxidase, the substrate was L-phenylalanine (1.125×10^{-5} g.mol.). As

there was no D-oxidase in the culture fluid, the equivalent amount of DL-phenylalanine could be used. In the later stages of purification, an extract of red blood cells (equivalent to 0.02 ml. blood) was added to supply catalase. In the case of the culture fluid or preparations obtained therefrom the rate of O_2 consumption was constant at least during the period of 5–25 min. after mixing unless more than 40 units of activity were present. The activity was calculated from this constant rate; one unit being defined as the amount of enzyme causing 1 $\mu\text{l. O}_2$ to be taken up in 10 min. The activity determined in this way was proportional to the amount of the enzyme preparation. The culture fluid did not absorb O_2 in the absence of added amino-acids.

Preparation of mycelia for measuring oxidase activity. The mycelia were briefly washed in distilled water. Extracts were prepared by grinding with sand and pyrophosphate buffer followed by centrifugation according to Horowitz (1944), and suspensions by disintegrating with 20 ml. water in a small metal cup fitted to the Waring blender. These preparations usually possessed a high endogenous respiration which precluded the exact assessment of the oxidase activity. In contrast to other strains the endogenous respiration of CMI 3411 and CMI 17836 was low, usually much less than 10% of the steady rate of O_2 consumption after adding phenylalanine; the activity of the mycelial preparations was calculated as for the culture fluid correcting for the endogenous respiration which was determined in a separate manometer vessel.

Flavin-adenine dinucleotide (FAD) was prepared from yeast by the method of Warburg & Christian (1938), but omitting the final fractionation of the Ba salt. The preparation was freed from riboflavin phosphate by ascending paper chromatography using the solvent mixture *tert*-butanol (60 vol.)–pyridine (25 vol.)–water (15 vol.). The paper was dried at 80° in the dark, the FAD band cut out and washed in ether. The flavin was eluted from the paper by water. The concentration of FAD in this solution was obtained from the light absorption at 450 m μ , assuming that the molecular extinction coefficient ($\epsilon_{450 \text{ m}\mu}$) is 1.13×10^4 (Warburg & Christian, 1938).

Riboflavin phosphate was obtained in aqueous solution by hydrolysing FAD with N-HCl at 100° for 10 min.

Apo-enzyme of kidney D-amino-acid oxidase was prepared according to Negelein & Brömel (1939). The protein component obtained after washing in 18% $(\text{NH}_4)_2\text{SO}_4$ solution at 0° was dissolved in 0.006 M-pyrophosphate buffer, pH 8.3; the pH of the solution was then 6.5. This material was stored at –10°.

Light absorption was measured in 10 mm. cells in a Beckman model DU quartz spectrophotometer.

Fluorescence measurements were made with exciting light of about 365 m μ , filtered from a mercury vapour lamp.

Non-dialysable N of enzyme preparations. The solution of enzyme was dialysed against running tap water until free from ammonium ions. 1.0 ml. was digested with 0.20 ml.

Table 1. *Strains of Neurospora examined*

Strain	Growth factor required (other than biotin)	Source
<i>N. crassa</i>		
CMI 3411	—	Commonwealth Mycological Institute, Ferry Lane, Kew
CMI 17836 (Beadle 37401)	Inositol	
CMI 19421 (Beadle 4545)	Lysine	
CMI 24298 (Beadle 9185)	Aneurin	
CMI 31288 (Beadle 1633)	<i>p</i> -Aminobenzoic acid	
Beadle 25 <i>a</i>	—	Dr N. H. Horowitz, California Institute of Technology, Pasadena, U.S.A.
Abbot <i>a</i> and <i>A</i>	—	Dr D. G. Catcheside, Botany School, Cambridge
Beadle 1 <i>A</i>	—	
Cambridge <i>a</i> (a spontaneous mutant of Beadle 25 <i>a</i>)	—	
Chilton <i>a</i>	—	
Emerson <i>a</i> and <i>A</i>	—	
Lindgren <i>a</i> and <i>A</i>	—	Dr J. R. S. Fincham, University College, Leicester
31223-8	α-Amino-acids	
47305	(see Fincham, 1950)	
<i>N. sitophila</i>		
CMI 34458	—	Commonwealth Mycological Institute
CMI 34457	—	
Beadle 299	Pyridoxine	Dr E. C. Barton-Wright, Whitbread and Co. Ltd., Chiswell Street, London, E.C. 1

50% H₂SO₄, 1% SeO₂ for 4 hr. in a test tube. The tube was cooled, 4-9 ml. water added and the solution mixed with a glass rod. Nessler's reagent (3 ml.) was added and the colour compared photometrically with standards.

Ammonia was estimated in the Parnas apparatus (Parnas & Heller, 1924).

Pyruvic acid was estimated by the carboxylase method (Krebs & Johnson, 1937).

RESULTS

*Factors influencing the yield of
L-amino-acid oxidase*

As already shown (Bender *et al.* 1949; Bender & Krebs, 1950) the yield varies from strain to strain and with the supply of biotin. It also depends on the presence of casein hydrolysate and the use of stationary culture as opposed to shaken culture. The highest yields of oxidase were obtained with strain CMI 3411 in stationary liquid culture on a sucrose, ammonium tartrate, inorganic salts medium supplemented by casein hydrolysate and 0.2-0.3 µg. biotin/l.

Table 2. *L-Amino acid oxidase in culture fluid*

(*N. crassa* grown 14 days at 25° on basal medium with 0.3% (dry) malt extract and 0.01% (dry) yeast extract.)

Strain	Oxidase activity (units/ml.)
Abbot <i>A</i>	2.3
Abbot <i>a</i>	3.3
Cambridge <i>a</i>	8
Lindgren <i>A</i>	7.6
CMI 3411	16
CMI 17836	4.7

Strain differences. Seventeen strains of *N. crassa* and three of *N. sitophila* (Table 1) were examined. They were grown in 1 l. Roux bottles at 25° on the

Fig. 1. Effect of L-phenylalanine on O₂ consumption by a mycelial extract (*N. crassa* strain Lindgren *a*). Manometer flasks contained: 2 ml. extract and 2 ml. 0.05M-pyrophosphate buffer, pH 8.3. Side bulb: 0.1M buffer (0.5 ml.) either alone (●) or with the addition of 1.88 mg. L-phenylalanine (○). Contents were mixed after 10 min. equilibration. Temp., 30°; gas, O₂.

basal medium (100 ml.) containing 0.3% (dry) malt extract and 0.01% (dry) yeast extract (compare Bender & Krebs, 1950). After 14 days' growth, 3 ml. samples of the clear culture fluid and 2 ml.

samples of mycelial extract were examined for L-amino-acid oxidase activity. Activity was detected in the culture fluid from six strains of *N. crassa*, but not from the remaining eleven strains nor the three strains of *N. sitophila*.

With mycelial extracts from strains CMI 3411, CMI 17836, Abbot *a* and Lindgren *A* the oxidation of L-phenylalanine (1.88 mg.) began after a lag period of a few minutes and was complete in less than 45 min. With extracts from other strains the addition of phenylalanine at first reduced the rate of O₂ consumption compared with the control, but after 30–150 min. the oxygen consumed with added phenylalanine overtook that of the control (Fig. 1); the extra consumption rose to about 127 μ l., corresponding to 1 atom of oxygen per molecule of phenylalanine. Whatever the initial effect of adding L-phenylalanine, about 127 μ l. extra oxygen was eventually consumed by extracts from every strain, indicating the presence of the L-amino-acid oxidase.

The initial inhibition of the endogenous respiration by phenylalanine may be connected with the inhibition of the L-oxidase by high substrate concentrations (see later).

Duration of growth. *N. crassa* CMI 3411 was selected for further study because of its high oxidase activity. Yields of oxidase obtained on the synthetic basal medium plus biotin (0.25 μ g./l.) were at least as high as those on the medium containing yeast and malt extracts. Maximum yields were obtained in 12 days (Table 3) on the synthetic medium with or without the addition of 0.2% casein hydrolysate. At this stage the total units of oxidase activity in the culture fluid were approximately the same as the total activity found in the mycelium.

Biotin supply. Bender *et al.* (1949) found that the yields were very much reduced with high concentrations of biotin (5 μ g./l.) in the culture medium. This effect has been studied in more detail (Table 4). Optimal yields of oxidase were obtained with

Table 3. *Duration of growth and yield of L-amino-acid oxidase*

(*N. crassa* CMI 3411 grown at 25° on 40 ml. basal medium with 0.3 μ g. biotin/l. in 250 ml. conical flasks.)

Days growth	Oxidase activity (units)			
	0.2% casein hydrolysate added to culture medium		No casein	
	Mycelial suspension	Culture fluid	Mycelial suspension	Culture fluid
4	700	200	530	130
6	1800	700	1040	400
9	2320	1700	1360	980
12	2550	2600	1500	1430
13	1700	2400	960	1330
14	900	2000	770	1030
16	630	1700	480	1000
19	500	1100	400	700

Table 4. *Effect of biotin and casein hydrolysate on the yield of L-amino-acid oxidase*

(*N. crassa*, CMI 3411 grown 10 days at 25° on 20 ml. medium in 50 ml. conical flasks. Dry weight determined on duplicate culture.)

Additions to growth medium			Oxidase activity (units)		
Biotin (μ g./l.)	Casein hydrolysate (%)	Mycelial dry wt. (mg.)	In culture fluid	In mycelial suspension	Total/mg. dry wt.
0	0	4.8	24	67	19
0.1	0	56.6	78	700	14
0.2	0	70.0	114	700	12
0.3	0	72.3	198	760	13
0.4	0	75.9	240	780	13
0.6	0	82.3	198	680	11
1.0	0	83.7	66	320	4.6
3.0	0	93.0	18	140	1.7
0	0.2	36.7	245	400	18
0.1	0.2	87.6	610	1080	19
0.2	0.2	98.9	1030	820	19
0.3	0.2	100.7	830	690	15
0.5	0.2	111.5	670	640	12
1.0	0.2	122.2	180	380	4.6
3.0	0.2	107.8	90	140	2.1

0.1–0.5 $\mu\text{g.}$ biotin/l. Growth was markedly reduced with less than 0.3 $\mu\text{g.}$ biotin/l. so that under these conditions the highest yields of L-oxidase per mg. mycelium were obtained. With 1 $\mu\text{g./l.}$ or higher concentrations of biotin the yields were low at periods of growth between 3 and 20 days. The oxidase itself is not inhibited by biotin even at 10^{-3}M.

Fig. 2. Effect of biotin on growth of *N. crassa*. Grown for 10 days at 25° on 20 ml. basal medium plus biotin in 50 ml. conical flasks. Strains Beadle 25a, Δ ; Cambridge a, $\circ$; and CMI 3411, $\bullet$.

Strain CMI 3411, which displayed the highest oxidase activity, required more biotin for optimal growth than two other strains, namely Beadle 25a and Cambridge a (Fig. 2).

Supply of essential nutrients other than biotin. Doubling the amounts of trace elements supplied did not influence the yields of oxidase from strain

CMI 3411, but when iron salts were omitted from the medium (1 $\mu\text{g.}$ biotin/l.) the mycelial dry weight was 80 % and yield of oxidase only 15 % of that on the complete medium. With strain CMI 17836, a mutant requiring inositol, on a medium containing 10 $\mu\text{g.}$ inositol/l., growth was 60 % and yield of oxidase 18 % of that obtained with 40 $\mu\text{g.}$ inositol/l.

Source of nitrogen. The addition of casein hydrolysate (0.2 %) to the growth medium containing 0.25 $\mu\text{g.}$ biotin/l. approximately doubled the total yields of the L-enzyme (Tables 3–5) without affecting the growth period or biotin supply required for maximum yields. The casein hydrolysate also increased the growth so that it has little effect on the yield of enzyme per mg. mycelium. The concentration of casein hydrolysate giving maximum yields was 0.2–0.4 % (Table 5); it was equally effective whether hydrolysed by HCl, H_2SO_4 or by HCl in the presence of titanous chloride, as in these experiments. An enzymic digest of casein ('Pronutrin', Herts Pharmaceuticals Ltd., Welwyn Garden City), was not as effective as the casein hydrolysate; the latter contains more of the sparingly soluble amino-acids (cystine and tyrosine) and less biotin than 'Pronutrin'. With casein hydrolysate as the sole source of nitrogen the yield of oxidase was similar to that with both inorganic nitrogen and casein hydrolysate (e.g. Table 5).

The yields of oxidase were low when the sole source of nitrogen was either nitrate or ammonium salts, but were higher on the basal medium which contained both; adding 1 % $(\text{NH}_4)_2\text{SO}_4$ to the basal medium also reduced the yield. As will be seen from Table 5, some of the changes in the composition of the medium affected the enzymic activity of the culture fluid more markedly than that of the mycelium.

Shaken cultures. Growth was more rapid in the culture flasks of Clayton *et al.* (1944) when shaken

Table 5. Nitrogen source and yield of L-amino-acid oxidase

(*N. crassa* CMI 3411 grown 9 days on 20 ml. medium (0.25 $\mu\text{g.}$ biotin/l.) in 50 ml. conical flasks. When ammonium tartrate was not present an equimolar amount of sodium potassium tartrate was substituted. Dry weight determined on duplicate culture.)

N source (%)				Oxidase activity (units)					
Ammonium tartrate	NH ₄ NO ₃	NaNO ₃	(NH ₄) ₂ SO ₄	Casein hydrolysate	Casein digest ('Pronutrin')	Mycelial dry wt. (mg.)	In culture fluid	In mycelial suspension	Total per mg. dry wt.
0.5	0.1	—	—	—	—	67.8	295	600	13.4
0.5	0.1	—	1.0	—	—	61.6	140	500	8.8
0.5	—	—	0.16	—	—	71.5	38	420	6.4
—	—	0.67	—	—	—	98.6	36	640	6.9
0.5	0.1	—	—	0.1	—	92.0	810	570	14.0
0.5	0.1	—	—	0.2	—	81.7	850	710	19.0
0.5	0.1	—	—	0.4	—	103	1020	960	19.2
0.5	0.1	—	—	0.7	—	118	220	1000	10.3
—	—	—	—	0.7	—	106	420	930	12.7
0.5	0.1	—	—	—	0.1	82.9	730	540	15.3
0.5	0.1	—	—	—	0.4	117	650	540	10.2
0.5	0.1	—	—	—	0.7	130	690	640	10.2

horizontally (120 oscillations/min.) than in stationary flasks, and the mould grew as small fluffy pieces about 5 mm. across. Under all conditions tested (varying growth between 2 and 10 days and varying the composition of the medium with respect to biotin and casein hydrolysate) the yields of oxidase were usually less than 1 unit/mg. mycelium.

Primary reaction catalysed by the oxidase

For each molecule of L-alanine oxidized by the culture fluid, one molecule of pyruvic acid was formed and half a molecule of O_2 consumed (e.g. 11.3 μ mol. L-alanine added, 10.6 μ mol. pyruvic acid formed and 5.45 μ mol. O_2 used). As the culture fluid contained a relatively high concentration of ammonium salts, the production of ammonia could not be accurately measured. The ammonium salts were removed without losing oxidase activity by dialysis against running tap water for 14 hr. One molecule NH_3 was formed from each molecule of L-alanine oxidized. The overall reaction is therefore

By analogy with other amino-acid oxidases it may be expected that hydrogen peroxide is formed in the primary reaction; this would be decomposed by the catalase present in the culture fluid. In fact hydroxylamine or hydrogen cyanide, which inhibit the catalase, increased the rate of O_2 uptake, and the final quantity of O_2 consumed rose to one molecule per molecule of L-amino-acid. With preparations of oxidase free from catalase (see below), one molecule of O_2 was consumed per molecule of amino-acid; addition of catalase halved the amount of O_2 consumed. It may therefore be taken that hydrogen peroxide is formed in the primary reaction. It should therefore be possible to couple the oxidation of ethanol with that of amino-acids in the presence of relatively high concentrations of catalase (cf. Keilin & Hartree, 1945). The culture fluid contained insufficient catalase for this reaction, but with the addition of red blood cells the O_2 consumption was increased to 0.90 molecule O_2 per molecule L-phenylalanine when ethanol was added.

Factors affecting the activity of the oxidase

Preparation of oxidase for kinetic studies. Strain CMI 3411 was grown for 12 days at 25° in 1 cm. depth of basal medium with the addition of 0.25 μ g. biotin/l. Culture flasks were those of Clayton *et al.* (1944) originally designed for the production of penicillin. The culture fluid at the end of the growth contained both L-amino-acid oxidase and catalase. The fall in activity on storage in the refrigerator with octanol as a preservative, was up to 50 % in a week but often less. The culture fluid contained about 11 mg. non-dialysable N/l.; no precipitate occurred

on adding trichloroacetic acid. A stable dry powder was obtained after concentrating the culture fluid to one-fifth the original volume at reduced pressure (bath temperature 40–50°). A precipitate appeared and was removed on the centrifuge. The clear golden-brown supernatant was cooled to 0°, 1.7 vol. acetone cooled to –5° was added with stirring and the suspension centrifuged. The precipitate was dried *in vacuo* in the centrifuge tubes over H_2SO_4 and solid NaOH. The precipitate was ground to a powder and stored *in vacuo* over H_2SO_4 and solid NaOH. It contained catalase activity and 50–60 % of the oxidase activity of the culture fluid; no activity was lost after storing for 6 months at room temperature.

Fig. 3. Substrate concentration curve. For procedure and calculation see text. L-alanine, ○; DL-alanine, ●; L-glutamate, □; DL-leucine, ●; L-phenylalanine, x.

For use the powder was dissolved in water; a small amount of insoluble residue was removed by centrifuging. In most preparations 1 mg. was equivalent to about 6 units of oxidase. The material consisted of the soluble constituents of the medium and contained less than 1 % protein. This preparation is a convenient form of the enzyme for use as an analytical reagent (e.g. Gibson & Wiseman, 1951).

Concentration of substrate. The relation between activity and concentration of substrate was studied for alanine, glutamic acid, leucine and phenylalanine at 30° and pH 8.2 with O_2 as the gas phase (Fig. 3). The main compartments of the manometer vessels contained 1.5 ml. 0.1 M-pyrophosphate buffer and the amino-acid in a total volume of 4 ml. The oxidase (5 mg. acetone-precipitated material in 0.5 ml. water) was added from the side arm when thermal equilibrium was established. To avoid

complications arising from the lag period in the first few minutes after mixing, the activities were obtained from the O_2 consumed in the period beginning 5 min. after mixing; the 5–25 min. period was used for concentrations higher than 0.02M, the 5–10 min. period for the lowest concentrations of phenylalanine and of leucine, and the 5–15 min. period for other values. The substrate concentrations plotted were the mean concentrations during the period used for the measurement of the O_2 uptakes. These were calculated from the initial substrate concentrations and the O_2 consumed, on the basis of equation (1).

Fig. 4. pH-Activity curve. Manometer flasks contained: 1.5 ml. 0.1M-buffer as below, 1.87 mg. L-phenylalanine and 2.5 ml. water. After thermal equilibration 0.5 ml. oxidase solution (5 mg. acetone-precipitated material) was added from side arm. Buffers: lactate, Δ ; acetate, $\bullet$; phosphate, $\circ$; pyrophosphate, $\times$. Temp., 30°; gas, O_2 .

It will be seen that the enzyme is inhibited by high (above 0.01M) concentrations of substrate, except in the case of glutamic acid. For alanine and leucine, the plot of activity against the logarithm of substrate concentration is bell-shaped as with certain other enzymes, e.g. L-amino-acid oxidase of snake venom (Zeller & Maritz, 1945) and cholinesterase (Alles & Hawes, 1940). The points obtained with DL-alanine lie on the same curve as those for L-alanine. With phenylalanine, the activity is almost independent of its concentration between 5×10^{-4} M and 3×10^{-3} M—and this amino-acid is therefore specially suitable for determining the activity of oxidase preparations. A theory of inhibition by high concentrations of substrate has been given by Haldane (1930).

pH. The pH activity curve shows a broad maximum between pH 6 and 9.5. At pH 4 the activity is approximately 70 % of the maximum (Fig. 4).

Hydrogen acceptors. Ferricyanide and reducible dyes can replace O_2 as a hydrogen acceptor. The rate of O_2 consumption at pH 8.2 in 0.033M-pyrophosphate was 4.5 times the rate of anaerobic reduction of ferricyanide measured manometrically at pH 7.4 in 0.025M- $NaHCO_3$ by the method of Quastel & Wheatley (1938). 2:6-Dichlorophenol indophenol (0.075 mg.) was decolorized in 3.3 min. when incubated anaerobically at 30° and pH 8.2 with 90 units of oxidase and 1.67 mg. DL-phenylalanine in 2 ml. 0.033M-pyrophosphate; in the absence of substrate the dye was decolorized in 50 min. The rate of reaction with O_2 was therefore about twenty times the rate with 2:6-dichlorophenol indophenol. Methylene blue was reduced much more slowly; the reduction of 0.075 mg. required 3 hr. in the presence of phenylalanine; no reduction occurring without added substrate.

The rate of oxidation in O_2 (1 atm.) was 3.4 times that in air.

Temperature. The optimum was about 45° when the activity was determined at pH 8.2 as already described (Table 6). Between 20 and 40° the rate of reaction was approximately doubled for every 10° rise in temperature. Heat stability is optimal between pH 6 and 7 and the substrate protects the enzyme against heat inactivation. After heating at 55° and pH 6 for 5 min. 55 % of the activity was lost; only 13 % was lost in the presence of 5×10^{-3} M-L-leucine.

Table 6. Effect of temperature on oxidase activity

(Manometer flasks contained: 1 ml. 0.1M-pyrophosphate buffer pH 8.2, 7.5 mg. acetone-precipitated oxidase and water to 4 ml. DL-phenylalanine (3.75 mg. dissolved in 0.5 ml. pyrophosphate buffer) was added from the side arm after 10 min. equilibration. Gas: O_2 .)

Temp. (°)	O_2 consumed (μl./10 min.)
20	10
30	20
35	28
40	40
45	51
50	39

Inhibitors. Of the substances tested on the acetone-precipitated material, the following did not affect either the rate of O_2 consumption or the final quantity of O_2 consumed: 2:6-dichlorophenol indophenol (1 mM final concentration); D-phenylalanine (6 mM); iodoacetic acid, *p*-chloroacetophenone, 8-hydroxyquinoline, 2:4-dinitrophenol, DL-lactic acid, kojic acid, benzoic acid and phthalic acid (10 mM); DL-N-methylleucine (20 mM), $(NH_4)_2SO_4$ (50 mM); sodium fluoride (100 mM) and octanol (saturated solution). Crystal violet, and methylene blue reduced the rate of reaction while 0.01M-HCN, azide or hydroxylamine increased the

O₂ consumption by inhibiting the catalase. The effects of these five substances and also atabrine, quinine and CuSO₄ were examined with a purified preparation of oxidase free from catalase (see below); the oxidase was not affected by 0.01M-HCN, hydroxylamine, or azide nor by 10⁻³M-quinine sulphate, but 0.01M-atabrine inhibited 15%, 10⁻³M-crystal violet inhibited 69% and 2 × 10⁻³M-methylene blue inhibited about 30%. In the absence of any buffer the inhibition by CuSO₄ was 63% at 10⁻³M and 15% at 10⁻²M. In the presence of 0.033M-pyrophosphate buffer there was no inhibition by 10⁻²M-CuSO₄.

Prosthetic group

Purification of oxidase. For bulk preparation the concentration of sucrose in the culture was reduced to 1.3% and that of ammonium tartrate to 0.2%; 0.2% NH₄Cl was added. This did not reduce the yield of oxidase. Strain CMI 3411 was grown at 25° in 1 cm. depth of 6.5 l. medium (0.25 μg. biotin/l.). After 12 days the culture fluid (5.6 l.) contained 150,000 units of oxidase (2400/mg. non-dialysable N). Preliminary experiments showed that the enzyme was adsorbed by cupric hydroxide, but not by alumina Cγ or calcium phosphate. To the culture fluid were added 214 ml. 10% CuSO₄·5H₂O and then 74 ml. 2N-NaOH with continuous stirring, and the precipitate of Cu(OH)₂ was centrifuged off. The amounts of copper present in the supernatant or precipitate did not affect the measurement of enzyme activity. Most of the activity remained in the supernatant, but was adsorbed by the precipitate formed on the addition of a further 118 ml. 10% CuSO₄. The precipitate of Cu(OH)₂ was centrifuged off and suspended in 100 ml. 0.1M-acetate buffer pH 6 (110,000 units of oxidase). After dialysing the suspension overnight against running water, most of the oxidase was still adsorbed to the insoluble Cu(OH)₂. This was centrifuged off and resuspended in 120 ml. water, and 2N-NH₄OH (15 ml.) was added slowly with continuous stirring until the Cu(OH)₂ was dissolved (66,000 units). This solution was dialysed overnight against running tap water (49,000 units) and centrifuged. The blue supernatant (39,000 units) was saturated with (NH₄)₂SO₄ by dissolving 175 g. in the 230 ml. solution. No precipitate was obtained at 80% saturation, but the material which was precipitated on complete saturation contained 32,000 units of oxidase. It was suspended in 20 ml. water, dialysed overnight and centrifuged. The clear, pale-yellow supernatant contained 25,000 units and 2.5 mg. non-dialysable N. To remove the last traces of Cu the enzyme was re-precipitated by saturation with (NH₄)₂SO₄. The precipitate (16,000 units) was collected by centrifuging, suspended in 15 ml. water, dialysed against running tap water for 15 hr. and centrifuged. The

24 ml. clear, pale-yellow supernatant contained 12,000 units of oxidase and 1.1 mg. non-dialysable N; it was free from catalase. The activity was slowly lost (30% in 2 weeks) on storing at -10°.

An alternative method of removing the Cu from the enzyme is to dissolve the Cu(OH)₂ fraction in 0.2M-pyrophosphate buffer pH 8. Most of the Cu can then be removed by dialysis. This procedure was sometimes, but not always, accompanied by large losses of the enzyme activity.

Presence of FAD in the purified oxidase. The enzyme preparation was examined for riboflavin, riboflavin phosphate and FAD by descending paper chromatography. Whatman no. 1 papers were washed with 0.1N-HCl, then with distilled water and dried before use. After developing the chromatograms they were dried at 80° in the dark; 0.03 μg. of each flavin could be located by its fluorescence in ultraviolet light. The *R_F* values of riboflavin, riboflavin phosphate and FAD were 0.8, 0.14 and 0.05 respectively, with a solvent mixture consisting of *n*-butanol (40 vol.)-acetic acid (10 vol.)-water (50 vol.) and 0.8, 0.19 and 0.08 with a solvent mixture of *tert*-butanol (60 vol.)-pyridine (25 vol.)-water (15 vol.). To concentrate the flavins in the oxidase preparation they were extracted by phenol as follows: saturated (NH₄)₂SO₄ solution (4 ml.) and 40% trichloroacetic acid (0.2 ml.) were added to 4 ml. of the purified oxidase solution and the mixture twice extracted by 1 ml. portions of phenol liquefied by adding water. The combined phenol extracts were shaken with 0.4 ml. water and 4 ml. CHCl₃ and centrifuged. About 0.2 ml. of the upper (aqueous) layer was then transferred to another tube and neutralized. When 30 μl. samples of this solution were examined chromatographically a single fluorescent spot (yellow-green) was observed; it had the same *R_F* values as FAD in both systems. Neither riboflavin nor riboflavin phosphate was detected.

The oxidase preparation showed a green fluorescence which was equivalent to 0.08 × 10⁻⁶M-riboflavin; it was increased to that of 0.53 × 10⁻⁶M-riboflavin by heating at 100° for 3 min. or by acidifying (one-tenth vol. N-HCl) and neutralizing to pH 7, suggesting that most of the FAD in the oxidase preparations was combined to protein, in a non-fluorescent form.

The fluorescence of riboflavin or riboflavin phosphate is 7.1 times that of FAD at pH 7 (Burch, Bessey & Lowry, 1948; cf. Singer & Kearney, 1950a). A solution of FAD was hydrolysed by heating at pH 0.6 (HCl) and 100° for 30 min.; after cooling and neutralizing the fluorescence was increased 8.1 times (from 0.13 to 1.05 times that of riboflavin). Similar treatment of the enzyme preparation increased the fluorescence to the equivalent of 4.4 × 10⁻⁶M-riboflavin, i.e. 8.3 times that after

only brief exposure to acid. This behaviour confirmed the identification of the oxidase flavin as FAD.

FAD can be identified and estimated using the protein component of kidney D-amino-acid oxidase (Warburg & Christian, 1938). The *Neurospora* L-amino-acid oxidase activated the kidney D-oxidase protein when the *Neurospora* enzyme had been heated at 100° for 3 min. or acidified (one-tenth vol. N-HCl) and neutralized. The untreated *Neurospora* enzyme did not activate the kidney D-amino-acid oxidase. The D-oxidase was not affected by the *Neurospora* enzyme in the presence of excess (2×10^{-8} g.mol.) FAD. As will be seen from Table 7, about 4×10^{-6} M-FAD was liberated by heating or acidifying the preparation of *Neurospora* L-amino-acid oxidase.

Table 7. Replacement of FAD in the D-amino-acid oxidase system by the L-amino-acid oxidase of *Neurospora*

(Manometer flasks contained: 1 ml. 0.2 M-pyrophosphate buffer, pH 8.2, 2 mg. D-alanine, red blood cells (0.01 ml. blood) as a source of catalase, and FAD or *Neurospora* L-oxidase preparation in a total volume of 3.3 ml. After equilibration, 0.2 ml. D-oxidase apo-enzyme was added from the side arm. Temp., 40°; gas, O₂.)

Addition		O ₂ consumed in 5-15 min. (μl.)	Free FAD in <i>Neurospora</i> oxidase (μM/l.)
FAD (g.mol. $\times 10^{-9}$)	<i>Neurospora</i> oxidase (ml.)		
—	—	14	—
1.42	—	51	—
2.13	—	63	—
2.84	—	70	—
—	1.0	12	0
—	(untreated)	—	—
—	0.50	58	3.6
—	(heated)	—	—
—	0.48	64	4.5
—	acidified and neutralized)	—	—

Effect of alanine and Na₂S₂O₄ on the absorption spectrum of the oxidase preparation. The purified oxidase (3 ml.) was mixed with 0.3 ml. 0.2 M-pyrophosphate buffer pH 7.5, and the absorption spectrum (A) was determined between 370 and 550 mμ. (Fig. 5). The solution was transferred to a Thunberg tube which was evacuated and 6 mg. solid DL-alanine was added from the stopper. After 10 min. the tube was filled with nitrogen and the solution transferred to the absorption cell: a stream of nitrogen was passed over the surface of the liquid until the absorption spectrum (B) had been measured. Curve C was obtained after 2 mg. Na₂S₂O₄ had been dissolved in the solution.

It will be seen from Fig. 5 that the DL-alanine decreased the light absorption of the oxidase preparation; when the spectrum (B) in the presence of

alanine was subtracted from the original (A), curve D was obtained with maxima at 385 and 470 mμ., and a minimum at about 415 mμ. This shows that some of the FAD in the oxidase can be reduced by the alanine. Na₂S₂O₄ further reduced the light absorption. The total decrease (A - C) in the extinction coefficient at 450 mμ. ($\Delta \log I_0/I_{(450 \text{ m}\mu.)}$) was 0.042. Assuming that this change was due

Fig. 5. Effect of alanine and Na₂S₂O₄ on absorption spectrum of L-aminoacid oxidase. For procedure see text. Curve A: oxidase preparation; curve B: oxidase + DL-alanine; curve C: oxidase + DL-alanine + Na₂S₂O₄; curve D: A - B.

entirely to the reduction of FAD and that the corresponding change in the molecular extinction coefficient ($\Delta \epsilon_{450 \text{ m}\mu.}$) is -1.05×10^4 , the oxidase contained $\frac{0.042 \times 3.3}{1.05 \times 10^4 \times 3.0} = 4.4 \times 10^{-6}$ M-FAD. The

value for $\Delta \epsilon_{450 \text{ m}\mu.}$ was obtained by measuring $\Delta \log I_0/I_{450 \text{ m}\mu.}$ when 2 mg. Na₂S₂O₄ was added to 3 ml. 10^{-5} M-FAD or riboflavin. When Na₂S₂O₄ was added to the acid-inactivated oxidase $\Delta \log I_0/I_{450 \text{ m}\mu.}$ corresponded to 4.3×10^{-6} M-FAD in the original oxidase preparation.

The values for the FAD content of the oxidase obtained by spectrophotometry agree with those obtained with the protein component of the D-amino-acid oxidase (4×10^{-6} M) and by fluorimetry (4.4×10^{-6} M).

Specificity

The relative rates of oxidation of the twenty amino-acids tested were similar both in the purified oxidase and in crude mycelial extracts. In addition to the thirty-three amino-acids previously found to be oxidized (Bender & Krebs, 1950), the enzyme attacks canavanine (private communication from Dr N. H. Horowitz), arginine, citrulline, glycine and

Table 8. *Rates of oxidation of amino-acids**

(Manometer flasks contained: 3 ml. culture fluid from strain CMI 3411, 1.0 ml. 0.1 M-pyrophosphate buffer, pH 8.3. Amino acid (2.25×10^{-3} g.mol.) weighed into side bulb and moistened with 0.5 ml. buffer. Temp., 30°; gas, O₂. The figures in the last column are comparable with the q_{O_2} values of Bender & Krebs, 1950.)

Amino-acid	O ₂ absorbed (μl.) after					Maximum rate (μl. O ₂ /10 min.)	Relative rate
	10 min.	20 min.	30 min.	90 min.	120 min.		
L-Phenylalanine	66	148	195	260	261	82	(52)
L-Arginine	12	34	68	227	228	34	22
L-Canavanine	44	101	176	260	264	75	47
L-Citrulline	31	88	158	265	265	70	45
α:ε-Diaminopimelic acid (natural compound)	60	134	213	252	254	79	50
Glycine	2	5	7	15	22	25	1.5

* Certain q_{O_2} values for the *Neurospora* L-oxidase given in Table 3 of the paper by Bender & Krebs were incorrectly calculated from the amounts of O₂ consumed. The corrected q_{O_2} values are phenylalanine: 52, tyrosine: 34, *p*-amino-phenylalanine: 32, β-6-methoxyquinolyl(4)alanine: 63, β-pyridyl(4)alanine: 60, and histidine: 75.

natural α:ε-diaminopimelic acid (Table 8). This last amino-acid probably has the *meso* configuration (Work, 1951).

Some observations on the formation of D-amino-acid oxidase by Neurospora

In previous experiments when the growth period was 14 days (Bender *et al.* 1949), the D-enzyme was found in some strains (e.g. Beadle 25*a*) but not in others (e.g. CMI 3411 and Cambridge *a*). Further experiments showed that the enzyme was formed by all strains when tested after 7 days' growth. The highest activities were obtained with strains Beadle 25*a* and Chilton *a*. The production of the D-oxidase by these strains was not affected by varying the concentration of biotin in the culture medium from 0.2 to 5 μg./l. With Chilton *a* grown on the basal medium plus biotin (5 μg./l.) the amount of D-oxidase per mg. dry weight found in mycelial suspensions was nearly doubled by the addition of 0.2% casein hydrolysate to the culture medium; no enzyme was found when 1% (NH₄)₂SO₄ had been added. The highest Q_{O_2} obtained in the suspensions (1) was much less than that obtained with L-phenylalanine and mycelial suspensions of CMI 3411 (115).

Distribution of L-oxidase in other mould species. Knight (1948) has described an L-amino-acid oxidase in the *Penicillia* and in *Aspergillus niger*. This enzyme differs from the *Neurospora* enzyme in its specificity and sensitivity to inhibitors. Knight examined *N. crassa*, but did not find an L-amino-acid oxidase, probably because his growth conditions (shaken cultures in a corn-steep liquor (2%) - lactose (2%) medium) were unfavourable for oxidase production. The oxidase is not destroyed by the acetone-drying procedure used by Knight.

Several mould species were grown under conditions which favour the production of the L-oxidase by *Neurospora* (14 days' stationary culture in the basal medium plus 0.01% yeast and 0.3% malt

extracts). L-Phenylalanine was readily oxidized by the mycelial extracts from *Melanospora destruens* and the culture fluid contained 1.2 units of oxidase activity per ml. The oxidase was not detected in the other species; L-phenylalanine or L-alanine did not affect the small endogenous respiration of mycelial extracts from *Chaetomium caprinum*, *Fusarium lini*, *Gibberella zeae*, *Nectria cinnabarina*, *Penicillium chrysogenum* Q 176, or *Trichoderma viride*. With *Aspergillus niger* CMI 594 and *Sordaria fimicola*, these amino-acids depressed the endogenous respiration.

DISCUSSION

Factors influencing the yield of L-oxidase. The most remarkable factor controlling the yield is the amount of biotin in the culture medium; the yield of enzyme per mg. mycelium is highest when growth is limited by the supply of biotin and is considerably reduced by more than 1 μg. biotin/l. In the absence of detailed information on the mechanism of action of biotin on growth generally it is hardly possible to offer an explanation of the observed effect.

Some data suggest a connexion between slow growth and high L-oxidase activity. The two strains which produce the most oxidase (CMI 3411 and Cambridge *a*) grow slower and sporulate less readily than other strains, especially Beadle 25*a* and Emerson *a*, which produce little oxidase. The oxidase activity is low when growth is stimulated by shaking the culture or by providing an optimal supply of biotin. However, this relation between growth and oxidase activity is not general; limitation of growth by the supply of iron to strain CMI 3411 or of inositol to the mutant, strain CMI 17836 decreases the oxidase activity per unit of weight of mycelium.

The yields of L-oxidase per mg. mycelium are similar with an inorganic source of nitrogen or with a completely organic source (casein hydrolysate).

This is in contrast to the findings with the L- and D-oxidases of *Penicillium* (Knight, 1948; Emerson, Puziss & Knight, 1950) and the D-oxidase of *Neurospora* where the yields of enzyme are considerably lower with an inorganic source of nitrogen than with an amino-acid source.

Prosthetic group. The purified oxidase contained 1 g.mol. bound FAD per 11,000 g. non-dialysable nitrogen, whilst riboflavin and riboflavin phosphate were not detectable. All preparations of the oxidase absorbed considerably more light below 480 m μ . than was expected from the FAD content. The change in absorption spectrum due to the reduction of FAD was therefore difficult to measure, but, on the addition of a substrate, a definite effect was found and this may be taken as strong evidence for the view that FAD is the prosthetic group of the oxidase.

FAD is also the prosthetic group of the L-amino-acid oxidase of snake venom (Singer & Kearney, 1950a), of kidney D-amino-acid oxidase (Warburg & Christian, 1938) and of glycine oxidase (Ratner, Nocito & Green, 1944). The prosthetic group of the kidney L-amino-acid oxidase described by Blanchard, Green, Nocito & Ratner (1945) is riboflavin phosphate.

Turnover number. The purified enzyme oxidized 2100 mol. phenylalanine/mol. FAD/min. at 30°. This is of the same order as the turnover numbers of the L-amino-acid oxidase of snake venom (Singer & Kearney, 1950b) and the mammalian D-amino-acid oxidase (Warburg & Christian, 1938), namely 3100 and 1440 respectively.

Comparison with some other oxidases. The more striking properties of the *Neurospora* oxidase include the ability to oxidize a wide range of L- α -amino-acids, the broad pH optimum and the inhibition by high substrate concentrations. The L-amino-acid oxidase of snake venom is also inhibited by high concentrations of substrate (Zeller & Maritz, 1945). The *Neurospora* oxidase is not affected by many substances such as benzoic acid, 2:4-dinitrophenol, iodoacetic acid or octanol which inhibit either the kidney D-amino-acid oxidase (Klein & Kamin, 1941), the amino-acid oxidases of *Penicillium* (Knight, 1948; Emerson *et al.* 1950) or the L-amino-acid oxidase of snake venom (Zeller & Maritz, 1945).

SUMMARY

1. Seventeen strains of *Neurospora crassa* and three of *N. sitophila* were examined for amino-acid oxidases. Under suitable conditions, which are described, both an L- α -amino-acid and a D- α -amino-acid oxidase were detected in all strains. The activity of both enzymes varied considerably from strain to strain. An L-oxidase was also found in *Melanospora destruens*; it was absent from several other species.

2. The highest yields of L-amino-acid oxidase were obtained with *Neurospora crassa* CMI 3411 in stationary culture on a liquid medium containing sucrose, ammonium tartrate, 0.2% casein hydrolysate, inorganic salts, and 0.1–0.5 μ g. biotin/l. The oxidase activity in the mycelium and also in the culture fluid was highest after 12 days' growth at 25°.

3. Concentrations of biotin above 1 μ g./l. considerably reduce the yield of L-amino-acid oxidase.

4. The kinetics of the L-amino-acid oxidase were studied. The pH-activity curve has a broad maximum between pH 6 and 9.5. High concentrations of substrate inhibit.

5. The specificity was not changed by 4.5-fold purification of the oxidase from the culture fluid.

6. Bound flavin-adenine dinucleotide (FAD) was present in the purified oxidase (1 g. mol./11,000 g. non-dialysable nitrogen) as shown by the activation of the D-amino-acid oxidase apo-enzyme, fluorimetry and paper chromatography. Riboflavin phosphate and riboflavin were not detected. Anaerobic addition of alanine decreased the optical density of the enzyme preparation between 380 and 500 m μ .; this change was that which is characteristic of the chemical reduction of flavo-proteins. It may therefore be taken that FAD is the prosthetic group of the L-amino-acid oxidase.

7. The turnover number was estimated to be 2100 molecules phenylalanine per minute at 30°.

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Studies in Carotenogenesis

2. CAROTENE PRODUCTION BY *PHYCOMYCES BLAKESLEEANUS*: THE EFFECT OF DIFFERENT AMINO-ACIDS WHEN USED IN MEDIA CONTAINING LOW CONCENTRATIONS OF GLUCOSE

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In a previous investigation (Garton, Goodwin & Lijinsky, 1951) it was shown that, using a medium containing 3% (w/v) glucose, carotene synthesis in *Phycomyces blakesleeanus* was independent of the amino-acid used as the nitrogen source with the exception that glycine enhanced carotene production. Amongst the amino-acids which had no effect was L-valine, from which a hypothetical five-carbon building unit such as β -methylcrotonaldehyde (Karrer, Helfenstein & Widmer, 1928) could conceivably arise and promote carotene synthesis. The only other naturally occurring amino-acid containing a terminal dimethyl grouping is L-leucine. Such a grouping would appear to be essential in an active repeating unit acting as a carotene precursor, but in media containing 3% (w/v) glucose L-leucine was as ineffective as L-valine.

It was possible, in media containing high concentrations of glucose, for stimulation of carotene synthesis by L-leucine or L-valine to be masked; it was therefore decided to investigate the action of these amino-acids in media containing sufficient glucose to produce a reasonable mycelial growth, but insufficient to produce more than small amounts of carotene. The observations of Garton *et al.* (1951) indicated that the most useful glucose concentration would be 1% (w/v); this produces about 75% of the maximal mycelial growth, whilst the carotene production is less than 10% of the maximal.

EXPERIMENTAL

The general cultural and analytical procedures employed were described in detail in the first paper of this series (Garton *et al.* 1951). The various media were made by

altering the glucose concentration of the standard medium, generally to 1% (w/v), and varying the N source according to requirements; the salt and aneurin concentrations were unaltered. All experiments were carried out under natural illumination.

Replacement cultures. Well-developed mycelia which could be easily handled and transferred to fresh media were obtained by culturing the fungus on filter paper (Whatman no. 50) in Petri dishes (11 cm. diameter). Small glass beads (2–3 mm. diameter) were added in sufficient numbers to cover the bottom of the dishes and 45 ml. of the medium added to each dish; this is just sufficient to cover the beads. The filter papers were then placed in position on the beads and the dishes sterilized. Inoculation was carried out by pipetting the spore suspension on to the filter paper. When the mycelia were at the appropriate stage of growth they were transferred to a new medium in another Petri dish by lifting out the filter paper and adhering mycelium, washing it with sterile water in an inoculating cabinet and placing it carefully on the new medium. Glass beads are not essential in the replacement media, but they are necessary in the first cultures in order to obtain reproducible mycelial growths. Glass beads are much better for supporting the filter paper than sand, which had previously been suggested for this purpose by Raaf (1941). Small amounts of sand inevitably get into the mycelia and, as they are impossible to remove completely, dry weight determinations cannot be carried out. After growth is completed, the filter paper can be removed by peeling it away from the fungal mat, which can then be examined in the usual way.

RESULTS

In the first experiment three media were used; all contained 1% (w/v) of glucose, whilst the N sources were either L-leucine, L-asparagine or L-valine (c, 0.35, 0.2 and 0.676% (all w/v) respectively). It